Viral Nucleoprotein Complexes In Cells Infected with the Parvovirus MVM

by

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A thesis presented for the degree of

Doctor of Philosophy

March, 1980

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Acknowledgements

I thank Professor R.M.S. Smellie and Professor A.R. Williamson for providing the facilities for undertaking this research, and I acknowledge the receipt of a Postgraduate Studentship from the Medical Research Council.

I am grateful to Dr. Robert Eason for his guidance and constant encouragement throughout this work.

I also thank the many members of the Biochemistry Department for helpful discussion and Mr. P. Ferry and Miss Paula Coia of the Wellcome Cell Culture Unit for invaluable assistance and advice on the culturing of cells. I also thank Dr. L.W. Coggins of the Beatson Institute for Cancer Research for the invaluable and expert advice on the techniques of electron microscopy.

Finally, I am indebted to Mrs. A. Strachan for typing the manuscript.

Abbreviations

Abbreviations used in this thesis are as laid down in the Biochemical Journal Instructions to Authors (revised 1978) with the following additions.

Ara-C	1-β-D-arabinofuranosylcytosine
BUdR	5-Bromodeoxyuridine
cpe	Cytopathic effect
DNase	Deoxyribonuclease
FA	Fluorescent antibody
FUdR	5-Fluorodeoxyuridine
HA	Haemagglutination
HAI	Haemagglutination inhibition
hpi	Hours post-infection
MVM	Minute Virus of Mice
pfu	Plaque forming units
PBS	Phosphate buffered saline
PPLO	Pleuropneumonia-like organisms
RF	Replicative Form
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SV40	Simian virus 40
TdR	Thymidine

Contents

Summary

Virus specific deoxyribonucleic acid (DNA) synthesis in BHK-21/C13 cells infected with the autonomous Parvovirus Minute Virus of Mice (MVM) can first be detected 8-10 hour post infection (hpi) and reaches a maximum rate of DNA synthesis between 14-16 hpi. Viral DNA was extracted from infected cells, between 16-18 hpi, using a method which preserved protein and DNA interactions. The mechanisms of viral DNA replication and the assembly process were studied, under these conditions.

A method was developed which quantitively and selectively extracted viral DNA from infected cells. This method relies on lysis of infected cells with the non-ionic detergent, NP40, followed by separation of the cells into cytoplasm and nuclei. The nuclei were then treated with various NaCl concentrations. Optimal release of viral DNA, without release of cellular DNA, occurred at a final concentration of 0.5M NaCl. The optimal pH for the extraction conditions was pH 7.0.

Two nucleoprotein complexes were detected. A fast sedimenting, approximately 100S, nucleoprotein complex, designated CI, was present in the cytoplasmic extract. CI had a buoyant density in CsCl slightly greater than that of mature virus and a sedimentation coefficient slightly less than mature virus. Mature MVM sediments at 110S. CI is 20% sensitive to micrococcal nuclease digestion. The second nucleoprotein complex, designated CII, sedimented at 22S with a leading edge towards higher sedimentation values. CII was located in the nucleus and constitutes the major viral DNA species in the infected cell. Protein was shown to be associated with the DNA by

the change in sedimentation coefficient upon digestion with SDS and pronase, the increased sensitivity of the DNA to digestion with DNase I after treatment with agents which destroy protein and DNA interactions, and the association of ^{35}S-L-methionine labelled protein with the DNA. Reconstruction experiments have indicated that the association of protein with the DNA is not due to an artefact of the extraction procedure.

The DNA components in the nucleoprotein complexes were analysed by neutral and alkaline agarose gel electrophoresis, NaI buoyant density analysis, sensitivity to digestion with S1 nuclease and hybridisation analysis. CI was shown to consist of single stranded DNA of the same molecular weight as MVM viral DNA. The DNA was demonstrated to be the viral strand and not the complementary strand by hybridisation with duplex MVM DNA synthesised *in vitro* from MVM viral DNA using DNA polymerase I. The main band of CII consisted of a duplex DNA molecule twice the molecular weight of MVM viral DNA and therefore represents monomer replicative form (RF) DNA. When analysed under alkaline conditions the DNA contained molecules up to twice genome length. This indicated that some of the DNA in the duplex consisted of covalently linked viral and complementary strands. Confirmation of this was obtained by the observation that some of the CII DNA molecules were capable of spontaneous renaturation. The fast-sedimenting region of CII contained DNA twice and four times the length of monomer RF DNA, but, only contained DNA up to twice genome length under alkaline conditions. CII was shown to be viral in origin by displacement hybridisation.

Pulse-chase experiments revealed that CII was a precursor to CI although no replicative intermediate could be isolated.

Preliminary electron microscopic studies revealed that the major DNA species was a linear double stranded DNA molecule of genome length. Potential replicative intermediates were identified by electron microscopy but no confirmatory data was obtained.

In conclusion, viral DNA replication occurs in the form of nucleoprotein complexes and a putative maturation product was identified.

The mechanisms of Parvovirus DNA replication and assembly are discussed.

1. Introduction

1.1 Nature of Viruses

Viruses were originally identified as causative agents of infectious diseases during the late part of the 19th Century and the early part of the 20th Century. At that time it was not possible to define in biochemical terms the nature of viruses, although Beijerinck in 1898 noted of Tobacco Mosaic Virus "a possibly water soluble molecule, able to replicate, but only when incorporated into the living protoplasm of the cell, into whose reproduction it is, in a manner of speaking, passively drawn." Since then more refined statements of this basic thought have been expressed. Luria and Darnell (1967) defined viruses as entities whose genomes are elements of nucleic acid, either DNA or RNA, that replicate inside living cells using the cellular synthetic machinery and causing the synthesis of specialised elements, the virions, that can transfer the viral genome to other cells. There are four processes, after penetration and uncoating, which are essential to the replication of all viruses: transcription of the genome to mRNA; translation of the messages into virus specific proteins; replication of the genome; and virus assembly and release. Distinguishing features that contribute to animal virus diversity are the kind of genome (DNA or RNA), the size and informational content of the genome, and the cellular sites of mRNA transcription and of genome replication (Bachrach, 1978). The classification of animal viruses is founded on the above facets of the replication cycle and also the morphology of the virion.

1.2 The Parvovirus Group

1.2.1. Classification

The generic designation, Parvoviridae, was first proposed by

Lwoff and Tournier (1966) and finally accepted by the Vertebrate Virus Subcommittee of the International Committee on Nomenclature of Viruses (ICNV) in 1970 (Andrewes, 1970). This group is characterised by possessing small (15-24 nm in diameter), non-enveloped, icosahedral particles composed of one to three proteins and a molecule of linear single stranded DNA. The viruses multiply in the cell nucleus. The virions are heat stable and ether resistant and have a relatively high buoyant density in caesium chloride (approximately 1.4 g/cm^3) owing to their high content of DNA (Wildy, 1971).

The family Parvoviridae can be separated into three genera (Table 1):

(A) Viruses of the largest subgroup, the genus Parvovirus, are the non-defective or autonomous Parvoviruses, in that, to elicit a productive infection they do not usually require the aid of a helper virus. Members of this subgroup package one unique strand of DNA. The term Parvovirus can be used to describe the entire family and not only this genus. In these circumstances the members of this genus would then be denoted as the non-defective or autonomous Parvo-viruses.

(B) The genus Adeno Associated Virus (AAV). The viruses in this subgroup all require the aid of a helper virus in order to produce progeny virions. As the name implies the helper virus is usually an Adenovirus. A further difference is that members of this group package approximately equal numbers of complementary single strands in separate virions.

(C) The genus Densovirus. Unlike the previous two subgroups which contained only vertebrate Parvoviruses, this subgroup only contains

Table 1 The Parvovirus Group

	Acronym	Full Name
	Nondefective Subgroup (Genus Parvovirus)	
a	KRV	Kilham Rat Virus
a	H-3	-
a	X-14	-
a	L-S	Lum - Schreiner virus
a	HER	Haemorrhagic Encephalopathy virus of Rats
a	KIRK	-
a	H-1	-
a	HT	-
b	FPV (or LV)	Feline Panleukopenia Virus (or Leopard Virus)
b	MEV	Mink Enteritis Virus
a	PPV	Porcine Parvovirus
a	KBSH	-
	MVM	Minute Virus of Mice
	BPV	Bovine Parvovirus (Haden)
	TVX	-
	HB	-
	MVC	Minute Virus of Canines
	LuIII	-
	RTV	-
	GHV	Goose Hepatitus Virus
	Defective Subgroup (Genus Adeno-Associated Virus)	
	AAV1	Adeno Associated Virus type 1
	AAV2	Adeno Associated Virus type 2
	AAV3	Adeno Associated Virus type 3
	AAV4	Adeno Associated Virus type 4
	$AAVX_7$	Bovine Adeno Associated Virus
	AAAV	Avian Adeno Associated Virus
	CAAV	Canine Adeno Associated Virus
	Anthropod Subgroup (Genus Densovirus)	
	DNV1	Densonucleosis Virus 1
	DNV2	Densonucleosis Virus 2

a Brackets include antigenically cross-reacting viruses.

b These cross-react antigenically and are probably the same virus.

Adapted from Tattersall and Ward (1978).

anthropod Parvoviruses. Densonucleosis viruses one and two (DNV1 and DNV2), which are the sole members of this group, replicate without the aid of a helper virus, but the individually packaged single strands are complementary. Verylittle is known concerning the replication of this group and therefore this thesis will concentrate on the mammalian Parvoviruses.

1.2.2 Biology

When KRV was first isolated by blind passage from tumour tissue it was thought likely that it may be the causative agent of these tumours. However, upon injection of KRV into adult animals no tumours or indeed any apparent disease developed (Kilham and Oliver, 1959).

The probable nature of Parvoviral infection was elucidated when hamsters were injected with H-1 either in utero or within 3 days of birth. The animals developed a mongoloid type deformity which was characterised by small size, flat face or microcephalic domed head, protruding eyes and tongue, abnormal teeth or absence of teeth, and bone fragility (Toolan, 1960a,b). A similar result was demonstrated for KRV, in that, KRV was determined to be infectious for suckling hamsters if inoculated from 1 to 4 days of age but two paths were open to the infection. In high doses, acute illness and death between 3 to 6 days, whereas, in low doses the animal survives but develops the "mongoloid type" deformity (Kilham, 1961). Further experiments with KRV demonstrated that when injected into newborn hamsters it was possible to produce cerebellar ataxia due to the virus selectively destroying the external germinal layer of the cerebellar cortex (Kilham and Margolis, 1964).

MVM can also produce these effects when inoculated into newborn hamsters. The hamsters were usually dead or moribund 6 days later but occasional hamsters surviving the effects of a low dose of MVM have had features of the mongoloid deformity described for KRV and H-1 (Kilham and Margolis, 1970). MVM will not infect rats and will only produce a mild infection in mice whose only apparent clinical sign is stunted growth. The cerebellum of susceptible animals was a special target for MVM but high titres were also found in urine and the gut (Kilham and Margolis, 1970)

The basic histopathological changes upon Parvovirus infection are a highly localised attack focussed upon germinal centres high in replicate activity of the neonate, particularly in the cerebellar external germinal layer, and a more diffuse and widespread attack upon replicating supporting structures in the neonatal nervous system, particularly upon vascular endothelium. The effects of Parvoviral infection are, however, pantropic rather than merely neurotropic (Margolis and Kilham, 1970).

H-1 injected intravenously, in high titre, into pregnant hamsters, resulted in a marked embryocidal effect and a wide spectrum of congenital malformations suggesting that the placenta is permeable to virus (Ferm and Kilham, 1965). In a similar study using LuIII it was shown that the foetus was most susceptible to virus infection if the mother was injected between the eighth and tenth day of the gestation period (Soike et al, 1976). Toolan (1978) confirmed these results, with H-1, but also noted a second peak of sensitivity to infection at day 13 of the 16 day gestation period, and suggested that there may be a correlation between the level of maternal hormones at the time of infection and a successful infection. The cause of this

biphasic distribution appears to be an alteration in the ability of the virus to cross the placenta, since H-1 is only detected in the foetuses, by cell culture analysis, at the most susceptible times (Toolan, 1979). In all of these experiments the mother did not show any significant signs of disease.

The adult animal is normally resistant to Parvovirus infection, but, if damage is done to a tissue which has the ability to regenerate then it is possible that a Parvovirus infection could result. Such a case was illustrated, whereby the partial hepatectomy of a normally resistant adult rat resulted in growth of the injected Parvovirus H-1 in the liver, causing hepatitis. Only the liver of the animal was affected (Ruffalo et al, 1966). The interaction of Parvoviruses with rapidly growing tissues, e.g. propensity to proliferate in the uterus, placentas and especially foetuses of pregnant animals, as well as regenerating tissue correlates with an interesting observation on the effect of H-1 on tumour production. Toolan (1967) noted that in "mongoloid type" deformed H-1 infected hamsters the natural occurrence of tumours was 23/10,000 whereas in uninfected animals the occurrence was 500/10,000. This prompted a more detailed study on the effects of H-1 on tumour production using the oncogenic virus, Adenovirus, to produce tumours in the hamsters (Toolan and Ledinko, 1968). H-1 hamsters which had survived the original H-1 infection either as mongoloid or non-mongoloid, but all with high titres of HAI and neutralising antibodies, had a much reduced level of tumour formation, i.e. 28% when compared to the 67% of control animals. The deformed H-1 infected animals had a 13% tumour incidence whereas the non-deformed H-1 infected animals had a 36% tumour incidence. The significance of these results on the

apparent reduction in tumour rate (whether spontaneous or induced by oncogenic viruses) and any similar effects in the natural population is unknown.

The ability of Parvoviruses to cause or at least be strongly implicated as the causative agent of still births and abortions in pigs and cattle correlates with their known laboratory effects (Cartwright et al, 1969; Lucas et al, 1974). Recently a possible role of Parvoviruses in inducing abortions or still births in humans was proposed. It is based on a statistically significant difference ($P < 0.001$ of the population being the same) of the occurrence of antibodies against H-1 and X-14 in women with repeated abortions or still births compared with levels in women with normal births. The percentages were for H-1 and X-14, respectively, 13.71% and 9.14% in women with repeated abortions or still births and 2.66% and 1.66% in the control sample (Guglielm et al, 1978).

The defective Parvovirus group, the Adeno Associated Viruses, have not been implicated in any known disease, although serological studies indicate the presence of AAV types 1, 2 and 3 in man (Blacklow et al, 1968a; Hoggan, 1966; Blacklow et al, 1968b).

1.2.3 Virion Morphology

The size and structure of a virion is a further determinant in the classification of viruses into groups. Parvovirus virions consist of small, non-enveloped, icosahedral particles. Table 2 illustrates the range in particle dimension of a selected number of Parvoviruses. As one can see the reported size range of these particles is between 16-30 nm, however, this may not reflect a real difference in the size of the particles but may be due to the different

Table 2 The size of a selection of mammalian Parvoviruses

VIRUS STRAIN	SIZE (nm)	REFERENCE
H-1	30	Chandra and Toolan (1961)
H-1	24-25	Toolan et al (1964)
H-1	30	McGeoch et al (1970)
H-1	21.5	Karasaki (1966)
KRV	28	McGeoch et al (1970)
KRV	16	Dalton et al (1963)
KRV	19	Karasaki (1966)
MVM	26	Crawford (1966)
MVM	19	Crawford (1966)
MVM	28	McGeoch et al (1970)
H-3	19	Karasaki (1966)
HT	21.5	Karasaki (1966)
HB	19	Karasaki (1966)
PPV	20-22	Mayr et al (1968)
$AAVX_7$	22	Luchsinger et al (1970)
AAAV	18-21	Yates et al (1973)
AAV-1	28	Crawford et al (1969)
AAV	min 18	Smith et al (1966)
	max 21	

methods of size determination. An indication that this is the case is given by the fact that the size of H-1 is reported as being between 21.5 and 30 nm and the size of KRV is reported at between 16 and 28 nm. These size differences may arise through two basic problems (Hoggan, 1971). Firstly, the method of measurement of particles which are not spherical. Smith et al (1966) observed for AAV that the virions were not circular and thus expressed their data as a set of two measurements. These measurements were the minimum and maximum dimension of each particle. Secondly, the method of sample preparation, since the stain used may affect the apparent size.

The elucidation of the fine structure of the virion has been hampered by their extremely small size. The basic method used to deduce the fine structure has been to compare negatively stained virus preparations, observed in the electron microscope, to various geometric models. It is agreed that the particles are naked, i.e. they do not contain a lipid envelope (Smith et al, 1966; Mayor and Mlenick, 1966; Karasaki, 1966) as has been confirmed by their resistance to lipid solvents (Kilham and Margolis, 1964; Atchison et al, 1965; Hoggan et al, 1966; Binn et al, 1970; Storz and Warren, 1970; Siegl et al, 1971). Vasquez and Brailovsky (1965) suggested that the virion structure of KRV could be represented by a pentagonal dodecahedron (a dodecahedron whose faces are all pentagonal pyramids) with thirty-two capsomeres. Karasaki (1966) for KRV, H-1, H-3, HT and HB proposed that the virions have 32 capsomeres arranged as an icosahedron or a pentagonal dodecahedron. The position with the AAV group is less clear, although AAV-4 and AAAV appear to possess icosahedral symmetry (Mayor et al, 1965; Yates et al, 1973), AAV has been reported as possessing a network-like arrangement of protein fibres (Smith et al, 1966). The final resolution of the capsid configuration must, however, await future studies.

1.2.4 Buoyant Density

All Parvoviruses have, for viruses, a comparatively high buoyant density in caesium chloride which is due to the high ratio of DNA to protein (e.g. approximately 1:3, DNA:protein, by weight, for MVM, Tattersall et al, 1976). The detailed buoyant density analysis of Parvoviruses has proved to be more complex than original observations implied.

Payne et al (1964) studying the morphology of the Parvovirus, X-14, reported two peaks of haemagglutinating activity when banded in caesium chloride. The densities of the bands were at 1.40 g/cm^3 and 1.31 g/cm^3 of which only the 1.40 g/cm^3 band was infectious. They suggested that the 1.40 g/cm^3 band consisted of full particles and that the 1.31 g/cm^3 band consisted of empty particles. Usategui-Gomez et al (1969) for H-1 also observed two peaks of haemagglutinating (HA) activity at 1.39 g/cm^3 and 1.30 g/cm^3. However, three peaks of HA activity were observed for MVM (Crawford, 1966) and KRV (Robinson and Hetrick, 1969). For MVM the densities were 1.43 g/cm^3, 1.38 g/cm^3 and 1.35 g/cm^3 and for KRV, 1.43 g/cm^3, 1.38 g/cm^3 and 1.32 g/cm^3. Similar results were also presented for three Parvoviruses which were isolated as contaminants of permanent human cell lines. These viruses, KBSH, LuIII and TVX displayed two major peaks of HA activity at 1.39 g/cm^3 and 1.31 g/cm^3, with a minor peak at 1.35 g/cm^3 (Siegl et al, 1971). Siegl (1972) analysed this property in more detail and showed that the particles banding at 1.39 g/cm^3 and 1.35 g/cm^3 had different DNA contents. The DNA from the 1.39 g/cm^3 band had a homogenous sedimentation profile in neutral sucrose gradients with a sedimentation coefficient of 24.7 $\pm$ 0.5S, whereas, the particles banding at 1.35 g/cm^3 contained DNA which was smaller than genome size

and sedimented heterogenously in sucrose gradients with a peak at approximately 10S. The 1.35g/cm^3 band was not infectious. The buoyant density for the infectious particles of the Adeno Associated Virus group ranged between 1.388 to 1.445 g/cm^3 for AAV types 1 to 4, AAV-2 being the lightest and AAV-4 the heaviest (Hoggan, 1971).

A further interesting feature concerning the buoyant density profile was noted for PPV, in that the buoyant density of the full particles was 1.38 g/cm^3 but some full particles banded at a density as high as 1.44 g/cm^3. These results which indicated full virus particles having a much higher buoyant density than the major band were observed for other autonomous Parvoviruses. Haden (Johnson and Hoggan, 1973), LuIII (Gautschi and Siegl, 1973), H-1 (Rhode, 1974a) and MVM (Clinton and Hayashi, 1975) all had full intact virus particles of much higher buoyant density than the main infectious band. This phenomenon was also observed for the defective AAV group. AAAV had a main band buoyant density of 1.39 g/cm^3 with some particles as dense as 1.42 g/cm^3 (Yates et al, 1973) and AAV X_7 had a main band buoyant density between 1.37 to 1.38 g/cm^3 with a minor band at 1.43 g/cm^3.

Clinton and Hayashi (1975) for MVM studied the relationship between these particles. They fractionated infected cell extracts on sucrose density gradients and noted the presence of two main peaks of HA activity sedimenting at 70S and 110S. The 110S particles were shown to incorporate ^{3}H thymidine and when banded to equilibrium in caesium chloride gradients they separated into two peaks, at 1.42 g/cm^3 and 1.47 g/cm^3. The particles were shown to contain the same ratio of DNA to protein. The light particle has a fivefold to tenfold greater HA activity than the heavy particle (Clinton and Hayashi, 1975) and also has a greater affinity to bind to the presumptive infective

cells than the heavy particles (Clinton and Hayashi, 1976). Consistent with this data is that the heavy particles are a precursor to the lighter particles (Clinton and Hayashi, 1975). An analysis of the protein composition of the virions at these two densities indicated that the major protein in the heavy species was protein B whereas the major protein in the lighter species was protein C (Clinton and Hayashi, 1975; Tattersall et al, 1976). See section 1.2.7 for a definition of the virion proteins. The heavy particles could be converted in vitro, to the lighter particles by treatment with 3 day old infected cell medium (Clinton and Hayashi, 1976) but although protein B could be converted to protein C, in vitro, by treatment of the denser virions with trypsin this did not alter the buoyant density of the particle (Clinton and Hayashi, 1976; Tattersall et al, 1977). Indeed, trypsin was not able to convert protein B in the empty particle to protein C (Tattersall et al, 1977). This suggests that although the processing event of the denser full virion to the lighter full virion is a proteolytic cleavage it is also dependent on a structural arrangement of the denser virion. Identical results on the kinetic relationship between heavy and light full particles have been obtained with H-1 (Kongsvik et al, 1978).

Recently, the non-ionic buoyant density medium, metrizamide, has been used to study the composition of the heavy and light full particles of H-1 (Kongsvik et al, 1979). On banding in metrizamide of both the heavy and light full particles, partially purified in caesium chloride gradients, they both formed into two main peaks, at 1.32 g/cm^3 and 1.20 g/cm^3, separated by a heterogenous region, which may be empty virus particles. Both peaks contained full length viral DNA and the 1.20 g/cm^3 particle was sensitive to micrococcal nuclease. The significance of this separation is unclear.

1.2.5 Haemagglutination

The original isolates of Parvoviruses were demonstrated to haemagglutinate various species of red blood corpuscles (Kilham and Oliver, 1959; Crawford, 1966). However, a comparison of haemagglutination results from laboratory to laboratory is not meaningful since the extent of haemagglutination will depend on the quantity and type (with regard to the density of the particle in caesium chloride buoyant density gradients) of virus particles and the condition of the red blood corpuscles (RBC's). For these reasons the most informative study would be that presented by Hallauer et al (1972), in which 10 Parvovirus strains were tested for haemagglutination activity against 16 different species of RBC's (Table 3). Some tentative general conclusions may be drawn from these results:-

(1) all virus strains react strongly with human (group O) and rat erythrocytes (as well obviously as guinea pig RBC's, since the viruses were all initially adjusted to give a HA titre of between 1:4096 to 1:8192 when measured against guinea pig RBC's) and also, though in some cases to a lesser degree, with mouse and hamster RBC's.

(2) virus strains KBSH and PPV (which belong to the same serotype) and MVM (serologically distinct) either failed to react or did so only very weakly with sheep, horse, pig, cat and dog RBC's, while TVX, LuIII, RTV (not serologically related) and the hamster osteolytic viruses (H-1, H-3, KRV, X14) show a much stronger reaction when tested with the same RBC's.

(3) all strains either failed to react or only did so very weakly with cattle and rabbit RBC's.

Hallauer et al (1972) noted that for all virus strains the haemagglutination reaction was temperature (between 4°C and 37°C) and

Table 3 Haemagglutination Spectra of selected Parvovirus Strains

VIRUS STRAINS	KBSH	TVX	LuIII	RTV	PPV	H-1	H-3	KRV	X14	MVM
RBC species										
Human (O)	+++	+++	+++	+++	+++	+++	+++	+++	++	++
Monkey	+++	+++	++	+++	++	++	++	++	++	-
Guinea Pig	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Mouse	+	+	+++	++	+	++	++	++	++	++
Rat	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Hamster	(+)	(+)	+++	+++	(+)	++	+++	++	++	++
Rabbit	-	-	-	-	-	-	(+)	-	(+)	-
Sheep	(+)	++	+	+	(+)	+	+	+	+	-
Horse	-	++	+++	++	-	(+)	++	+	++	-
Cattle	-	(+)	(+)	-	-	-	-	-	-	-
Pig	-	+++	+	++	-	+++	++	(+)	+	-
Cat	(+)	++	++	++	+	++	++	+	+	-
Dog	(+)	+++	+++	+++	(+)	+++	+++	+	+	++
Chicken	++	++	++	+	++	+	(+)	(+)	(+)	(+)
Goose	++	++	++	++	+	++	+	-	-	(+)
Frog	-	-	NT	NT	NT	++	+	NT	-	NT

Each virus strain was adjusted to a similar haemagglutination titre (1:4906 to 1:8192) for Guinea Pig RBC's. The degree of haemagglutination is expressed as the virus dilution giving partial (approximately 50%) haemagglutination: ::: (1:1024-8192); ++ (1:128-512); + (1:16-64); (+) (1:2-8); - (no reactivity in indicated range of virus dilutions).

NT : not tested.

Adapted from Hallauer et al (1972).

pH (between pH 6.6 to 8.5) independent. Also the HA titre could be reduced twofold by incubating at pH 9.0 (which was reversible by lowering the pH) or destroyed completely by neuraminidase treatment.

A similar though much less extensive study of the haemagglutination reaction was reported by Hoggan (1971). Table 4 illustrates these results and perhaps the most significant difference between these results and those of Hallauer et al (1972) is that KRV and MVM do not haemagglutinate human (group O) RBC's under the conditions of Hoggan (1971). Another interesting observation is that certain members of the AAV group do not haemagglutinate guinea pig, human (group O) or rat RBC's. Indeed, even when haemagglutination titre is detected with, e.g. AAV-4 the reaction is reversible when the temperature is increased from 4°C to 37°C (Ito and Mayor, 1968). AAV X_7 will also haemagglutinate both guinea pig and human (group O) RBC's in a temperature reversible reaction. Thus, there is a significant difference in haemagglutination properties between the autonomous and AAV subgroups of the genus, Parvoviridae.

1.2.6 DNA structure

1.2.6.1 Autonomous Parvoviruses

The autonomous Parvoviruses have not had as intensive an analysis of the physical structure of their genomes as the members of the AAV group (section 1.2.6.2). The reasons for this are twofold. Firstly, since the virions only contain one type (with regard to complementarity) of DNA strand, the DNA once extracted cannot form a duplex structure, therefore, cannot be analysed by the use of restriction endonucleases, and, secondly, there has been, until recently, an inability to obtain sufficiently large quantities of viral DNA for structural analysis.

Table 4 Haemagglutination Spectra of selected Parvovirus Strains

VIRUS STRAIN	RBC species		
	Guinea Pig	Human (O)	Rat
KRV	+++	-	++
H-1	++++	+++	+
MVM	+++	-	+
AAV-1	-	-	-
AAV-2	-	-	-
AAV-3	-	-	-
AAV-4	++	++	+

Each virus strain was adjusted to contain a fixed amount of complement fixing units of virus specific antigen. The degree of haemagglutination is expressed as the virus dilution giving complete haemagglutination at 4°C: ++++ (1:1024); +++ (1:256-1024); ++ (1:32-128); + (1:2-16); - (no reactivity in indicated range of virus dilutions).

Adapted from Hoggan (1971).

The nature of the genome of autonomous Parvoviruses was demonstrated to be single stranded by the reaction of virion DNA (i.e. the DNA still encapsidated) with formaldehyde (for MVM see Crawford, 1966). A second criterion was the properties of the isolated DNA:-

(1) reaction of formaldehyde with the DNA which demonstrated the presence of free amino groups and thus indicates the presence of single stranded regions (Robinson and Hetrick, 1969; Usategui-Gomez et al, 1969; Salzman and Jori, 1970).

(2) the same buoyant density whether the DNA was native or denatured (Usategui-Gomez et al, 1969; May and May, 1970).

(3) the base composition, which indicated that A did not equal T and that G did not equal C (Crawford et al, 1969; Salzman and Jori, 1970).

(4) sensitivity to digestion with single strand specific exo- and endonucleases (Salzman et al, 1971; Siegl, 1973; Tattersall et al, 1973; Bourguignon et al, 1976; Salzman, 1977).

(5) thermal denaturation profiles which did not show a sharp melting temperature (Usategui-Gomez et al, 1969; Salzman and Jori, 1970).

(6) electron microscopy which indicated single stranded and linear DNA with a molecular weight range between 1.2×10^6 to 2.0×10^6 daltons (Crawford et al, 1969; Salzman et al, 1971; Siegl, 1973; Rose, 1974; Bourguignon et al, 1976; Singer and Rhode, 1977b; Salzman, 1978).

Thus autonomous Parvovirus DNA is a linear single stranded DNA molecule. The extent of contamination of the viral DNA preparations with DNA strands of the opposite polarity, i.e. complementary strands, has been demonstrated to occur at about a level of 1 per 400 DNA strands (Singer and Rhode, 1977b).

Several lines of evidence indicated that the autonomous Parvovirus genomes have a small amount of secondary structure, i.e. double stranded regions. Hydroxyapatite chromatography, ethidium bromide fluorimetry, and S1 nuclease digestion all revealed that 5 to 6% of MVM viral DNA was in a double stranded form (Bourguignon et al, 1976). Similar results were obtained by digestion of KRV DNA (Salzman, 1977), LuIII DNA (Siegl and Gautschi, 1976) and H-1 and H-3 DNA (Chow and Ward, 1978) with single strand specific nucleases. By labelling the 5' end of internally ^{3}H labelled MVM DNA with ^{32}P, Bourguignon et al (1976) were able to show that while 90% of the ^{3}H label was solubilised by S1 nuclease, the majority (80%) of the terminal ^{32}P label remained acid precipitable, indicating a hairpin-like structure at the 5' end. The S1 nuclease resistant fragment was approximately 130 bases in length. A similar result has been obtained with KRV DNA (Salzman, 1977). Although unable to show a stable hairpin structure at the 3' end of the MVM DNA molecule it was possible to demonstrate the existence of, at least, a transient 3' hairpin. This was demonstrated by the formation of a completely duplex unit length molecule by the action of DNA polymerase I which requires a 3' OH group to act as a primer (Bourguignon et al, 1976). Similar results have been reported for KRV DNA (Salzman et al, 1978). However, later experiments, using S1 nuclease, have demonstrated the existence of a 3' hairpin structure at the termini of MVM DNA of approximately 110 nucleotides in length (Chow and Ward, 1978). The ability of DNA polymerase I to synthesise a completely duplex DNA molecule from viral single stranded DNA has made it possible to construct restriction maps of the Parvoviral genome (Salzman et al, 1978).

The recent development of rapid DNA sequencing methods (Maxam and Gilbert, 1977) have enabled the 3' ends of KRV, H-1, H-3 and MVM DNA to be sequenced (Astell et al, 1979a; Astell et al, 1979b; Salzman and Fabisch, 1979). The sequences of the four DNA's are illustrated in Fig. 1 in their most highly base-paired configuration. The sequences are very homologous although there are some minor differences. Of the first 115 nucleotides, 102 can be base paired to create a stable Y-shaped hairpin structure. Thus these data confirms that the 3' end of Parvovirus DNA exists as a stable hairpin. These results also demonstrate that the sequence at the 3' end is unique, a situation which does not occur with AAV DNA where two sequence orientations are present (see section 1.2.6.2).

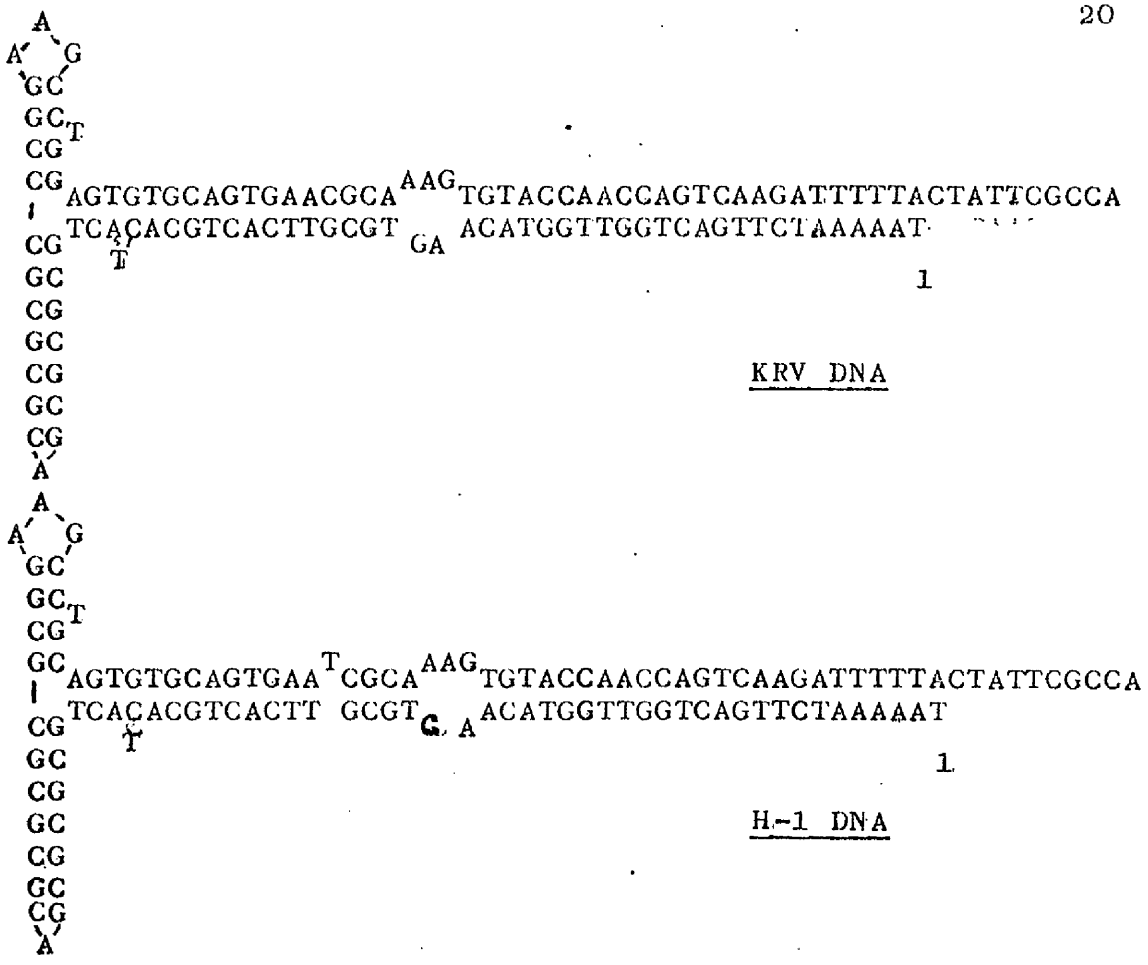
Although the ends of autonomous Parvoviral DNA have some unusual features, they do not possess inverted terminal repetitions (Bourguignon et al, 1976; Singer and Rhode, 1977b). This was demonstrated by the inability of either MVM or H-1DNA to form circles upon extensive renaturation.

1.2.6.2 Adeno Associated Virus Group

When first analysed the genomes of the AAV group were thought to consist of double stranded DNA molecules (Rose et al, 1966; Parks et al, 1967) although previous results using staining of viral preparations with acridine orange had argued for the single stranded nature of the genome (Mayor and Melnick, 1966). The criterion used to conclude that the genomes were double stranded was based on the properties of the isolated DNA. The sharp melting temperature, the base composition, the increase in buoyant density upon exposure to denaturing agents, and the correlation between the molecular weight as determined by sedimentation coefficient and the contour length

Fig. 1

The nucleotide sequences of the 3' ends of KRV, H-1, H-3, and MVM DNA's shown in their most highly base-paired configurations. Number 1 indicates the position of the 3' end of the DNA molecule.


AGTGTGCAGTGAACGCAAAGTGTACCAACCAGTCAAGATTTTTACTATTCGCCA
TCACACGTCACTTGCGTGA ACATGGTTGGTCAGTTCTAAAAAT
KRV DNA
AGTGTGCAGTGAATCGCAAAGTGTACCAACCAGTCAAGATTTTTACTATTCGCCA
TCACACGTCACTT GCGTG A ACATGGTTGGTCAGTTCTAAAAAT
H-1 DNA
AGT GTGCAGTGATCGCAAAGTGTACCAACCAGTCAAGATTTTTACTATTCGCCA
TCATCACGTCACTTGCGTG A ACATGGTTGGTCAGTTCTAAAAAT
H-3 DNA
AGT-GTGCAGTGAATGCAAAGTGTACCAACCAGTCAAGATTTTTACTATTCGCCA
TCATCACGTCACTTACGTG A ACATGGTTGGTCAGTTCTAAAAAT
MVM DNA

measured by electron microscopy all indicated the presence of double stranded DNA (Rose et al, 1966; Parks et al, 1967). However, when the similarity between the physical properties of the virions of MVM, ØX174 and AAV were pointed out, there was then an apparent paradox in the size and nature of the genome (Crawford et al, 1969). All three viruses have a similar size, buoyant density in caesium chloride and sedimentation coefficient. This led Crawford et al (1969) to propose the "unlikely hypothesis" that single, complementary strands of DNA are present in different AAV particles. A similar argument was raised by Mayor et al (1969). The authors determined the molecular weight of the virion from hydrodynamic properties to be 5.4×10^6 daltons (they measured the sedimentation coefficient and diffusion coefficient and calculated the partial specific volume from chemical data). There was thus a discrepancy if the genome had a molecular weight of 3×10^6 daltons and the virion was 26.5% (by weight) DNA, as determined by chemical analysis (Parks et al, 1967).

It was soon demonstrated, by the elegant use of BUdR substitution, that the AAV genome did indeed consist of a single stranded DNA molecule (Rose et al, 1969). The basic methodology was to separately prepare BUdR substituted and unsubstituted virions then extract the DNA from appropriate mixtures of the virions, followed by buoyant density equilibrium centrifugation in caesium chloride. The authors were thus able to show the existence of hybrid molecules and hence that the double stranded DNA resulted from re-annealing during the extraction procedure. This result was confirmed by the use of extraction conditions which prevented re-annealing and also did not cause double stranded DNA to denature (Mayor et al, 1969; Berns and

Rose, 1970). The two complementary strands of AAV DNA, prepared by the above mentioned conditions, could be separated on caesium chloride buoyant density gradients if they had been previously substituted with BUdR in place of thymidine (Berns and Rose, 1970). It was later demonstrated that the two types of particles, each type containing the complementary DNA strand, could be separated by the judicious use of BUdR substitution (Berns and Adler, 1972).

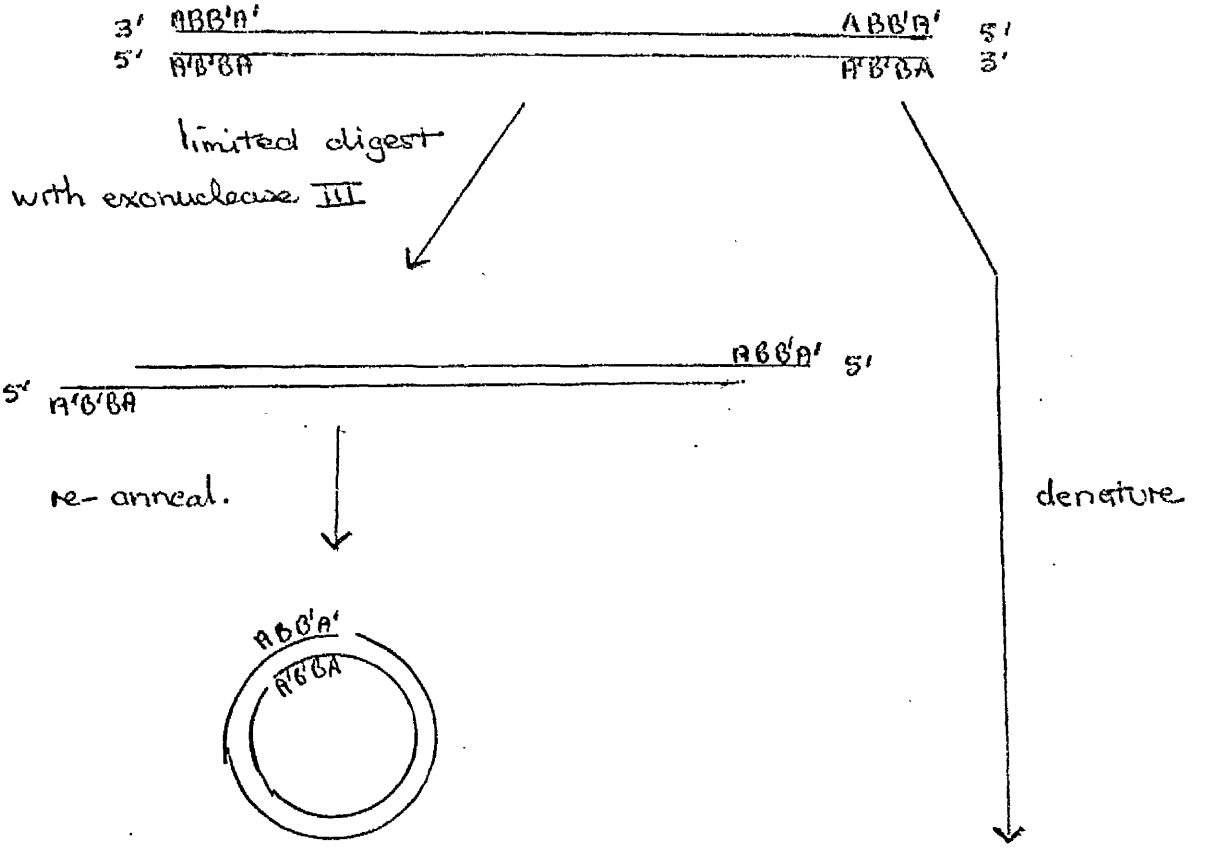
While analysing the pattern of transcription of AAV DNA it was observed that the single stranded, ^{32}P labelled, BUdR substituted minus strands (minus with regard to transcription and heavy with regard to density) appeared to be 56% double stranded as measured by hydroxyapatite chromatography, yet these strands were less than 5% contaminated with plus strands (Carter et al, 1972). Digestion with the single strand specific nuclease S1 revealed that 12% - 14% of the minus strands were resistant to digestion. This result suggested that AAV DNA contained some degree of secondary structure (Carter et al, 1972). When either purified plus or minus strands were spread for electron microscopy the presence of both linear and circular single stranded DNA molecules was noted. There was up to a maximum of 70% circular molecules in the preparation (Kocz ot et al, 1973). The circular molecules were generated by intramolecular re-annealing between the terminal sequences as determined by denaturation followed by analysis by electron microscopy at various times during the re-annealing process and confirmed by the sensitivity of the circles to exonuclease III digestion (Kocz ot et al, 1973). The arrangement of the terminal nucleotide sequences would thus be as an inverted terminal repetition. Identical results on the formation of circles and an estimation of the length of the repetition at 1.5% of the genome was

presented by Berns and Kelly (1974). Koczot et al (1973) further observed that when duplex AAV DNA was prepared by denaturation of the extracted virion DNA followed by renaturation, and analysed by electron microscopy, double stranded circles were present at a frequency of between 5% to 15%. A more intensive analysis of this phenomenom revealed that the DNA prepared in this manner sedimented heterogenously in a sucrose gradient with one major peak and two minor peaks (Gerry et al, 1973). The major peak (consisting of approximately 50% of the total DNA) contained linear duplex monomers while the two minor peaks contained varying amounts of circular duplex monomers or linear duplex dimers. These species all formed monomer length single strands after treatment with alkali. The linear duplex monomers isolated from neutral sucrose gradients could form circular duplex monomers or linear duplex dimers after denaturation and then renaturation, however, extensive renaturation of the duplex linear monomers, without prior denaturation, would not result in the formation of circular duplex monomers or linear duplex dimers. Thus, there appears to be two different subsets of molecules, which are interchangeable, possessing or not possessing cohesive termini, i.e. a limited sequence permutation (Gerry et al, 1973). The linear duplex monomers could be made to form circles by treatment with exonuclease III (1% digestion), followed by re-annealing, thus demonstrating the existence of a natural terminal repetition. As illustrated in Fig. 2 the implication of possessing both a natural and inverted terminal repetition is that upon denaturation to produce single strands, the single strands can themselves form hairpin structures at the termini by intramolecular re-annealing (Gerry et al, 1973). The existence of such a structure was demonstrated by end-labelling the DNA, then,

Fig. 2

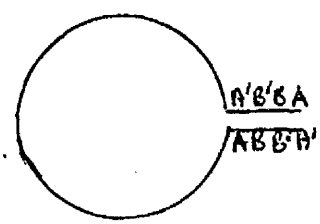
Sequence organisation at the termini of duplex AAV DNA.

Duplex AAV DNA digested with exonuclease 111 is able to re-anneal to form circular structures. This is indicative of a natural terminal repetition. If duplex AAV DNA is denatured, then re-annealed, circular single stranded DNA molecules are formed. This is indicative of an inverted terminal repetition. However, if AAV DNA is denatured and quench cooled then hairpin structures are observed at the ends of the single stranded DNA molecules. A nucleotide sequence consistent with all these phenomena is illustrated ie ABB'A'. A and B represent nucleotide sequences and A" and B' represent, respectively, their complementary sequences.



and (a) quench ecol.

or (b) re-anneal.



after denaturation, digesting with a single strand specific endonuclease and isolating the resistant material. The resistant material consisted of two fragments (one major and one minor) when analysed by polyacrylamide gel electrophoresis, the major fragment being between 120-160 nucleotides in length (Fife et al, 1977).

The terminal sequences of AAV DNA are therefore ordered in a specific manner which may be of importance in the replication of the virus. Further studies of the organisation of the terminal sequences have involved the extensive use of bacterial restriction endonucleases and more recently DNA sequencing. Essentially the strategy employed when utilising restriction endonucleases has been to prepare a physical map of the AAV genome and then to analyse the terminal fragments in more detail. In the initial studies the DNA used was the linear duplex monomer material previously discussed, although in later studies the linear duplex DNA employed had the potential to form linear duplex circles or linear duplex dimers upon renaturation. A physical map of AAV DNA is illustrated in Fig. 3 and as can be seen Hind II + III makes four cleavages (Berns et al, 1975) and ECoRl makes two cleavages (Carter and Khoury, 1975; Berns et al, 1975). Essentially the same results were obtained when the dimeric and circular forms of the DNA were used for the digestion (Carter and Khoury, 1975). When analysing the terminal fragments from the Hind II + III digest on polyacrylamide gels it was observed that they could be resolved into two species which would be consistent with a limited permutation of two. A similar result was obtained with the restriction endonucleases Pst-1, BamHl and HaeIII (de la Maza and Carter, 1976; Carter et al, 1976). A more detailed analysis of these species revealed that the change in mobility was not due to

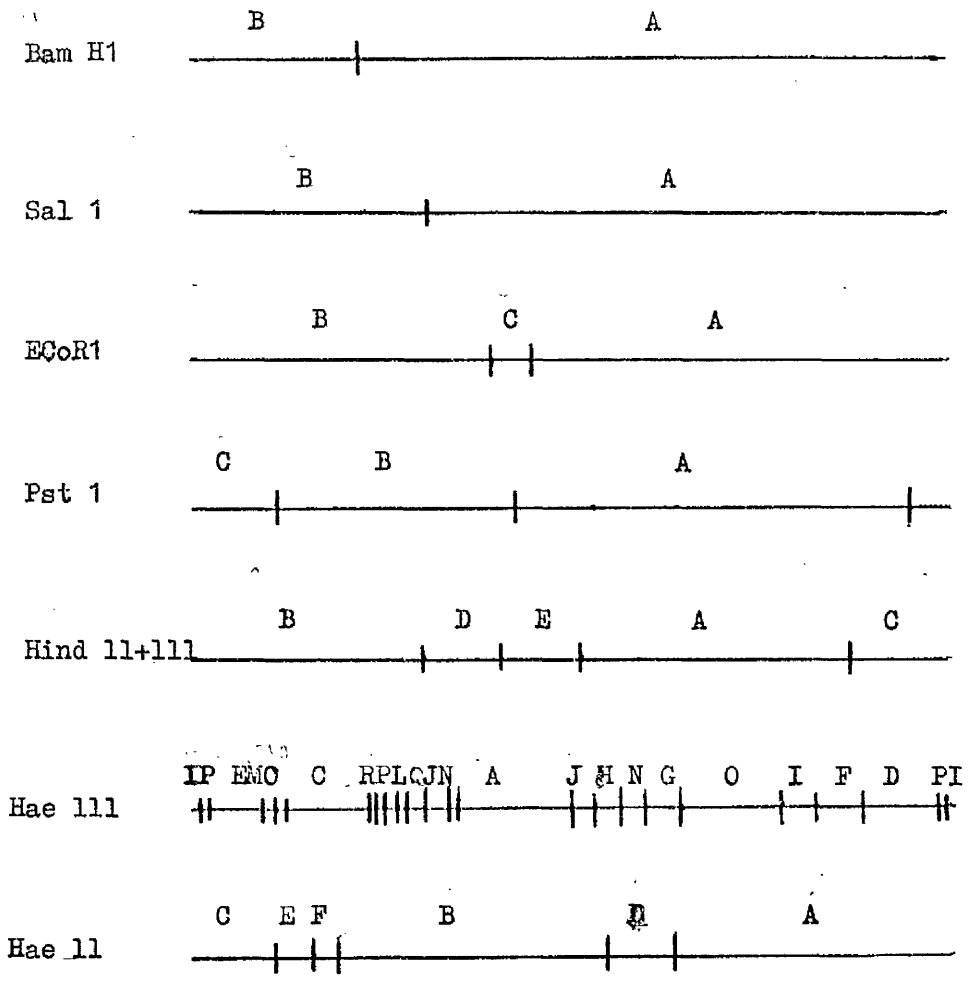


Fig. 3 Restriction endonuclease cleavage maps for AAV2 DNA. Adapted from Berns and Hauswirth (1978).

a molecular weight difference (as would be the case for a limited permutation of the nucleotide sequence) but was due to conformational effects (Denhardt et al, 1976; Spear et al, 1977; de la Maza and Carter, 1977). Denhardt et al (1976) using HaeIII analysis of duplex AAV DNA showed that there were three terminal fragments produced (the same fragments were produced from each end of the molecule). The authors also noted that the same fragments were produced by single stranded circular AAV DNA. One of the species isolated had an aberrant secondary structure.

A possible explanation for all the observed terminal fragments involved a change in the sequence orientation (not sequence permutation) at the termini (Spear et al, 1977). This was postulated on the basis of the HpaII restriction endonuclease analysis of end labelled Bam Hl terminal regions. As has been stated previously Bam Hl B consists of two species, B_1 and B_2. Spear et al (1977) showed that B_1 and B_2 were interconvertible upon denaturation followed by renaturation suggesting that the difference was due to a conformational change. Upon digestion of terminally labelled AAV DNA with HpaII 6 bands were observed (of which one was a doublet), labelled ter 1 to ter 6. Ter 1 originates from the right end and ter 2 from the left end and therefore these fragments extend beyond the terminal repetition. It was further suggested that all the terminal fragments except ter 6a and ter 6b (the doublet band previously mentioned) had an aberrant secondary structure. A possible resolution of the nature of the ends of AAV DNA has come from the results of direct nucleotide sequence analysis (Berns et al, 1978). The results of the sequence analysis is illustrated in Fig. 4. The terminal repetition is 145 nucleotides in length and within the

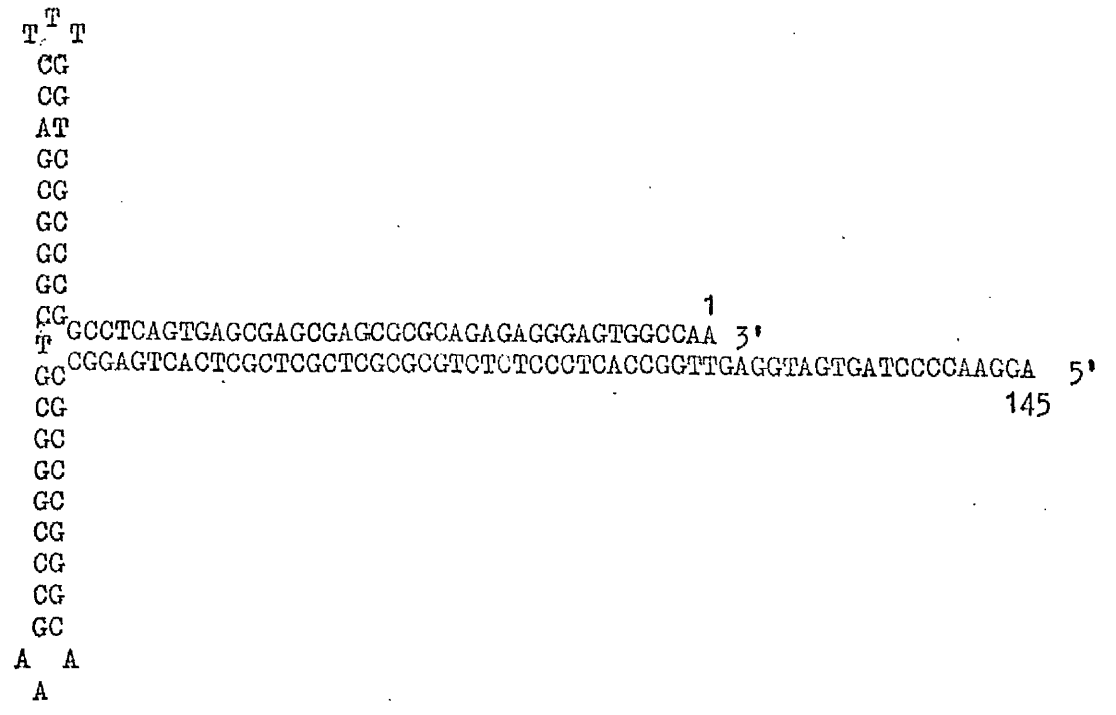
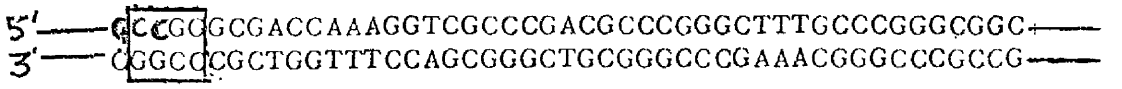


Fig. 4. The nucleotide sequence of the 3'end of AAV DNA. The terminal repetition of 145 nucleotides of AAV DNA is represented in its most highly base paired configuration. Adapted from Berns et al.(1978).

terminal 125 nucleotides there is a considerable degree of self complementarity: (1) Nucleotides 1-41 can base pair with nucleotides 125-85, (2) nucleotides 42-50 can base pair with nucleotides 62-54 and (3) nucleotides 64-72 can base pair with nucleotides 84-76. Furthermore, there are two possible nucleotide sequence orientations, i.e. the first 125 nucleotides can be arranged either as 1 - 125 or 125 - 1. Since nucleotides 1 - 43 are identical to nucleotides 125 - 83, it is only nucleotides 44 to 82 which are arranged in a different order. Interestingly, all the potential HpaII cleavage sites are within this region. By hybridising the complementary strands of AAV DNA it can be seen that the molecules formed can be either perfect duplexes at the ends or slightly mismatched duplexes depending on the sequence orientation of the hybridising molecules. For the perfectly matched molecules there will be two HpaII terminal fragments of differing length which should correspond to ter 6a and ter 6b (Fig. 5). If a terminally mismatched duplex is formed there will be little hybridisation between the HpaII region of each molecule, therefore, no cleavage sites within the terminal repetition and hence produce fragments ter 1 and ter 2. The molecules (i.e. duplexes) could hybridise with each other at the terminal regions to produce oligomeric and/or circular duplex molecules. However, a third possibility does arise and this is that intramolecular re-annealing by the individual strands within the mismatched duplex as illustrated in Fig. 6 and 7 could occur. These figures show the possible conformations and also the resulting HpaII terminal fragments. The fragments are all of total length 144 bases but with different lengths of bases in each strand, different conformations and different amounts of single stranded regions. One can see that the 6 possible fragments fall

Orientation 1.



Orientation 2.

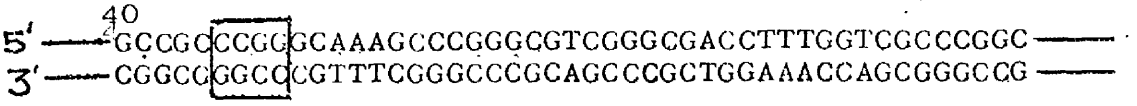


Fig. 5. A representation of the two sequence orientations of AAV DNA. Perfectly matched duplex AAV DNA prepared by hybridisation of virion AAV DNA has two possible sequence orientations at the ends of the molecules. Only nucleotides 44 to 82 are arranged in a different order between the two sets of molecules. Nucleotides 40 to 86, numbered from the ends of the molecules, are illustrated. The box indicates the recognition sequence of the restriction endonuclease Hpa 11.

Fig. 6

A representation of the three possible structures at the ends of duplex AAV DNA formed due to the hybridisation of terminally mismatched virion AAV DNA. Only nucleotides 40 to 86 are represented. Nucleotide 40, numbered from the end of the molecule, is at the left end of each diagram. This diagram represents the hybridisation of the 5' end of orientation 1 with the 3' end of orientation 2, as depicted in Fig. 5.

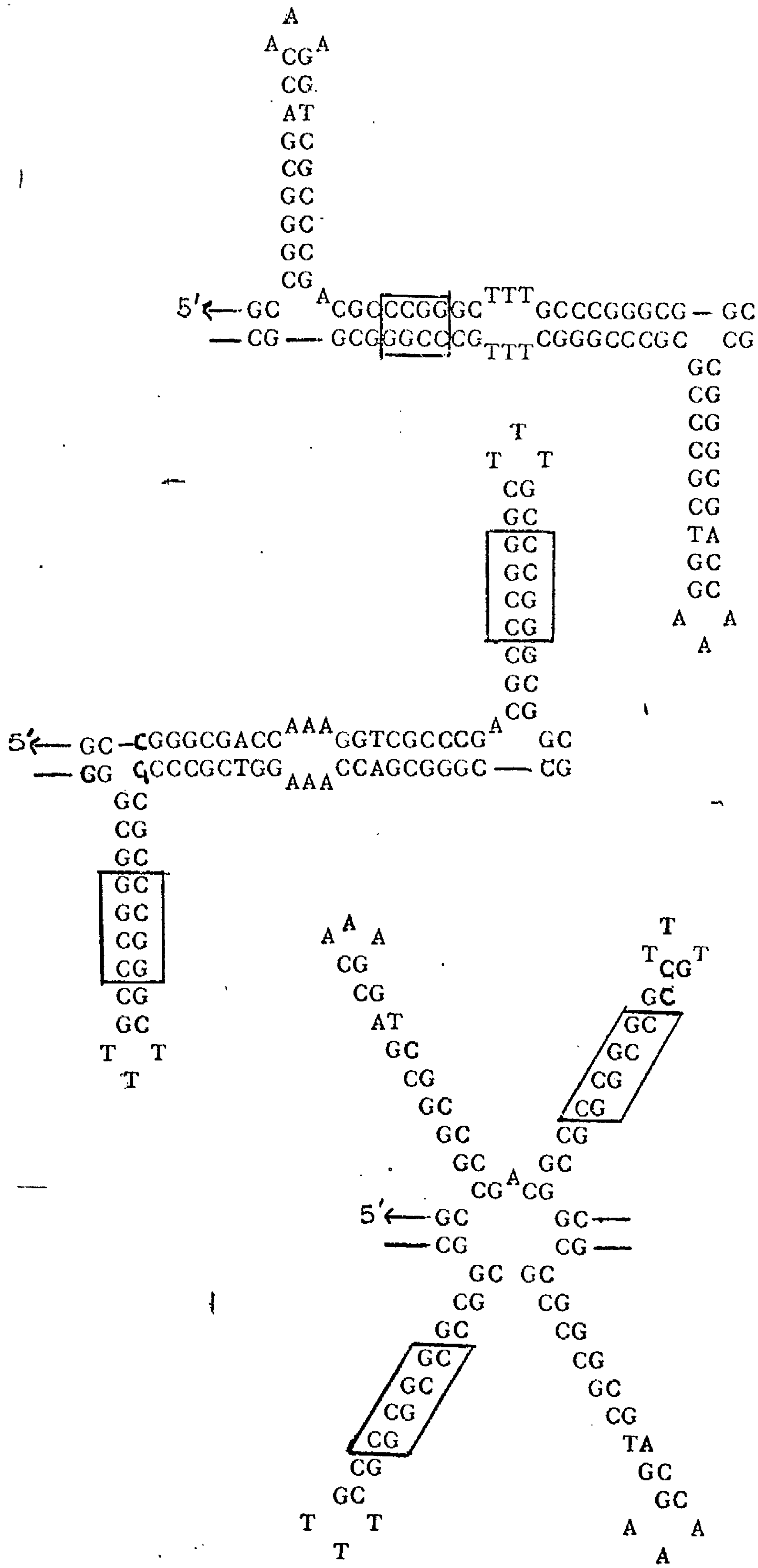
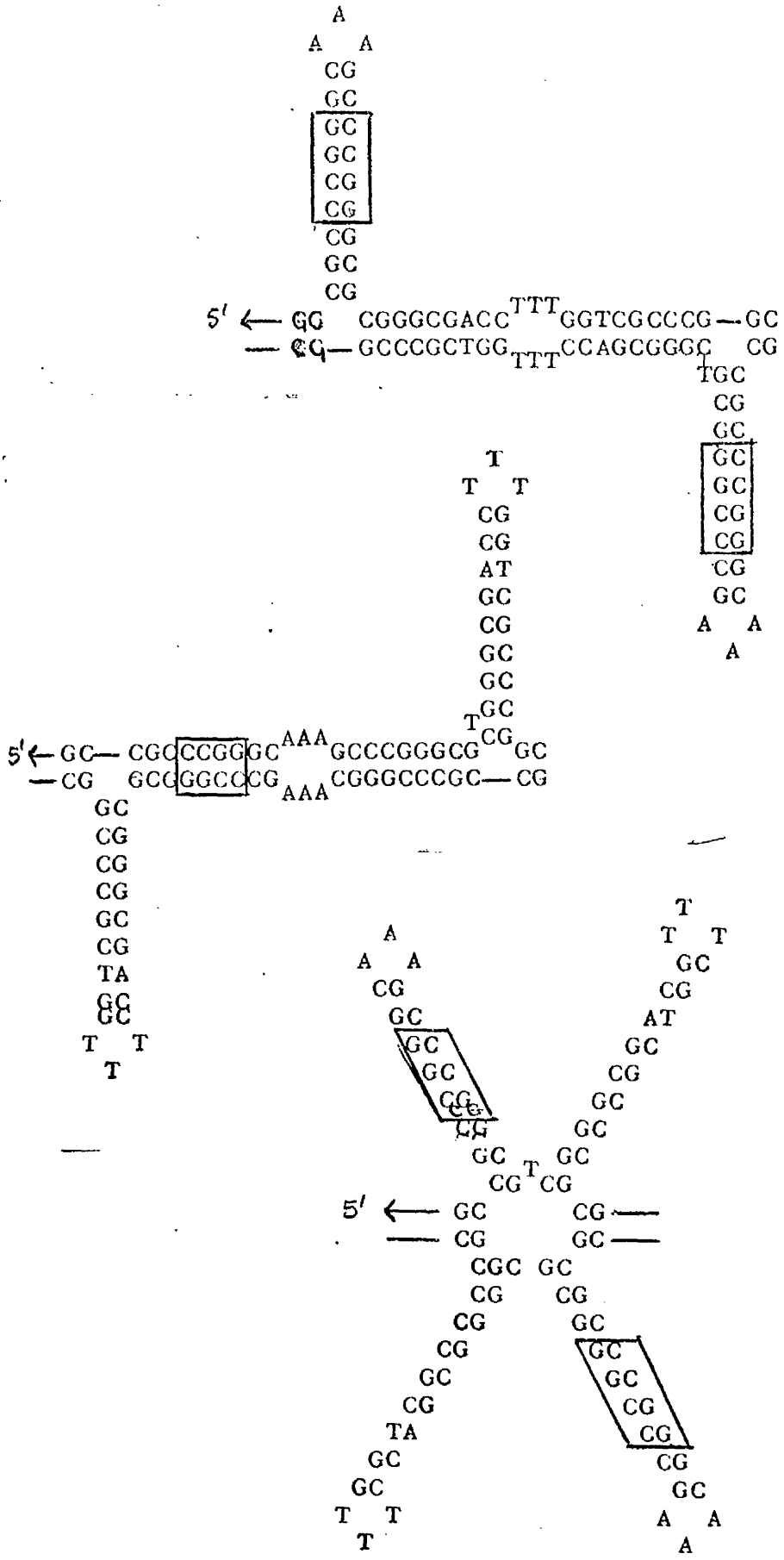

5'← GC ACGGCCGCGC TTT GCCCGGGCG — GC
— CG — GCGGGCCCG TTT CGGGCCCGC CG
5'← GC —CGGGCGACC AAA GGTCGCCCG A GC
— GG GCCCGCTGG AAA CCAGCGGGC — CG
5'← GC
— CG

Fig. 7

A representation of the three possible structures at the ends of duplex AAV DNA formed due to the hybridisation of terminally mismatched virion AAV DNA. Nucleotides are arranged as indicated in the legend to Fig. 6. This diagram represents the hybridisation of the 5' end of orientation 2 with the 3' end of orientation 1, as depicted in Fig. 5.


5′ ← GC CGGGCGACC TTT GGTCGCCCG — GC
— CG — GCCCGCTGG TTT CCAGCGGGC CG
5′ ← GC — CGC CCGG GC AAA GCCCGGGCG CG GC
— CG GCG GGCC CG AAA CGGGCCCGC — CG
5′ ← GC CG
— CG GC
CGC GC
CG CG
CG GC
GC
CG
GC
TA
GC
GC
T T
T

into three classes and thus these structures could be the terminal fragments ter 3 to ter 5. Each class would give rise to two bands on denaturing polyacrylamide gels if the 5' ends had been labelled, and one of the bands would have the same length, i.e. 45 bases, as one of the HpaII terminal fragments from the perfect duplexes. This situation has been observed (Spear et al, 1977).

In conclusion, it can be said that the structure of the terminal regions of AAV DNA is rather unusual and that any mechanism to explain the replication of the virus must explain the occurrence of certain of these structures, notably the two sequence orientations.

1.2.7 Proteins

As outlined in section 1.2.4, the buoyant density profile of Parvoviruses in caesium chloride is quite complex, in that there are up to a possible 4 bands at individual densities of 1.30 - 1.32 g/cm^3, 1.35 - 1.37 g/cm^3, 1.39 - 1.42 g/cm^3 and 1.45 - 1.47 g/cm^3. The major infectious bands are the two most dense bands. The polypeptide content of these virion particles has been analysed by SDS polyacrylamide gel electrophoresis of either labelled or unlabelled samples (the latter being detected by coomassie blue staining of the protein). Table 5 illustrates the results of this analysis on a variety of Parvovirus strains. To enable some degree of comparison between the viruses a nomenclature has been chosen such that the proteins are lettered (A, B, etc) in order of decreasing molecular weight.

There are some general points which one can deduce from the Table. Firstly, there are three or possibly four proteins associated with all groups of Parvoviruses. The authors who have only observed two bands on their gels could have done so because of the close proximity of bands B and C and due to the technique of detection,

Table 5 Parvovirus Structural Polypeptides

Virus	Particle type[a]	Particle density[b]	Structural polypeptides			
			A	B	C	D
KRV	LF	1.40-1.41	72[c](13)[d]	62(76)		55(11)
H-1	F	ND	92 (15)	72(75)		56(10)
H-1	F	ND	92	72	69	56
H-1	F	ND	92(20)	72(80)		
BPV	LF	1.40-1.42	86(10)	77(8)	67(83)	
	E	1.30-1.31	86(10)	77(8)	67(83)	
LuIII	LF	1.41	74(16)	62(85)		
	I	1.35	74(14)	61(82)		
	E	1.32	76(9)	63(87)		
MVM	HF	1.46	92(12)	72(86)	69(2)	
	LF	1.41	92(12)	72(27)	69(61)	
	E	1.32	92(14)	72(79)	69(7)	
MVM	LH-HF	1.42-1.47	83(14)	64(15-76)	61(7-70)	
	E	1.32	84(17)	64(83)		
X-14	LF	1.40-1.43	82(14)	68(86)		
AAV1	LF	1.38-1.42	87(8)	73(5)	62(86)	
AAV2	LF	1.38-1.42	87(8)	73(5)	62(86)	
AAV3	LF	1.38-1.42	87(8)	73(5)	62(86)	
AAV3	LF	1.40-1.41	92(11)	78(11)	66(79)	
	HF	1.47	93(7)	80(7)	69(85)	
AAV3	E	1.34	92(10)	80(10)	66(80)	
AAV1	LF	1.38-1.40	84(10)	70(12)	56(78)	
AAV4	LF	1.43	83(7)	71(10)	58(83)	

ND Not determined.

a LF, "light" full; HF, "heavy" full; I, intermediate; E, empty; F, full particle of unspecified density.

b Buoyant density in CsCl (g/cm^3).

c Molecular Weight x 10^{-3}.

d Figures in parenthesis are weight percents of total virion protein.

Adapted from Tattersall (1978).

e.g. slicing and counting of the gel or the sensitivity of the gel system employed (e.g. compare Kongsvik et al, 1974a and Kongsvik and Toolan, 1972), have not resolved the species into two separate bands. In the autonomous Parvovirus group there is occasionally a protein of approximately 55,000 daltons observed, which appears to be dependent on the cell line in which the virus is propagated and therefore may possibly be of cellular origin (Kongsvik et al, 1974b; Tattersall et al, 1976). All Parvoviruses, whether autonomous or defective, possess a protein in the molecular weight range around 85,000 daltons which is present at a constant level, approximately 15%. There is a distinction, however, between the autonomous and the defective virus groups and that is the presence of a doublet protein band at around 70,000 daltons. As has been discussed previously (section 1.2.4), there appears to be a correlation between the amount of protein B and protein C in the virion and the buoyant density of the full virions, such that protein B is thought to be proteolytically cleaved to yield protein C, on processing of the mature virion from 1.46 g/cm^3 to 1.42 g/cm^3 (Clinton and Hayashi, 1975, 1976; Tattersall et al, 1976). However, protein B is present only at a similar level to protein A even in heavy full particles of AAV3 (Johnson et al, 1971) and the major protein component is protein C. Also AAV3 has an identical protein composition of empty and full virions (Johnson et al, 1978).

In view of the presumptive relationship between polypeptides B and C of the autonomous group and the fact that the combined molecular weights of the 3 structural polypeptides exceeds the coding capacity of the genome for both autonomous and defective viruses various studies were undertaken to determine the sequence homology, if any, of the

three polypeptides. In a preliminary study, Johnson et al (1972), demonstrated for AAV3 an immunological relationship between proteins A and B. A more intensive study revealed considerable and specific, as shown by immunocompetitive studies, immunological cross reactivity between the three polypeptides. There were also unique regions of antigenic nonhomology (Johnson et al, 1977). For MVM, peptide maps of iodinated SDS polyacrylamide gel electrophoresis purified proteins have revealed several interesting results (Tattersall et al, 1977). Firstly, the tryptic and chymotryptic maps of proteins B and C are almost identical, giving further evidence to the product precursor relationship, and, secondly, the entire sequence of protein B can be contained within protein A. A similar situation appears to exist for AAV. Using SDS PAGE purified virion polypeptides, Lubeck et al (1979) demonstrated that the amino acid content of polypeptides B and C could be contained within polypeptide A and that the amino acid content of polypeptide C could be contained within polypeptide B. Furthermore peptide mapping studies indicated a considerable homology between all three polypeptides.

This situation immediately raises the question as to whether protein A is a precursor, either in the virion or before assembly into the virion, i.e. as a free molecule, to protein B or whether a similar situation as exists between large T and small t antigens in SV40 occurs, i.e. the difference is not at the level of protein cleavage but at the level of RNA splicing (Rigby, 1979). It appears that protein A has a distinct function in the virion, e.g. protein A is stable to digestion with proteolytic enzymes which cleaves protein B either in vivo (Clinton and Hayashi, 1975) or in vitro (Clinton and Hayashi, 1976; Tattersall et al, 1977). Also protein A is at a

constant level in all Parvovirus strains so far analysed (Table 5), even under chase conditions (Clinton and Hayashi, 1975; Kongsvik et al, 1974b). Therefore protein A can either be proteolytically cleaved to yield protein B which is then incorporated into the virion or they are synthesised from distinct species of mRNA.

Salzman and White (1970) reported that the major viral protein, protein B, was responsible for the haemagglutination activity of the virus. This was demonstrated by purifying the virion proteins by excision from SDS-polyacrylamide gels and testing for haemagglutination activity and haemagglutination inhibiting properties of the individual proteins. Johnson and Hoggan (1973) and Salo and Mayor (1977) for BPV and X14, respectively, could not demonstrate any haemagglutination activity from their proteins, purified by identical methods. Thus it appears, at least in some cases, that the haemagglutinating properties are associated with the assembled virion and not an individual protein.

1.3 The Infectious Cycle

The development of cell culture systems for the growth of Parvoviruses have made it possible to analyse the events of a single replication cycle, i.e. adsorption, penetration and uncoating, DNA synthesis, RNA transcription, protein synthesis and, finally, assembly into mature virions. It has also provided, in the case of AAV, a means to elucidate the nature of the helper function supplied by Adenovirus. The biology of the autonomous and AAV groups possess similarities and differences in terms of the events at each stage of the replication cycle and therefore a comparison will be made between the two groups.

1.3.1 Adsorption, Penetration and Uncoating

The initial events in the infectious cycle of all mammalian viruses are adsorption penetration and uncoating, however, little is known about this stage of the Parvoviral infectious cycle. It is known, however, that for MVM there are specific binding sites on the cell surface of susceptible cells and that cells which are not susceptible to MVM infection appear to lack these specific binding sites. Indeed, MVM resistant clones of mouse A9 cells (a normally susceptible host cell with, approximately, 5×10^5 specific MVM binding sites per cell) exhibited low levels of nonsaturable virus binding but lacked the MVM specific binding sites (Linser et al, 1977). These studies were performed at $4^{\circ}C$, but when the temperature is increased to $37^{\circ}C$ there is uptake of the virus into the cell. The process of uptake appears to be separated into two stages and is not cell cycle dependent. After uptake the particles are rapidly associated with the nucleus (Linser et al, 1979). Similar, though less detailed results of Siegl and Gautschi (1973a) for LuIII, suggested that the uptake of virus into the cell was also cell cycle independent. In the case of AAV the adsorption process was also rapid (75% complete within 30 min) and the particles became associated with the cell nucleus after uptake. These events occurred with equal efficiency whether an Adenovirus helper was present or not (Rose and Koczot, 1972). Therefore, AAV replication is not defective at this stage which agrees with the fact that AAV DNA (duplex molecules) was only infectious if an Adenovirus helper virus was also present (Hoggan et al, 1968). Thus, it appears likely that the initial events in the infectious cycle are similar for all Parvoviruses.

1.3.2 DNA synthesis

The initiation of Parvoviral DNA synthesis appears to involve a function which is not coded for by the Parvovirus genome. Autonomous Parvovirus replication is cell cycle dependent, in that viral DNA synthesis will not commence until the infected cell has reached late S phase or early G_2 phase of the cell cycle (Tennant et al, 1969; Hampton, 1970; Tennant and Hand, 1970; Tattersall, 1972; Siegl and Gautschi, 1973a; Rhode, 1973). Indeed, although one MVM particle is responsible for infection, the plaque size is dependent on the amount of cell growth (Tattersall, 1972). However, there also appears to be a genetic component of plaque size (Hoggan et al, 1978). There is a slight decrease in host DNA synthesis and an inhibition of mitosis during autonomous Parvoviral infections (Hampton, 1970; Tennant, 1971; Tattersall, 1972; Siegl and Gautschi, 1973a). AAV requires coinfection with either Adenovirus (Rose and Koczot, 1972) or Herpesvirus (Boucher et al, 1971; Rose and Koczot, 1972) in order to initiate AAV DNA replication. The helper virus must commence its DNA replication before AAV DNA synthesis can begin (Boucher et al, 1969) although temperature sensitive, DNA synthesis negative, Adenovirus mutants will still support AAV DNA replication at the non-permissive temperature (Handa et al, 1976; Straus et al, 1976a). There does not require to be any species relationship between the AAV and the Adenovirus helper (Casto et al, 1967) but AAV DNA replication reduces the yield of Adenovirus in the coinfected cultures (Hoggan et al, 1966; Casto et al, 1967). The infectious unit of AAV appears to be a single particle as was suggested by titration studies of AAV infectivity in the presence of excess Adenovirus (Blacklow et al, 1967).

The early events in the study of the DNA replication of viruses can be analysed by the use of radioactively labelled virions (labelled specifically in the DNA component). However, in the case of the Parvovirus group the study of the fate of the parental viral strand has been hampered by the high particle:infectivity ratios, in the order of 500:1 (Tattersall, 1972; Linser et al, 1979), therefore any results obtained from this method have to be punctuated by the knowledge that the radioactively labelled DNA of the virus which is being monitored, may not be infectious. Salzman and White (1973) reported, for KRV, that 28% - 42% of the input labelled viral strand was converted into a double stranded form, of molecular weight twice that of the input viral strand, within the first hour of infection, and maintained at this level throughout the course of the infection. Ward and Dadachanji (1978) observed slightly different results with MVM, in that the maximum amount of the double stranded form of undetermined molecular weight, was 10% of the input and that the time of conversion was from 4 to 12 hpi and mimicked the time course of viral DNA replication. A further observation was that the double stranded form isolated from 6 to 8 hpi hybridised, after denaturation, with zero order kinetics, indicating a hairpin like structure, i.e. a covalent link between the viral and the complementary strand (Ward and Dadachanji, 1978). The formation of an integrated form of MVM DNA with the host DNA does not appear to occur as either an early or late event in the replication cycle of MVM (Richards and Armentrout, 1979).

The existence of a duplex molecule of approximately double the molecular weight of the viral single stranded DNA has also been shown by radioactive labelling studies with precursors of DNA synthesis, usually thymidine, at the peak of viral DNA replication, around 14-16 hpi,

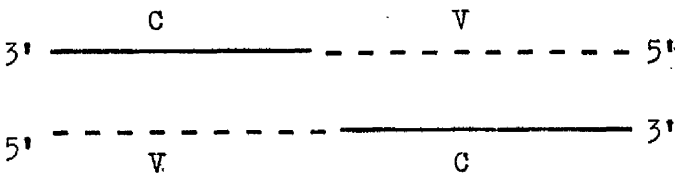
although the duplex is present at all times in the replication cycle, after detectable commencement of viral DNA replication. This duplex DNA of monomer length, i.e. the same length as viral DNA, will be referred to as monomer replicating form (monomer RF) DNA and has been isolated from MVM (Tattersall et al, 1973; Dobson and Helleiner, 1972), KRV (Gunther and May, 1976; Salzman and Fabisch, 1978; Lavelle and Li, 1977), LuIII (Siegl and Gautschi, 1976), H-1 (Rhode, 1974a,b) and AAV (Mayor et al, 1974; Handa et al, 1976; Straus et al, 1976b; Hauswirth and Berns, 1977, 1979). Monomer RF, which constitutes the major labelled species (Salzman and Fabisch, 1978), replicates semiconservatively (Rhode, 1974a) and consists of basically two DNA moieties, one being a normal extended duplex, i.e. non-hairpinned and the other being a duplex which has a covalent linkage between the viral and complementary strands, i.e. hairpinned. This has been demonstrated for KRV (Gunther and May, 1976; Hayward et al, 1978), MVM (Ward and Dadachanji, 1978), H-1 (Rhode, 1977a) and AAV (Straus et al, 1976b; Hauswirth and Berns, 1977). Reconstruction experiments have demonstrated that the method (Hirt, 1967) which is used to extract viral DNA from the infected cell does not result in renaturation of complementary single strands to form a duplex structure, thus monomer RF appears to be a true replicative intermediate (Straus et al, 1976b). The location of the hairpin structure, i.e. the covalent linkage between viral and complementary strands, in H-1 monomer RF has been achieved by the use of restriction endonucleases (Rhode, 1977a). Restriction endonuclease, ECoRI, cleaves H-1 monomer RF DNA once thereby producing two fragments, A and B, of genome fractional length 0.78 and 0.22 respectively. It was also noted that fragment B could be resolved into two species B_1 and B_2 (B_1 migrates

more slowly than B_2) which had an apparent molecular weight difference equivalent to approximately 60 base pairs. When the monomer RF DNA which had been digested with ECoRI was alkali denatured, then neutralised, and immediately assayed for double strand content by EDC-chromatography, the rapidly annealing species was found to be fragment B. The size of this fragment was identical to B_2 (Rhode, 1977a). This data suggests that B_1 has a normal duplex terminal region and that B_2 is the fragment which contains the covalent linkage between the V and C strands. The covalent linkage has been shown to occur at the arbitrarily designated left end which contains the 3' end of the viral strand (Rhode, 1977b). This was demonstrated by hybridising 5'-^{32}P end labelled viral single stranded DNA to denatured monomer RF DNA then digesting with restriction endonucleases and hence locating the radioactive fragment which contains the 5' end of the viral strand. An identical conclusion as to the location of the covalent linkage of the viral to complementary strand was shown for MVM (Ward and Dadachanji, 1978). In this study a small amount of covalent linkage between the viral and complementary strand at the right end of the monomer RF DNA was also detected. A more detailed physical map of the H-1 genome has revealed some abnormalities in structure at the right end of monomer RF DNA but whether it is due to a covalent linkage or some other feature of the terminal region is as yet unknown (Rhodes, 1977b). These studies on the H-1 monomer RF DNA replication have all utilised a temperature sensitive mutant, tsl H-1, which when incubated at the restrictive temperature is unable to synthesise progeny DNA (Rhode, 1976). This mutant thus allows the possibility to study monomer RF DNA replication without the added complication of concomitant progeny DNA synthesis, although there is the possible

disadvantage that the mutation may result in a different pathway of DNA replication. Electron microscopic studies of the replication of monomer RF DNA of tsl H-1 have revealed some interesting results. Firstly, the replicative intermediate appears to be a completely double stranded molecule with three branches, i.e. a Y-shaped molecule, similar to those observed during bacteriophage T7 DNA replication, except that no eye structures are observed (Singer and Rhode, 1977a). Secondly, by the use of denaturation mapping, it has been possible to locate the origin of monomer RF DNA replication at the arbitrary right end of the molecule, i.e. the end which contains the 5' end of the viral strand and that there is unidirectional replication (Singer and Rhode, 1977b). That this end may be the origin of DNA replication for progeny DNA synthesis as well as monomer RF DNA replication was implied from some preliminary labelling studies (Rhode, 1977a). The rationale behind these experiments is that if a molecule is labelledfor the time of one round of DNA replication and the mature DNA species isolated, then the region containing the highest concentration of radioactivity will be at the terminus of replication. Therefore, digestion of the mature DNA species (in this case monomer RF DNA) with restriction endonucleases and orientation of the fragments with respect to a physical map of the genome will enable the origin and terminus of DNA replication to be determined. Similar, though more detailed experiments of this nature were performed on AAV (Hauswirth and Berns, 1977). In this case two origins for DNA replication were identified. One origin is at the 5' end of the plus strand and the other is at the 5' end of the minus strand as was determined by measuring the radioactivity present in the separated strands of the restriction fragments of

monomer RF DNA. The origin does not appear to be exactly at the terminus, but is between 35 to 65 nucleotides inside the ends of the mature DNA molecules. The finding of two origins for AAV DNA replication is in contrast to that for H-1 DNA replication but perhaps reflects the symmetry of the replication products, i.e. complementary strands in separate virions, of AAV DNA replication compared to the asymmetry of H-1 DNA replication products.

Apart from duplex monomer RF DNA in the infected cells there are also concatemeric forms of Parvoviral DNA, i.e. duplex DNA's which are integer multiples, in terms of molecular weight, of duplex monomer RF DNA. These species have been identified for KRV (Gunther and May, 1976; Salzman and Fabisch, 1978; Hayward et al, 1978), MVM (Ward and Dadachanji, 1978), H-1 (Rhode, 1977a), LuIII (Siegl and Gautschi, 1976) and AAV (Straus et al, 1976b; Hauswirth and Berns, 1979). These concatemers which have been identified up to tetrameric duplex RF's are tail to tail arrangements of monomeric RF's (Gunther and May, 1976; Straus et al, 1976b; Rhode, 1977a; Ward and Dadachanji, 1978). They are present in much smaller amounts than the duplex monomer RF's and, indeed, only for MVM has it been demonstrated that forms greater in size than the dimer RF's exist (Ward and Dadachanji, 1978). The structure of the dimeric RF has been partially elucidated for KRV (Gunther and May, 1976), AAV (Straus et al, 1976b; Hauswirth and Berns, 1979), H-1 (Rhode, 1977a) and MVM (Ward and Dadachanji, 1978). Gunther and May (1976) demonstrated that the dimeric RF consists of a normal duplex structure with, after alkaline denaturation, dimeric length single strands consisting of a covalent linkage between the V and C strand, i.e.



In approximately 50% of the dimeric RF's the duplex dimer was formed by monomer length single stranded DNA suggesting a nick close to the V-C junction on one or both of the strands of the duplex. Similar results have been obtained with AAV (Straus et al, 1976b; Hauswirth and Berns, 1979), H-1 (Rhode, 1977a) and MVM (Ward and Dadachanji, 1978), although there are differences concerning the amount of dimer length single strands in the duplex dimer RF DNA. That the linkage between the V and C strands was internal, in H-1, was demonstrated by digesting the dimer RF DNA with restriction endonuclease, ECoRI, then analysing for rapidly re-annealing species by BDC chromatography as previously outlined. If the linkage was internal a fragment twice the length of fragment B would be obtained, but if the linkage was at the termini then a fragment twice the length of fragment A would be obtained since the dimer had been shown to consist of a tail to tail linkage of monomer RF DNA. A fragment twice the length of fragment B was obtained (Rhode, 1977a). Identical results on the structure of MVM dimer RF DNA have also been obtained (Ward and Dadachanji, 1978).

Kinetic studies involving pulse-chase techniques (usually pulse-labelling with ^{3}H thymidine for short periods of time followed by chasing with an excess of unlabelled thymidine to study the fate of the labelled species) have not convincingly revealed any precursor-product relationship between dimer RF and monomer RF with either wt H-1 (Rhode, 1977a) or MVM (Ward and Dadachanji, 1978), although with MVM both dimer and monomer RF decrease as the amount of single stranded

DNA increases. In AAV, dimer RF DNA may be a precursor to monomer RF DNA (Straus et al, 1976b). Also in AAV dimer RF DNA the amount of dimer length single strands decreases as the chase time increases (Hauswirth and Berns, 1979) which is also consistent with a precursor-product relationship between dimer length single strands and monomer length single strands in the total population of both monomer RF and dimer RF (Straus et al, 1976b). A possible explanation for the concatemers is that concatemeric forms of monomer RF DNA are formed by random re-annealing of monomer RF DNA during extraction. BUdR labelling experiments do not rule out this possibility, but the results are also consistent with different replication times for monomer RF and dimer RF in H-1 (Rhode, 1977a). The existence of internal, covalent, linkages between viral and complementary strands in duplex dimer RF argues against random association during extraction unless ligation is occurring in the presence of 0.6% SDS (Rhode, 1977a; Ward and Dadachanji, 1978).

A temperature sensitive mutant of H-1, ts14H-1, was temperature sensitive with regard to plaque formation and the production of progeny virus, and is defective in RF DNA replication at both the permissive and non-permissive temperatures (Rhode, 1978a). This mutation appears to be in a regulatory sequence for a number of reasons. Firstly, ts14H-1 cannot be rescued by wild type H-1, i.e. cis dominant (Rhode, 1978a). Secondly, ts14H-1 inhibits the RF DNA synthesis of H-1, and H-3, in the presence of a predominance of ts14 protein and higher proportions of ts14 DNA, i.e. the mutation is trans acting. Thirdly, ts14H-1 can rescue RF DNA replication defective, defective interfering (DI) virions, suggesting that the protein required for RF DNA replication is functional in ts14H-1 mutants (Rhode,

1978b). Another interesting feature of ts14 H-1 is that there is an unexpectedly high amount of progeny DNA replication (28% of wild type levels), considering the extremely low levels of RF DNA replication (4% of wild type levels) (Rhode, 1978a). Therefore it appears that not only is there a viral specified protein required for progeny DNA synthesis in H-1 (Rhode, 1976) but that there is also a viral specified protein required for RF DNA replication and that this protein recognises a specific sequence in the RF DNA (Rhode, 1978a,b). This is in contrast to the situation with AAV DNA replication. Cycloheximide does not affect AAV DNA synthesis even if the drug is present from 0 hpi, relative to AAV infection, in cells which have been pre-infected by Adenovirus 10 hours before AAV coinfection (Handa et al, 1976). This suggests that an AAV function is either not required for DNA synthesis or that the AAV function is only required in catalytic amounts. In H-1, cycloheximide can prevent RF DNA replication if the drug is added at 5-6 hpi but not if it is added at 12 hpi suggesting that a protein of either viral or cellular origin is required for the initiation of RF DNA replication (Rhode, 1974b). In AAV this function may be coded for by the Adenovirus helper.

The mechanism of production of progeny single stranded viral DNA for the autonomous Parvoviruses must be an extremely asymmetric process since only about 1 in 400 of the total single stranded DNA population are complementary strands (Singer and Rhode, 1977a; Green et al, 1979).

Numerous studies have indicated that a replicative intermediate of progeny DNA synthesis is a duplex DNA molecule which is partially single stranded. Tattersall et al (1973) with MVM

demonstrated that a short pulse (2 min) with ^{3}H thymidine, late in infection, labelled almost exclusively a molecule which was double stranded as tested by chromatography on hydroxyapatite but contained single stranded regions as detected by BND chromatography. Unfortunately no information was given as to the size of these molecules. Lavelle and Li (1977) with KRV noted that after a 10 minute pulse with ^{3}H thymidine the label was incorporated into monomer RF and also into molecules sedimenting slightly faster. After various chase times with an excess of unlabelled thymidine the DNA was chased into single stranded progeny DNA. Those molecules sedimenting slightly faster than monomer RF had a greater proportion of single stranded regions and also the kinetic properties of a replicative intermediate. Monomer RF DNA when labelled with ^{3}H BUdR late in infection preferentially incorporates the precursor into the viral strand of the monomer RF DNA (Rhode, 1974a,b). In agreement with this finding is some preliminary electron microscopic evidence that duplex monomer RF DNA in wild type H-1 may contain single stranded branches on the duplex structure (Rhode, 1978c). Another preliminary electron microscopic study indicates that the concatemeric forms of LuIII DNA contain single stranded branches which could indicate that progeny DNA synthesis was occurring on the large forms of DNA in the infected cell (Siegl and Gautschi, 1976). As mentioned previously, Ward and Dadachanji (1978) for MVM have demonstrated that monomer and dimer RF DNA are precursors to progeny single stranded DNA. The intermediates in this study were not characterised. In AAV DNA replication the monomer duplex RF appears to be the precursor to the progeny single stranded DNA molecule (Straus et al, 1976b). In summary, it can be said that the events leading to the production of single stranded progeny DNA

are unclear and that the template for the reaction is unknown, though both monomer RF DNA and duplex concatemeric DNA's have been implicated. However, at no time during Parvovirus DNA replication has a circular DNA molecule been implicated as a replicative intermediate.

These previously described experiments have all been concerned with the in vivo DNA replication of Parvoviruses, however, some preliminary experiments have been attempted in vitro. Handa and Shimojo (1977a) developed an isolated nuclei system which was capable of synthesising AAV DNA. When the AAV DNA was extracted by the SDS, high salt procedure (Hirt, 1967) without digestion with proteolytic enzymes, the unit length DNA molecules (monomer RF) were linked by a protein to form faster sedimenting species. Further investigation of the DNA protein complex revealed that by utilising less severe extraction procedures, i.e. lower salt concentration and non-ionic detergents, it was possible, under appropriate conditions, for the DNA-protein complex to continue DNA replication in vitro (Handa and Shimojo, 1977b). The AAV DNA-protein complex could be extracted from both Adenovirus and Herpesvirus coinfected cells although there were differences between the DNA-protein complexes extracted from the different coinfected cells (Handa and Carter, 1979). In the HSV-1 coinfected cells the AAV DNA-protein complex had a greater sedimentation coefficient and contained the HSV-1 induced DNA polymerase with lesser amounts of cellular DNA polymerases α and γ, whereas in the Adenovirus 5 coinfected cells the major DNA polymerase activity associated with the AAV DNA-protein complex was the cellular DNA polymerase γ. However, none of these in vitro DNA synthesising systems made unit length AAV DNA when analysed by alkaline sucrose

gradient centrifugation, thus, an essential factor for AAV DNA replication is not present. Those systems do stress, however, the possible importance of DNA-protein interactions in Parvoviral DNA replication.

1.3.3 RNA synthesis

The RNA synthesis of Parvovirus genomes has to be separated into two sections, since the techniques used to deduce the structure of the autonomous Parvovirus transcription pattern and those used to deduce the structure of the AAV transcription pattern are radically different.

Some background information is necessary to explain some of the terms used in the subsequent chapter. A recent discovery has shown that many genes have their coding sequences interrupted by one or more non-coding sequences. These split genes have been shown to exist for many eukaryotic genes, e.g. β-globin (Tilghman *et al*, 1978) and also for the genes of eukaryotic viruses, e.g. Adenovirus (Berget *et al*, 1977; Chow *et al*, 1977). The primary transcript includes the non-coding regions which are then removed and the two coding regions ligated. This process is known as "splicing" and has been shown to occur for both eukaryotic genes (Tilghman *et al*, 1978) and eukaryotic viruses (Nevins and Darnell, 1978).

1.3.3.1 Autonomous Parvovirus RNA synthesis

Very little is known about RNA synthesis in cells infected with autonomous Parvoviruses. In KRV infected cells, viral RNA synthesis commences at between 2-4 hpi and reaches a plateau at between 12-24 hpi, concomitant with a progressive inhibition of host

cell RNA synthesis (Salzman and Redler, 1974). The viral RNA has a base composition similar to that predicted for the complementary strand and had a broad distribution in denaturing sucrose gradients with a peak at 18S. The fact that RNA synthesis occurred before RF DNA replication suggested that RNA synthesis may be required for RF DNA replication. In H-1 infected cells, α-amanatin and actinomycin D, as well as the inhibitor of protein synthesis, cycloheximide, inhibited RF DNA replication when the drugs were added at 5 hpi to 6 hpi but did not cause an inhibition when they were added at 12 hpi suggesting that a viral induced function may be required for RF DNA replication (Rhode, 1974b). More extensive studies of viral transcription in H-1 infected cells have used more refined techniques than those described previously (Green et al, 1979). This group also observed that greater than 80% of the H-1 genome is transcribed and that the transcription is mainly, if not exclusively, from the viral strand. The RNA had a broad sedimentation profile with a peak around 19-20S. Using the S1 nuclease mapping techniques (Berk and Sharp, 1977, 1978), Green et al (1979) demonstrated that there were two major cytoplasmic RNA's of size 3000 and 2800 bases (the lower molecular weight RNA being present in greater abundance). There were also a collection of smaller cytoplasmic RNA's, the most reproducible being at 1450 and 1300 bases in length. The presumptive precursor to these molecules was a 4700 base, nuclear RNA which like all the previously mentioned RNA molecules was polyadenylated. The 4700 base RNA was transcribed from a non-contiguous DNA template of 2600 bases and 2200 bases. The 2800 and 3000 base cytoplasmic RNA's were also non-contiguous with a major segment of an identical size to the 2600 base fragment, mentioned previously (Green et al, 1979). The occurrence of spliced

cytoplasmic RNA's in Parvovirus infected cells has also been indicated from studies involving MVM (Tal et al, 1979). The comparison of poly A containing nuclear and cytoplasmic RNA by electron microscopy after hybridisation with viral DNA has revealed that the nuclear RNA is transcribed from a contiguous region (although small discontinuities may not be resolved) covering almost the entire length of the genome whereas the cytoplasmic RNA is composed of two non-contiguous regions, a short leader (8.8 ± 1.2% of genome length) and a long body (61.8 ± 2.3%), separated by a presumptive spliced out region, presumably caused by processing of the nuclear RNA, of 29.3 ± 2.4% (Tal et al, 1979). The long body of the mRNA would be approximately 2800 bases in length if one assumes a length of 4400 bases for the viral genome (Bourguignon et al, 1976).

1.3.3.2 AAV RNA synthesis

The availability of double stranded AAV DNA with, therefore, the ability to produce physical maps of the genome by restriction endonuclease digestion, has allowed the construction of transcription maps.

RNA transcription is solely from one strand, designated the minus strand or heavy strand, with regard to buoyant density in caesium chloride, with approximately 75% of this strand being transcribed into stable RNA (Carter and Rose, 1972; Carter et al, 1972; Carter et al, 1976). The RNA synthesised, is identical, as determined by competitive hybridisation, whether Adenovirus 2 or Herpesvirus 1 is used as a helper virus (Carter and Rose, 1972) and is transcribed by RNA polymerase II (Bloom and Rose, 1978). The size of the stable RNA is approximately 20S when measured in either denaturing sucrose

gradients or denaturing polyacrylamide gels (Carter and Rose, 1972, 1974). This would correspond to a molecule of between 0.85×10^6 to 0.9×10^6 daltons.

More refined analysis, using restriction endonuclease fragments, has revealed the transcription unit illustrated in Fig. 8 (Carter et al, 1975, 1976). The basic technique was to purify radioactively labelled restriction endonuclease fragments, denature, then re-anneal in the presence of excess unlabelled RNA under conditions in which there is little, if any, DNA-DNA hybridisation yet there is still RNA-DNA hybridisation and then measure the percentage double stranded either by S1 nuclease or hydroxyapatite chromatography. The percentage double stranded, under these conditions, will be directly related to the percentage of transcription from that region. The RNA used in these studies was poly A containing whole cell RNA. However, AAV RNA does not all appear to be polyadenylated. The poly A containing RNA in both the nucleus and cytoplasm comprised, predominantly, 20S molecules, whereas the poly A lacking AAV RNA contained mainly, 4S to 18S heterogeneous species (Carter, 1976). These fractions both contained sequences complementary to 70-75% of the minus DNA strand. The heterogeneous small molecules (4S to 18S which lack poly A) are metabolically less stable and are non-polysomal (Carter and Rose, 1974). The 20S AAV RNA is present in both nucleus and cytoplasmic polysomes.

Short radioactive pulses with ^3H uridine did not show the existence of a larger precursor molecule, in the nucleus, to the 20S AAV poly A containing cytoplasmic RNA (Carter and Rose, 1974). Recent evidence, however, using restriction fragments have demonstrated that a slightly larger, poly A containing, precursor molecule may be present

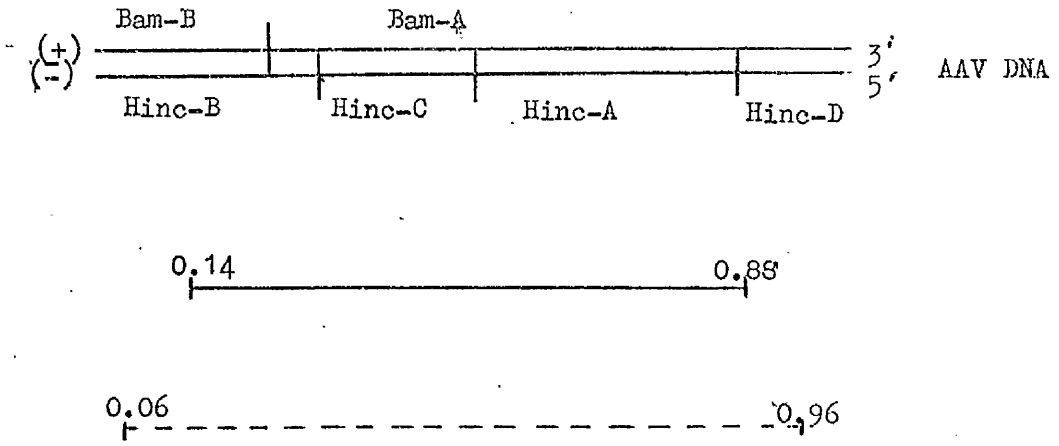


Fig. 8. AAV transcription map. The indicated restriction endonuclease fragments have been mapped (Berns et al.,1975; Carter et al.,1976). The region of the AAV genome contained in stable poly(A+) RNA is represented by the solid line. The presumptive map of the precursur RNA molecule to the stable AAV RNA is indicated by the broken line. Transcription is only from the minus strand. Adapted from Jay et al.(1978).

in the nucleus (Jay et al, 1979). No evidence has been presented to show the existence of non-contiguous mRNA molecules because the techniques used to elucidate the structure of the transcribed regions are not sensitive enough to detect this phenomenon.

1.3.4 Protein Synthesis

Studies on viral protein synthesis, its kinetics and location have employed fluorescent antibody (FA) staining, haemagglutination titre or radioactive labelling. Using synchronised cell cultures, Siegl and Gautschi (1973a) for LuIII noted the presence of a cytoplasmic fluorescence at 3-4 hpi which was independent of the cell cycle position and an intranuclear fluorescence, which was dependent on cell cycle position, commencing at 8-10 hpi, under optimal conditions. The early antigen, in the cytoplasm, did not depend on de novo DNA synthesis and was not inhibited by α-amanatin, although it was inhibited by actinomycin D (Siegl and Gautschi, 1973b). The appearance of the intranuclear fluorescence was prevented by specific inhibitors of DNA synthesis (FUdR, ara C, mitomycin C) and RNA synthesis (actinomycin D, α-amanatin) which is consistent with the fact that viral DNA replication precedes the appearance of intranuclear antigen by 2 hr (Siegl and Gautschi, 1973b). Rhode (1973) for H-1 observed similar results with respect to the production of haemagglutination activity. Cell associated haemagglutination activity commences at 10 hpi, at the same time as FA staining nuclei were observed, and its appearance is prevented by the inhibitor of DNA synthesis, ara C. Also, the synthesis of RF DNA is blocked by inhibitors of protein or RNA synthesis (Rhode, 1974a,b).The appearance of MVM intranuclear antigen is also blocked by the inhibition of DNA replication (Parker et al, 1970). These results can be interpreted

in two ways. Firstly, a direct inhibition of viral DNA synthesis results in a cessation of viral protein synthesis, or secondly, since viral DNA replication relies on a specific cellular event then the prevention of cellular DNA replication results in an inhibition of viral protein synthesis because viral protein synthesis relies on that event directly or indirectly, because viral DNA replication is inhibited by inhibition of expression of the cellular function.

The three structural polypeptides of AAV virions are synthesised in the presence of either Adenovirus or Herpesvirus acting as a helper virus (Johnson et al, 1972; Salo and Mayor, 1979). The polypeptides were synthesised in the cytoplasm and transported to the nucleus as demonstrated by immunofluorescence studies (Johnson et al, 1972) and confirmed by radioactive labelling with ^{35}S-L-methionine and SDS polyacrylamide gel electrophoresis to detect the structural polypeptides (Salo and Mayor, 1979). A similar synthesis and transport event occurs for LuIII polypeptides and indeed the LuIII structural polypeptides were found associated with the non-histone chromosomal fraction of nuclei mainly in the form of empty capsids (Gautschi et al, 1976). Kongsvik et al (1974a) for H-1 demonstrated by pulse chase experiments that viral structural protein synthesis occurred at a peak between 8 to 16 hpi.

A precursor polypeptide to the major structural polypeptides of AAV was demonstrated in Adenovirus coinfected cells (Johnson et al, 1977). This polypeptide (molecular weight, 120,000 daltons) was suggested to be the translation product of the primary AAV transcript. This data was not confirmed during pulse chase experiments by both Salo and Mayor (1979) and Buller and Rose (1978a). Various protease inhibitors did not change the ratios of the structural polypeptides,

although an amino acid analogue, L-canavanine did prevent the synthesis of polypeptides C and D but no larger precursor was observed. An *in vitro* translational system using AAV selected mRNA did not detect a large precursor molecule (Buller and Rose, 1978b). Apart from the three structural proteins found in AAV infected cells, two non-structural polypeptides, D and E, are detected, of molecular weight, 24.9×10^3 and 15.8×10^3 daltons, respectively (Buller and Rose, 1978a). They are synthesised at approximately the same time post infection as the structural polypeptides (Buller and Rose, 1978a) and are detected in the *in vitro* cell free translation system using AAV mRNA (Buller and Rose, 1978b). No large precursor polypeptide has been reported for the autonomous group nor has the presence of the two non-structural polypeptides been discovered.

In the autonomous Parvovirus group the existence of a temperature sensitive mutant in progeny virus synthesis has indicated that at least one of the virally coded proteins is essential for single stranded DNA synthesis (Rhode, 1976). In the AGMK cell line, Adenovirus will only provide a partial helper function for AAV, in that AAV DNA and RNA are produced but not AAV polypeptides. If SV40 is infected into these cells together with Adenovirus, then AAV will produce single stranded DNA and infectious virions (Buller *et al*, 1979). These data suggest that at least one AAV coded function is required for progeny DNA synthesis.

1.3.5 Assembly

A final stage in the assembly of Parvoviruses is the conversion of the 1.46 g/cm^3 (heavy) particle to the 1.42 g/cm^3 (light) particle by proteolytic cleavage (Clinton and Hayashi, 1975, 1976; Richards

et al, 1977). The site of virus assembly appears to be in the nucleus, as determined for MVM by biochemical studies (Richards et al, 1977) and H-1 by electron microscopic studies of infected cells (Singer and Toolan, 1975). The processing of the heavy to the light particle also occurs in the nucleus, but, transport to the cytoplasm does not appear to have any specificity with regard to particle type (Richards et al, 1977).

The assembly of replicative intermediates into virus particles is rapid both in vivo and in vitro for LuIII (Siegl and Gautschi, 1976; Gautschi and Reinhard, 1978). Indeed, using Brij 58 lysed LuIII infected cells as an in vitro system it has proved possible to demonstrate the conversion of in vitro and in vivo labelled replicative intermediates into virions (Gautschi and Reinhard, 1978; Gautschi et al, 1978). Virus assembly appears to require DNA synthesis in vivo and probably in vitro as well (Richards et al, 1977; Gautschi and Reinhard, 1978). As yet no intermediates in the conversion of replicative intermediates to mature virions have been isolated either in vivo or in vitro.

AAV appears to be potentially defective at this stage in the replication cycle. On coinfection with Herpesvirus, AAV is able to synthesise RNA, DNA and protein but does not produce progeny virus (Rose and Koczot, 1972; Boucher et al, 1971; Salo and Mayor, 1979). Boucher et al (1971) demonstrated that non-encapsidated infectious AAV DNA was present indicating that the level of defectiveness was either the assembly process or the synthesis of progeny single stranded DNA.

1.4 DNA Replication - General

The replication cycle of Parvoviruses appears to consist of three stages: stage 1 is the conversion of the input viral strand to a duplex; stage 2 is the replication of this duplex; and stage 3 is the production of single stranded progeny virions. In this respect the asymmetry of the replication process (at least for the autonomous group) can be compared and contrasted with the mechanism of ØX174 DNA replication. Also, the replication of the linear double stranded DNA molecules formed during stage 2 will have the same problems in DNA replication to overcome as bacteriophage T7 and Adenoviruses. Thus, the DNA replicative mechanisms of bacteriophage ØX174, bacteriophage T7 and Adenoviruses will be presented in order to compare with the intermediates present during Parvoviral DNA replication. Also, the existence of DNA-protein complexes during the replication of the eukaryotic viruses, SV40 and Adenoviruses, and their importance in the replication mechanisms of these viruses will be presented in this section.

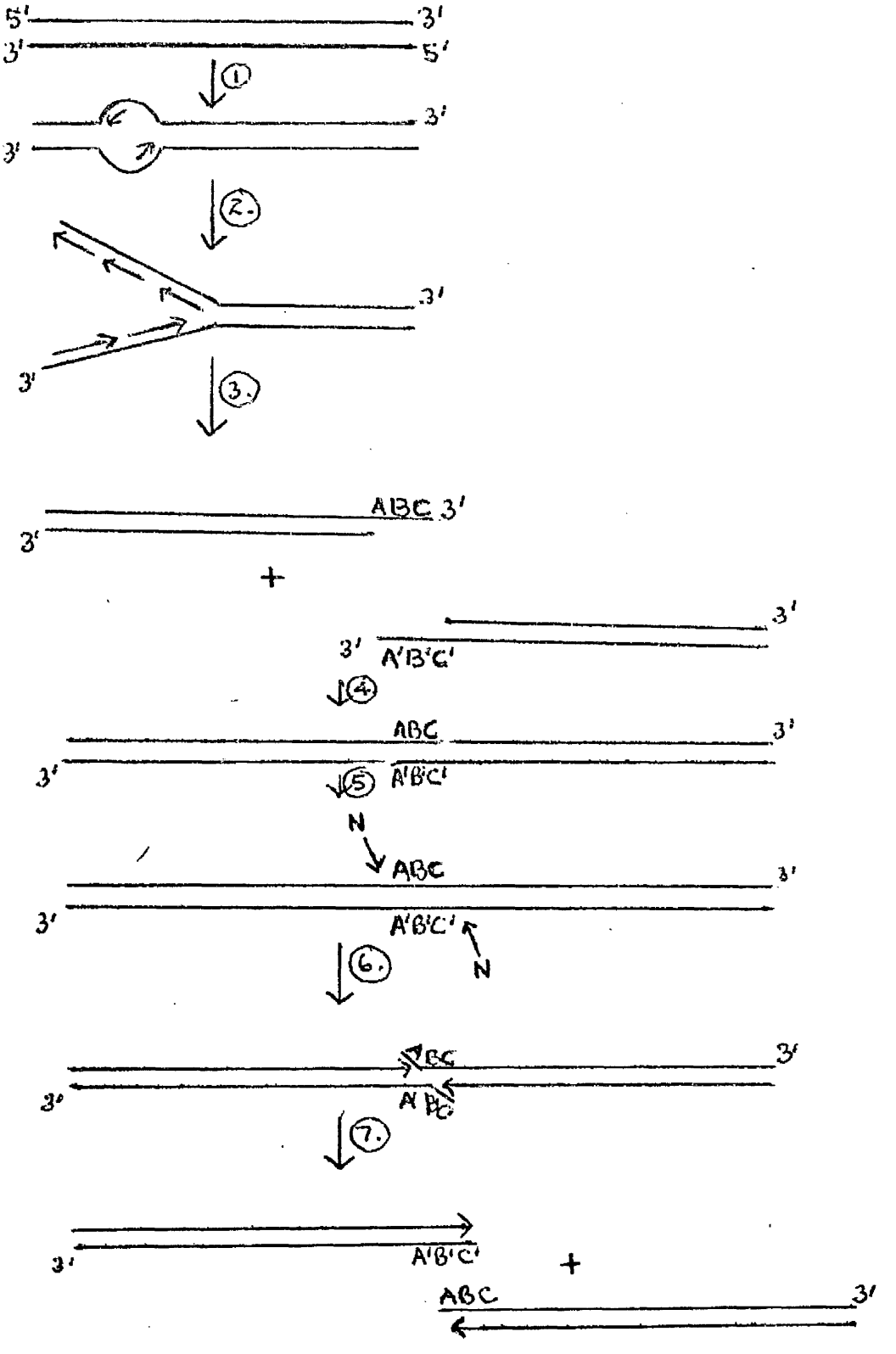
1.4.1. Bacteriophage T7

The model for T7 DNA replication is illustrated in Fig. 9 and is adapted from the original model proposed by Watson (1972). In stage 1 there is initiation of DNA synthesis at a point approximately 17% of the genome length from the genetic left end of the molecule with the formation of a replication bubble. DNA synthesis is bi-directional and when the left end of the replication bubble reaches the left end of the genome a Y-shaped molecule, similar to the structure observed during H-1 RF DNA replication, is formed, i.e. stage 2 (Wolfson *et al*, 1971; Dressler *et al*, 1972; Wolfson and

Fig. 9

Mechanism of T7 DNA replication.

RNA primed DNA synthesis commences at the origin, which is located at a point 17% from the genetic left end of the molecule, forming a replication bubble (stage 1). Replication is bidirectional and when the left end of the replication bubble reaches the end of the molecule a Y shaped molecule is formed (stage 2). Replication is now unidirectional towards the right end of the molecule. Once completed two daughter linear duplexes are formed and after removal of the RNA primer a gap is left at the 5' end of the newly synthesised strand (stage 3). T7 DNA is terminally redundant. A,B and C represent nucleotide sequences and A',B' and C' represent their respective complentary sequences. Therefore, the two daughter duplexes can hybridise to form a concatemeric molecule (stage 4). The dimer molecule is then made completely duplex by gap fill synthesis and ligation (stage 5). Two single strand scissions are then made in the duplex molecule by cleavage with a site specific endonuclease (N) at points just outside the terminal repetition in the middle of the molecule. This scission will give rise to a 3'OH group capable of elongation with a DNA polymerase (stage 6). Extension of the internal 3' ends results in the disruption of the H bonds, keeping the duplex molecule intact, thus forming two monomer DNA molecules whose 5' ends can now be completed (stage 7).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A'B'C'
N
⑥
BC
3'
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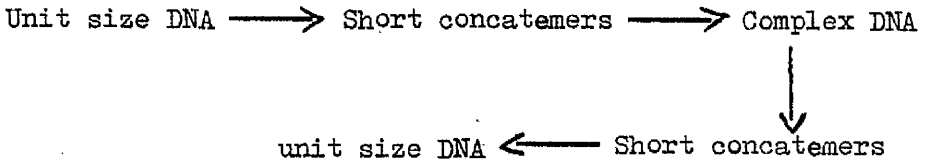
A'
⑦
3'
A'B'C'
+
ABC
3'

Dressler, 1972a). On the completion of the first round of replication (stage 3), one can see that when the RNA primer is removed at the extreme 5' end of the newly synthesised strand, on each molecule, a gap is left which cannot be filled by the action of DNA polymerase because DNA polymerase cannot initiate synthesis, de novo, without a 3' OH group. Since the ends of T7 DNA are terminally redundant (Ritchie et al, 1967) there is the potential to form a concatemeric DNA molecule by hybridisation of the single stranded regions (stage 4). The genomes of the autonomous Parvoviruses are not terminally redundant, however, the members of the AAV group are terminally redundant. The gap could be sealed with DNA polymerase and polynucleotide ligase and then a site specific endonucleolytic cleavage at each end of the terminally repetitious region (as shown in stage 5) would result in the production of a free 3' OH group to act as a primer for DNA polymerase (stage 6). DNA synthesis will result in the displacement of a 5' ended tail and when the growing 3' ends meet there will be no more hydrogen bonds holding the molecule together and they will fall apart (stage 7). Each separate molecule can now complete the terminal structure. This model agrees with the experimental data which indicate the following pathway of T7 DNA replication,



(Center, 1972, 1973; Schlegel and Thomas, 1972; Stratling et al, 1973; Serwer, 1974; Paektou et al, 1977; Langman et al, 1978). Complex DNA is very large concatemeric forms (head to tail arrangements of T7 DNA molecules) consisting of possibly the entire amount of

T7 DNA in the infested cell. Large concatemeric molecules can be formed by re-initiation of either replicating molecules (stage 2) or newly completed molecules (stage 3). Concatemeric molecules are formed during Parvovirus DNA replication but so far the largest found is a tetramer and also the concatemers are tail to tail arrangements of monomer RF DNA. Thus the formation of concatemeric DNA molecules is a mechanism by which the 5' ends of newly synthesised duplex DNA molecules are completed.

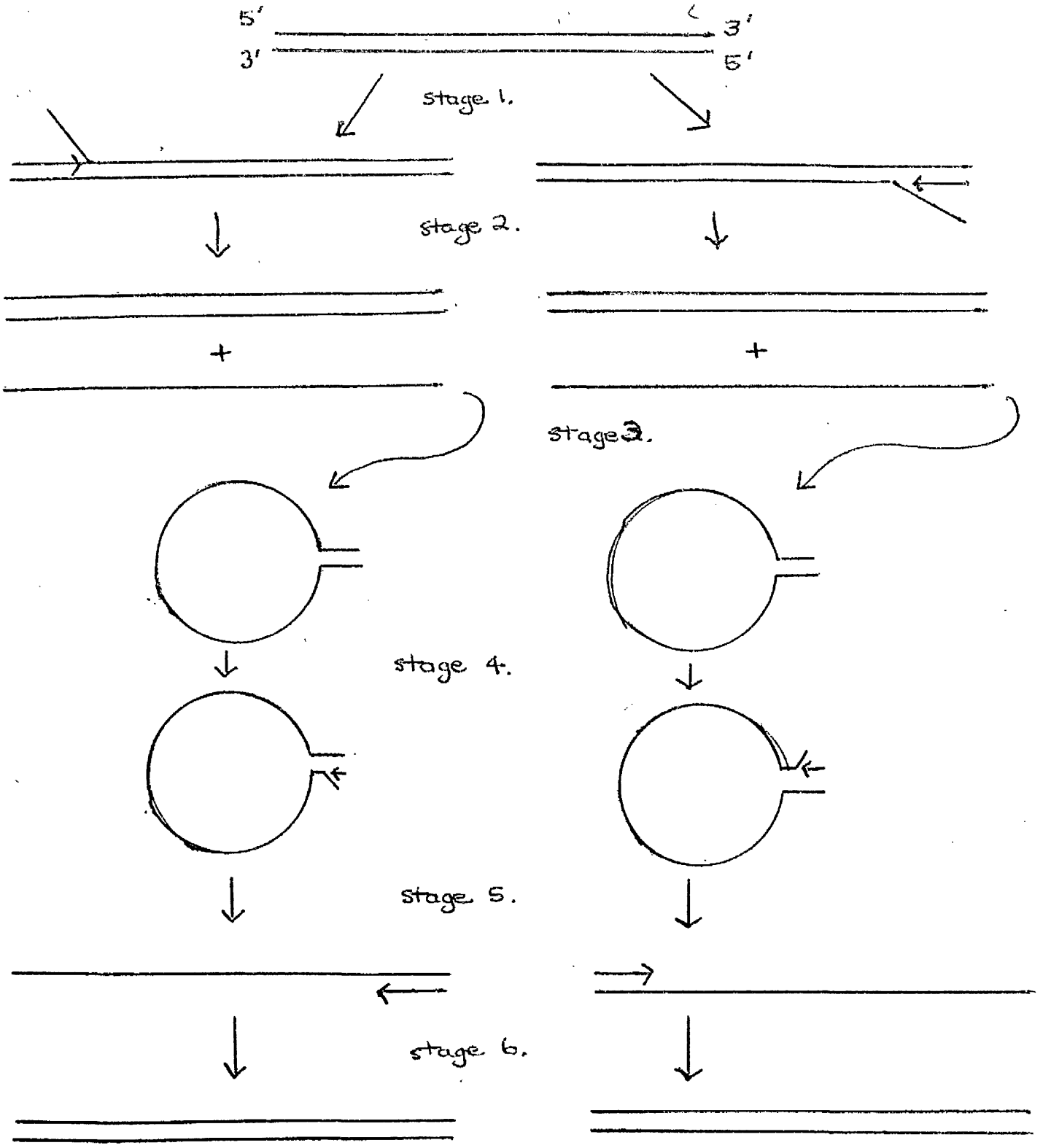
1.4.2. Adenovirus

The model of Adenovirus DNA replication is illustrated in Fig. 10. The first step is an initiation event which can occur with equal probability at either end of the DNA molecule. The origins for DNA synthesis of AAV are also at both ends of the molecule. DNA synthesis then proceeds by a displacement mechanism which need not be discontinuous, since synthesis is in the 5' to 3' direction. The molecules formed at this stage contain extensive single stranded regions (Bellet and Younghusband, 1972; Pettersson, 1973; Robin et al, 1973; van der Eb, 1973; Flint et al, 1976). Step 2 results in the formation of a completed duplex and a single stranded linear molecule. The single stranded molecule can form a circle due to the inverted terminal repetition (Garon et al, 1972; Wolfson and Dressler, 1972b) and the duplex region thus formed will be identical to the terminal region of completely duplex Adenovirus DNA. AAV single stranded DNA can also form circular structures. The next step is the initiation of this circular molecule to yield a partially single stranded and partially double stranded molecule, which can thus form the completed duplex Adenovirus DNA. All these intermediates have

Fig. 10

Mechanism of Adenovirus DNA replication.

An initiation event which can occur with equal frequency at either end of Adenovirus DNA results in displacement DNA synthesis (stage 1). Continued DNA synthesis results in the formation of a completely duplex progeny DNA molecule and a single stranded DNA molecule (stage 2). The single stranded DNA molecule can form a circle due to the inverted terminal repeat which exists at the termini of Adenovirus DNA (stage 3). The duplex region has the same sequence as the ends of duplex Adenovirus DNA, thus the initiation event can now occur on the single stranded circular DNA (stage 4). Continued DNA synthesis results in the formation of a partially double stranded linear DNA molecule (stage 5). Extension of the double stranded region results in the formation of a completely duplex progeny DNA molecule (stage 6).


5'
3'
3'
5'
stage 1.
stage 2.
+
+
stage 3.
stage 4.
stage 5.
stage 6.

been observed by electron microscopy (Lechner and Kelly, 1977). Also this model predicts two origins of DNA replication which have been observed (Horwitz, 1976; Weingartner et al, 1976).

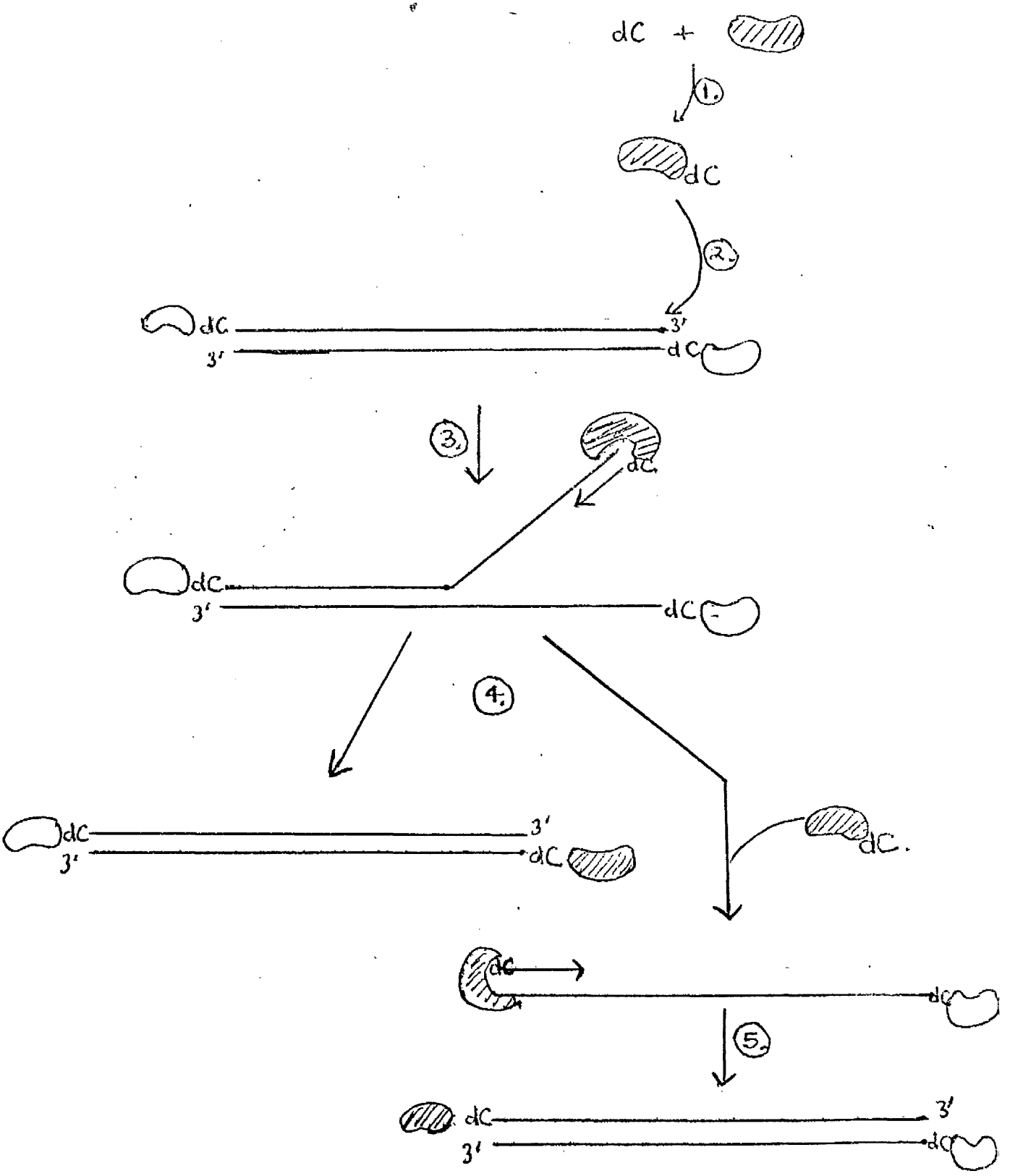
A major problem which this model does not solve is the mechanism of initiation of DNA replication which is the same problem as existed for T7 DNA replication, i.e. how to complete the ends of the DNA molecule. Initial theories implicated a hairpin priming mechanism (Wu et al, 1977 modification of the model of Cavalier-Smith, 1974). However, later experimental data suggested that this mechanism did not operate (Steenbergh et al, 1977; Stillman et al, 1977; Sussenbach and Kuijik, 1978). Hairpin priming is thought to occur during AAV DNA replication and also possibly during autonomous Parvovirus replication.

There is a protein covalently attached to the 5' end of each strand of Adenovirus DNA isolated from virions and it has been suggested that the function of this protein is to initiate Adenovirus DNA replication (Rekosh et al, 1977). The model is illustrated in Fig. 11. During DNA replication the terminal protein containing a deoxycytidine residue can bind strongly, but non-covalently, to the 3' end of the Adenovirus DNA remaining on the outside of the phosphodiester chain. The protein would become attached covalently to the 5' terminal deoxycytidine of the progeny strand, possibly by acting directly as a primer for DNA synthesis. One can see that this method would either result in de novo initiation of a new strand or could act to initiate a small region of DNA synthesis to repair the gap left by the excision of a possible RNA primer. No conclusive evidence has yet been presented to suggest that this is the function of the terminal protein, although recent experiments have demonstrated that a terminal protein is

Fig. 11

Protein initiation of Adenovirus DNA replication.

A deoxycytidine (dC) residue is bound covalently to the 55,000 dalton protein molecule (stage 1). This protein molecule then binds strongly, but non-covalently, to the 3' end of Adenovirus DNA. Mature Adenovirus DNA contains a protein covalently attached to each 5' end of the duplex DNA molecule (stage2). This protein molecule, which is strongly bound to the 3' end of Adenovirus DNA, then primes DNA synthesis by using the dC residue to prime for DNA synthesis resulting in displacement DNA synthesis (stage 3). Continued DNA synthesis results in a completely duplex DNA molecule containing a covalently attached protein molecule to the 5' end of the newly synthesised strand and a single stranded DNA molecule to which another protein molecule can attach (stage 4). The newly attached protein molecule on the single stranded DNA molecule can prime DNA synthesis in a similar manner as before (stage 4) resulting in a completely duplex progeny DNA molecule (stage 5).


dC +
1.
dC
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dC

attached to replicating DNA (Robinson et al, 1979; Stillman and Bellet, 1978). Also parental DNA, which had undergone replication, as determined by BUdR labelling, had a terminal protein attached at late times in infection (Straus et al, 1979). Thus, another solution to the problem of replication of the ends of linear chromosomes has been put forward to explain Adenovirus DNA replication.

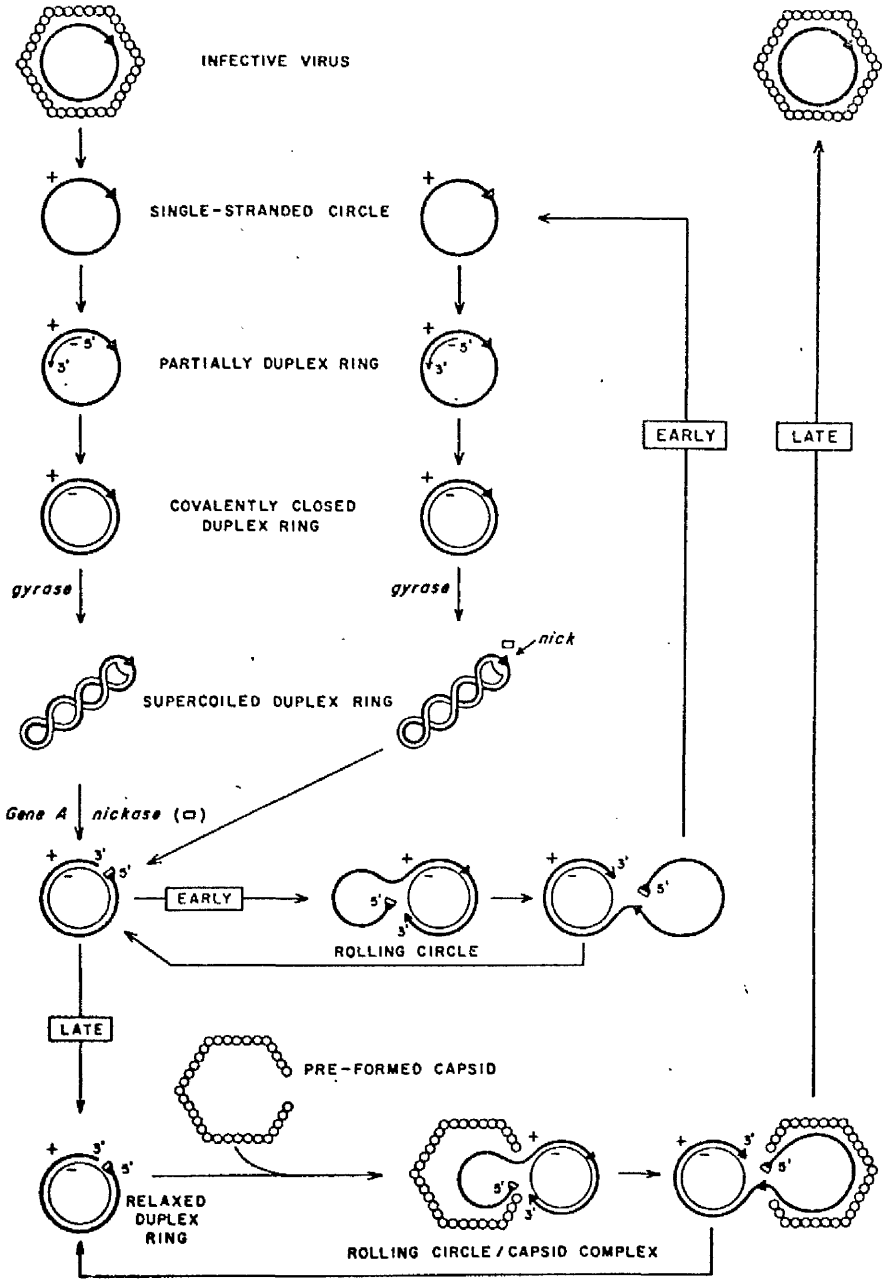
1.4.3 Bacteriophage ØX174

The model for bacteriophage ØX174 DNA replication is illustrated in Fig. 12. In the first step the input viral strand is converted to a duplex molecule by synthesis of the complementary strand. The synthesis of this strand is discontinuous and does not commence at a specific origin (Eisenberg et al, 1975; McFadden and Denhardt, 1975). However in other single stranded circular DNA bacteriophages synthesis is initiated from a specific origin (Martin and Godson, 1977). In all Parvovirus infected cells a duplex DNA molecule is formed from the input single stranded DNA. The second step in the replication cycle is the replication of this duplex circle. The second stage in the replication cycle of all Parvoviruses is the replication of the duplex linear DNA. The duplex circle is cleaved by the bacteriophage ØX174 gene A product such that a free 3' OH group is now present which can act as a primer for DNA synthesis. DNA synthesis would then proceed by extension of the viral strand, at the 3' end of the nick, thus displacing the viral strand from the 5' end of the nick. After initiation of DNA synthesis, resulting in displacement of the viral strand, two routes are open to this "rolling circle" molecule. At early times in infection displacement synthesis will continue and via, possibly, the action of the bacteriophage gene A

Fig. 12

Mechanism of Bacteriophage ϕX174 DNA replication.

After entry of the bacteriophage into the bacterium the single stranded circular DNA molecule is converted to a duplex circle by discontinuous DNA synthesis. The duplex circle is then converted into a supercoiled DNA molecule by the action of DNA gyrase. The supercoiled DNA is cleaved once, in the viral strand, with the bacteriophage gene A product which results in the formation of a nicked circular molecule with a free 3'OH group and the gene A product covalently attached to the 5' end of the nick. The 3'OH group functions as a primer for DNA synthesis. Extention of the 3'OH group causes displacement of the viral strand. At early times in infection displacement synthesis continues resulting in formation of a single stranded circle and a duplex circle. The single stranded circle can then undergo discontinuous DNA synthesis to form a duplex in an identical manner to the input DNA. At late times during infection the displaced viral strand is recognised by the pre-formed capsid structure. As replication continues a duplex circle and a maturation product en route to mature virion are formed. The gene A product may function to cleave and ligate the single stranded viral DNA produced during displacement DNA synthesis.


INFECTIVE VIRUS
SINGLE-STRANDED CIRCLE
PARTIALLY DUPLEX RING
COVALENTLY CLOSED DUPLEX RING
gyrase
gyrase
nick
SUPERCOILED DUPLEX RING
Gene A nickase (□)
EARLY
ROLLING CIRCLE
LATE
PRE-FORMED CAPSID
RELAXED DUPLEX RING
ROLLING CIRCLE / CAPSID COMPLEX
EARLY
LATE

protein, one will produce a single stranded circle and a duplex circle. The single stranded circle can then undergo complementary strand synthesis similar to that observed for the infecting single stranded viral circle. Evidence for this mechanism comes from biochemical (Dressler and Wolfson, 1970; Schroder and Kaerner, 1972; Eisenberg and Denhardt, 1974a,b; Eisenberg et al, 1975; McFadden and Denhardt, 1975; Fukada and Sunsheimer, 1976; Baas et al, 1978) and electron microscopic studies (Koths and Dressler, 1978; Keegstra et al, 1979). At late times in infection the protruding viral single strand of the rolling circle will be recognised by the pre-formed capsid and thus when synthesis is complete the viral single strand will be associated with viral capsid proteins and thus be unable to act as a template for complementary strand synthesis. Evidence for this mechanism of single strand progeny virion production includes biochemical (Gilbert and Dressler, 1968; Komano et al, 1969; Knippers et al, 1969; Dressler, 1970; Fujisawa and Hayashi, 1976; Sims et al, 1978) and electron microscopic data (Knippers et al, 1969; Dressler, 1970; Koths and Dressler, 1978).

The first step in the replication cycle is the formation of the duplex circle and this only requires host coded functions. However, in the next step a viral coded function, gene A protein, is essential for the replication of the duplex circle. The fate of this replicative intermediate, i.e. whether to produce duplex rings or single stranded viral circles depends on the presence of phage coded proteins (Denhardt, 1975, 1977). In H-1 infected cells progeny single strand production requires functional viral proteins. Thus the asymmetry of the replication process in ØX174 is determined by phage coded proteins and also is a consequence

of the fact that the viral strand is synthesised in a continuous manner which is caused by the use of the gene A protein to initiate, indirectly, unidirectional DNA replication by the creation of a 3' OH group.

1.4.4 DNA-protein complexes

DNA exists as a complex with proteins, histones, in the eukaryotic cell and replicates in this structure (Sheinin et al, 1978). The eukaryotic viruses, SV40 and Adenovirus, also exist and replicate as DNA-protein complexes within the infected cell (White and Eason, 1971; Shaw et al, 1979).

The SV40 DNA-protein complex has a structure very similar to that of eukaryotic chromatin, in that, when analysed by electron microscopy it has the typical chromatin structure, i.e. nucleosomes (Griffith, 1975; Griffith and Christiansen, 1977) and contains, as judged by polyacrylamide gel electrophoresis, a full complement of histones, i.e. H1, H2A, H2B, H3 and H4 (Varshavsky et al, 1976; Griffith and Christiansen, 1977). The repeat unit in chromatin, the nucleosome, as proposed by Kornberg (1974), consists of a well defined length of DNA associated with an octamer aggregate of pairs of the histones H3, H4, H2A and H2B and probably one molecule of the fifth histone H1 (reviewed by Kornberg, 1977; Felsenfeld, 1978). Confirmation of the nucleosomal structure in SV40 was gained by digestion of nuclei from SV40 infected cells with micrococcal nuclease resulting in an identical digestion pattern of SV40 DNA when compared to cellular DNA (Shelton et al, 1978). The SV40 virion is assembled by a gradual addition of capsid proteins to the SV40 nucleoprotein complex and subsequent organisation of the capsid into a salt resistant shell (Fernandez-Munoz et al, 1979; Coca-Prados and Hsu, 1979).

The Adenovirus DNA-protein complex has been extracted from infected cells but does not appear to be in a nucleosome-like structure (Brison et al, 1977; Kedinger et al, 1978; Shaw et al, 1979). The structure is a linear molecule, slightly shorter than naked Adenovirus DNA, 2 nm thick. The DNA replicates in this structure in vivo (Kedinger et al, 1978; Shaw et al, 1979) and can continue to replicate under appropriate conditions in vitro (Brison et al, 1977). Thus DNA-protein complexes appear to be necessary for the DNA replication of animal viruses and also for the final steps in the maturation process of these viruses.

Aims

As described in the Introduction, the events of Parvovirus replication and assembly are unclear. Therefore in order to study the complete set of virus replication and assembly events in MVM-infected cells a mild extraction procedure of the virus infected cells was sought. In this way the synthesis of viral DNA and its subsequent packaging and maturation into virion structure from replicative intermediates could be followed.

2. Materials and Methods

2.1 Materials

2.1.1 Virus

MVM was plaque-purified (Tattersall, 1972) 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/mmole
2-^{14}C thymidine	57 mCi/mmole
^{32}P-orthophosphate	10 mCi/ml
^{35}S L-methionine	600 Ci/mmol
α-^{32}P thymidine 5' triphosphate	350 Ci/mmol

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 (1M solution in methanol)	Fison, London

2.1.5 Enzymes

Pronase (B grade)	Calbiochem, Los Angeles, California
Deoxyribonuclease I (DNase I)	Sigma Chemical Co., London
Ribonuclease-A (RNase A)	"
Micrococcal nuclease	"
S1 nuclease	Miles Laboratories Inc., Stoke Poges, England
R.ECoRI	"
R.Hind III	"
DNA polymerase I, large fragment	Boehringer Mannheim, London.

2.1.6 Nucleic Acids

Calf thymus DNA	Sigma Chemical Co., London

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

ØX174 am 3 infected *E. coli* cells were obtained from Microbiological Division, Porton Down.

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.
Calf serum	" "
MEM powder	Flow Laboratories Ltd., Irvine, Scotland
Penicillin	Flaxo Pharmaceuticals, London
Streptomycin	" "
Sephadex G25 and G50 (medium)	Pharmacia, Uppsala, Sweden
CsCl	Mappin and Williams Ltd., Chadwell Heath, Essex
NaI	" "
Agarose	Sigma Chemical Co., London
Thymidine	" "
2'-deoxycytidine	" "
Sarkosyl	" "
Ethidium bromide	Calbiochem, Los Angeles, California
Plastic petri dishes	Nunc Ltd., Denmark
Whatman 3MM 2-5 cm discs	M. Reeve-Angel and Co. Ltd., London
Nonidet-P 40 (NP40)	Shell Chemical Co. Ltd.
Triton X-100	Packard Instrument Co. Inc., Illinois
Formvar-Carbon Coated Grids (3 mm)	LKB-Produkter, Sweden
Kodirex X-ray film	Kodak Ltd.
Millipore filters	Millipore (U.K.) Ltd., London

2.2 Standard Solutions

2.2.1 Cell Culture Solutions

A) Balanced Salt Solution (BSS) Earle (1943) BSS consisted of 0.116M 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) $NaHCO_3$.

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

As defined in Flow catalogue.

Spinner culture medium as defined in Flow catalogue.

EFC 10 Eagle's MEM supplemented with 10% foetal calf serum.

EFC 5 " " " " 5% " " "

ETC 10 Eagle's MEM containing 10% tryptose phosphate and supplemented with 10% calf serum.

ETC 5 Eagle's MEM containing 10% tryptose phosphate and supplemented with 5% calf serum.

C) Phosphate Buffered Saline (PBS)

PBS was composed of 3 separate solutions mixed immediately before use.

PBS (a) 170 mM 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
PBS (b) 1
PBS (c) 1

2.2.2 Buffers

Agarose gel electrophoresis buffers

(a) neutral

36 mM tris-HCl, 30 mM Na_2HPO_4, 1 mM EDTA pH 7.7

(b) alkaline

30 mM NaOH, 2 mM EDTA

SSC 0.15M NaCl, 0.015M sodium citrate pH 7.0.

2.2.3 Enzymes

Pronase: Pronase (B grade), 20 mg/ml in 0.02M tris-HCl, 0.02M EDTA pH 8.6 was heated at 80°C for 5 min and self digested for 2 hr at 37°C to destroy latent DNase activity. Stored at -20°C.

S1 nuclease: S1 nuclease, 1000 units/ml in 0.1M NaCl, 0.03M sodium acetate, 2 mM $ZnSO_4$ pH 4.5 containing 50% glycerol. Stored at -20°C.

DNase I: DNase I, 0.1 mg/ml in 50 mM tris-HCl, 0.1M NaCl, 0.01M $MgCl_2$, 0.001M $CaCl_2$, pH 7.4. Stored at -20°C.

Micrococcal nuclease: Micrococcal nuclease, 1000 units/ml, in 50 mM tris-HCl, 0.1M NaCl, 0.01M $CaCl_2$ pH 7.4. Stored at -20°C.

DNA polymerase I, large fragment: DNA polymerase I, large fragment, i.e. DNA polymerase I lacking the exonuclease activity. 1250 units/ml in 50 mM tris-HCl, 10 mM mercaptoethanol 10% glycerol pH 7.5.

ECoRI: Restriction endonuclease, ECoRI, 4000 units/ml in 0.1M tris-HCl, 0.05M NaCl, 0.01M $MgCl_2$, 0.005M β-merceptoethanol, 10% glycerol, pH 7.5.

Hind III: Restriction endonuclease, Hind III, 500 units/ml in 7 mM tris-HCl, 60 mM NaCl, 7 mM $MgCl_2$, 10% glycerol, pH 7.5.

2.2.4 Scintillation Spectrometry Solutions

A) Toluene PPO scintillation fluid is 0.5% (w/v) PPO in toluene.

B) Triton/toluene scintillation fluid is 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

BHK-21/C13 cells and A9 cells were grown in monolayers on either 80 oz roller bottles or Roux bottles. The BHK-21/C13 cells

were grown in ETC10 at 37°C in an atmosphere of 5% CO_2 in air. The A9 cells were also grown at 37°C in an atmosphere of 5% CO_2 in air but the medium was EFC10. The cells, both BHK-21/C13 and A9, were grown to confluence and subcultured by trypsinisation. The medium was removed and the cells washed with 10 ml of a solution of trypsin: versene (1:4). 5 ml of trypsin:versene was added and the bottle containing the cells placed at 37°C until the cells were observed to detach from the glass. 20 ml of either ETC10 or EFC10, dependent on the cell line, was added to the bottle and the medium, now containing the cells, pipetted until a single cell suspension was obtained. The cells were counted by haemocytometer. The A9 cells were also grown in suspension at 37°C in an atmosphere of 5% CO_2 in air. The suspension cell medium is that defined by Flow (see 1978 catalogue) supplemented with 10% foetal calf serum. A9 cells were maintained at a concentration of between 2 x 10^5 to 8 x 10^5 cells/ml.

2.3.2 Contamination Checks

The cells were routinely screened, after each subculture for monolayer cultures and every 6 or 7 days for suspension cultures, 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.

A second method relies on the binding of a fluorescent dye to nucleic acid. 10^5 cells were inoculated onto coverslips and grown at 37°C, in an atmosphere of 5% CO_2 in air, overnight. The medium was removed from the coverslip culture and the cells were fixed by acetic acid-methanol (1:3). Between 1 to 2 ml of the fluorescent stain, Bisbenzamid fluorochrome (0.005% (w/v) Haechot 33258 Bisbenzamid fluorochrome in PBS(a) was diluted 1:1000 in PBS(a) and used immediately) was added to each culture and incubated at 37°C for 1 hr. The stain was removed and the cells rinsed twice with deionised H_2O and then mounted for UV microscopy. Mycoplasma are detected by a distinctive cytoplasmic fluorescence which is granular in nature.

2.3.3 Propagation of MVM in either BHK-21/C13 cells or A9 cells

BHK-21/C13 cells or A9 cells were seeded in 80 oz roller bottles at 20 x 10^6 cells in the appropriate medium. After 20 hr the medium was removed and the cells were infected with MVM, at a low multiplicity (0.001 to 0.01 pfu/cell) in 20 ml of BSS. Adsorption was allowed to continue for between 60 min to 90 min, after which 100 ml of either ETC5 for BHK-21/C13 cells or EFC5 for A9 cells was added, after removal of the virus suspension. After 7 days, by which time cpe was advanced, the infected cells were shaken into the medium. Virus stocks were prepared by sonicating the infected cell medium (5 minutes in an ultrasonic cleaning unit,

Burndept. Ltd., Kent) followed by three cycles of freezing and thawing. The cell debris was removed by centrifugation at 10,000 g for 30 minutes and the supernatant fraction assayed for infectivity. The MVM usually had a titre between 5×10^7 to 5×10^8 pfu/ml.

2.3.4 Titration of MVM

2.3.4.1 Plaque Assay (Tattersall et al, 1976)

A9 cells from logarithmically growing suspension cultures were seeded in EFC10 at 5×10^5 cells in 50 mm plastic petri dishes, and incubated at 37°C for 20 hr. The medium was removed and the cells infected with 0.5 ml samples of serially diluted MVM. Adsorption was allowed to continue at 37°C for 90 minutes after which time the virus dilution was removed and the cultures overlaid with 5 ml of Eagle's medium containing 5% foetal calf serum in 0.75% Noble agar (overlay medium). This medium was kept at 41°C before addition to the cultures which were then incubated at 37°C for 7 days in an atmosphere of 5% CO_2 in air. After incubation the living cells were stained by adding 2 ml of overlay medium (without serum) containing 0.008% neutral red. The plaques were counted after overnight incubation at 37°C.

2.3.4.2 Haemagglutination Assay (Crawford, 1966)

Samples for measurement (60 µl to 200 µl) were diluted with an equal volume of PBS (a) and mixed thoroughly. Half the total volume was removed and diluted with an equal volume of PBS(a) and mixed thoroughly. This procedure was repeated until desired dilutions were obtained. To each dilution was added an equal volume of 1% (v/v) Guinea-Pig red blood cell suspension in PBS(a). The end point

of the agglutination was read after incubation for 3 to 4 hr at 4°C. Titres of haemagglutination (HA) units are expressed as the logarithm to the base 2 of the reciprocal of the dilution causing complete agglutination.

2.3.5 Infection of cells

Logarithmically growing A9 cells, in suspension, were collected by low speed centrifugation (450 g for 5 min) and infected by resuspension in an appropriate virus dilution in BSS to give a multiplicity of 5 pfu/cell and a concentration of 5×10^6 cells/ml. After 90 min adsorption at 37°C, with constant stirring, the non-adsorbed virus was removed, by centrifugation, and the cells resuspended at a concentration of 5×10^5 cells/ml in EFC5. Control cells were mock-infected with BSS.

Monolayer cultures of BHK-21/C13 cells (seeded at 1×10^6 cells per 90 mm plastic petri dish) or A9 cells (seeded at 2×10^6 cells per 90 mm plastic petri dish) were grown at 37°C, in an atmosphere of 5% CO_2 in air, for 2-3 days, by which time confluence had not been reached. The cells were infected with virus in 2 ml of BSS at a multiplicity of 5 pfu/cell. After 90 min adsorption at 37°C the virus dilution was removed and the cultures were overlaid with 10 ml of ETC5 or EFC5, dependent on the cell line. Control cells were mock infected with 2 ml of BSS.

2.3.6 Labelling of cells

In monolayer cultures, on 90 mm plastic petri dishes, at the appropriate time post infection, the medium was either reduced to a volume of 2 ml and the appropriate amount of isotope added or replaced with 2 ml of medium containing an appropriate amount of

isotope. The isotope in this case was either ^{3}H-thymidine or ^{14}C thymidine. When labelling with ^{32}P orthophosphate the medium was removed and the isotope added to medium containing 10% of the normal phosphate concentration. When ^{35}S L-methionine was the radioactive precursor, the medium was removed at an appropriate time post infection, and methionine free medium containing 5% dialysed serum added and incubation continued for 30 min at 37°C. This medium was then removed and medium containing 5% dialysed serum and 2% of the normal methionine concentration and appropriate amounts of radioactive methionine added. Incubation was continued at 37°C for the required time period.

For suspension cultures the radioactive precursor (either ^{3}H TdR or ^{14}C TdR) was added after the cells were concentrated approximately 10 fold. If incubation in the radioactive precursor was for longer periods than 2 hr the cells were not concentrated and the radioactive precursor was added to the medium directly. When ^{32}P orthophosphate was the label the medium contained 10% of the normal phosphate concentration.

2.3.7 Pulse-Chase Experiments

After infection the cells were overlaid with medium (either ETC5 or EFC5 dependent on the cell line) which was buffered with Hepes instead of bicarbonate and incubation was in air in a 37°C hot room. Pulse labelling of 90 mm plastic petri dishes containing MVM-infected or mock-infected cells at the appropriate time post-infection was performed by removing the medium and adding 2 ml of radioactive medium at 37°C. After incubation for the appropriate time the radioactive medium was removed and the cells washed once with 10 ml of PBS(a) at

37°C. Zero time chase was obtained by adding 10 ml of ice cold PBS(a) and the cells processed accordingly. If the cells were to be chased, 10 ml of medium, either ETC or EFCS, also at 37°C, containing unlabelled thymidine (100 μg/ml) and 2'-deoxycytidine (10 μg/ml) was added and incubation continued at 37°C for the appropriate time.

2.3.8 Preparation and purification of MVM virus (Tattersall et al, 1976)

BHK-21/Cl3 cells or A9 cells were seeded at a concentration of 15 x 10^6 cells/80 oz roller bottle or 25 x 10^6 cells/80 oz roller bottle, respectively, and infected 20 hr later with MVM at a multiplicity of between 0.001 to 0.01 pfu/cell and grown in either ETC5 or EFC5 for 1 week, by which time cpe was pronounced. For the preparation of radioactively labelled virus the cells were grown, after seeding, for 2-3 days at 37°C, by which time confluence had not been reached, and infected with MVM at a multiplicity of 5 pfu/cell. The radioactive precursor was added at 10 hpi to a concentration of either 2 μCi/ml for ^{3}H thymidine or 100 μCi/ml for ^{32}P orthophosphate. Incubation was continued till 48 hpi by which time cpe was pronounced. Virus was purified by the same method for either labelled or unlabelled preparations. All operations were carried out at 4°C or on ice as quickly as possible. The medium was removed and cells collected by low speed centrifugation. 20 ml of BSS was added to each 80 oz roller bottle and the cells were scraped into the BSS and collected by low speed centrifugation (450 g for 5 min). The cells were washed once in PBS(a) and once in TNE (0.15M NaCl, 50 mM Tris-HCl, 0.5 mM EDTA pH 7.5). The cells were then washed once in hypotonic

buffer, TE (50 mM Tris-HCl, 0.5 mM EDTA pH 8.7) and resuspended in TE at a concentration between 5 x 10^6 to 25 x 10^6 cells/ml and homogenised for a total of 1 minute, in short bursts, at medium speed. The nuclei were then removed by centrifugation at 450 g for 15 min. The supernatant fraction, containing the virus, was decanted and centrifuged at 15,000 g for 30 min in an MSE Hi-Speed to remove cell organelles. Calcium chloride was then added to a final concentration of 25 mM and the sample kept on ice for at least 30 min. This procedure precipitates the virus. The precipitate was collected by centrifugation at 10,000 g for 10 min. The pellet was resuspended in 50 mM Tris-HCl, 20 mM EDTA pH 8.7 by gentle sonication in an ultrasonic cleaning unit (Burndept Ltd., Kent). The insoluble material was removed by centrifugation at 10,000 g for 10 min. The supernatant fraction was removed and layered in 10 ml amounts on top of 2 ml of 30% sucrose in TE which had itself been layered, immediately before this, on top of 5 ml of caesium chloride (density = 1.40 g/cm^3) in TE. Centrifugation was in a Beckman SW27.1 rotor at 82,000 g for 20 hr at 4°C in a Beckman L5-50 centrifuge. After centrifugation, for unlabelled preparations, two main bands are visible when viewed in vertical light. The lower band (full particles) and the upper band (empty particles) were collected by side puncture and dialysed against TE. For labelled preparations the gradient was harvested by direct puncture and dripped into test tubes. An aliquot was removed for measurement of acid precipitable radioactivity and the appropriate fractions either dialysed against TE, or desalted using a Sephadex G25 column into TE.

2.3.9 Preparation of ØX174 phage (Galibert et al, 1974)

One to three grams wet weight of ØX174 am3 infected E. coli

cells were resuspended in 12 ml of 0.1M sodium tetraborate with 0.1 ml chloroform. 1 ml of lysozyme (4 mg/ml) in 0.25M tris-acetate (pH 8.0), 0.1M EDTA was added and the solution was incubated at 37°C for 30 min followed by three cycles of freezing and thawing (solid CO_2/acetone, 37°C water bath); after which 0.35 ml of 1M $CaCl_2$ was added followed by micrococcal nuclease (20 mg/ml in 0.1M sodium tetraborate) to a final concentration of 0.5 mg/ml. The solution was incubated for 30 min at 37°C with intermittent swirling. Two ml of 0.2M EDTA (pH 8.0) were added and the solution centrifuged at 10,000 g for 10 min in an 8 x 50 rotor in an MSE Hi Speed 18. The supernatant fraction was decanted and saved. The pellet was re-extracted by dispersal in 5 ml of 0.1M sodium tetraborate solution and recentrifuged as above. The combined supernatants were adjusted by the addition of solid caesium chloride to a density of 1.40 g/cm^3 and placed in 5 ml amounts in cellulose nitrate SW50.1 centrifuge tubes. Centrifugation was in a Beckman SW50.1 rotor for 40 hr at 80,000 g at 4°C. After centrifugation one opaque band which contained ØX174 phage particles was observed and harvested by side puncture. The sample was dialysed into 50 mM tris-HCl, 0.5 mM EDTA pH 7.5.

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 were washed once in PBS (a) and resuspended at a concentration of between 5 x 10^6 to 10 x 10^6 cells/ml in 20 mM tris-HCl, 150 mM NaCl,

10 mM EDTA pH 7.5 and an equal volume of 1.2% SDS in the same buffer added. 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, 5M NaCl was added to a final concentration of 1M and mixed by gently inverting the tube, 10 times. After standing on ice overnight, the lysate was centrifuged at 15,000 g for 60 min at 0°C in an 8 x 50 rotor in a MSE Hi Speed. The supernatant fluid containing the viral DNA was decanted and stored at -20°C.

B) Triton X-100 Method (White and Eason, 1971)

Infected cell cultures on 90 mm dishes were washed once with PBS(a) and 0.9 ml of 0.25% triton X-100 in 10 mM tris-HCl, 10 mM EDTA pH 7.9 was added to each dish. After incubation at 20°C for 1 hr, 0.1 ml of 2M NaCl was added and the lysate scraped into centrifuge tubes and centrifuged at 450 g for 15 min at 4°C. The supernatant fluid containing the viral DNA was stored on ice. Alternatively, infected cells were resuspended in 0.25% triton X-100 in 10 mM tris-HCl, 10 mM EDTA pH 7.9 at a concentration of between 15 x 10^6 to 30 x 10^6 cells/ml and incubated on ice for 30 min before addition of NaCl to a final concentration of 0.2M. The lysate was then centrifuged at 450 g for 15 min at 4°C and the supernatant fraction decanted and stored on ice.

C) Whole cell lysate method

a) neutral

Infected cells were washed twice in SSC and resuspended at a concentration of 5-10 x 10^5 cells/ml in SSC. The cell suspension, 0.180 ml, was gently mixed with 0.020 ml of 10% SDS in SSC. After

15 min at room temperature the DNA extracts were layered onto a 4.0 ml linear sucrose density gradient (15-30% (w/v) in 0.25M NaCl, 0.001M EDTA, 0.01M tris-HCl, pH 7.4 containing 0.5% SDS) formed over a 0.5 ml cushion of 70% sucrose in SSC. The gradients were centrifuged in a Beckman SW50.1 rotor at 215,000 g (average) for 3 hr at 25°C. The gradients were harvested from the top using an MSE gradient harvester and 8 drop fractions were collected on Whatman 3MM discs (25 mm diameter) and acid insoluble radioactivity measured.

b) alkaline

4.0 ml sucrose density gradients, 15 to 30% (w/v) in 0.5M NaCl, 0.25M NaOH, 0.001M EDTA, 0.01% SDS containing a 0.5 ml 70% sucrose cushion were prepared and 0.1 ml of 0.3M NaOH, 0.001M EDTA, 0.5% SDS was layered onto the gradients. A cell suspension of 0.150 ml (as previously described) was carefully pipetted into the upper layer. The tubes were sealed in a SW50.1 rotor and kept for at least 8 hr at room temperature. The gradients were centrifuged at 215,000 g for 3 hr at 25°C. After centrifugation the gradients were harvested and assayed for radioactivity as before.

D) Preparation of nucleoprotein complexes (NP40 - NaCl method)

All steps were carried out at 4°C or on ice. Cells, either MVM infected or mock infected were washed twice in PBS(a) and once in 1/6xRSB and resuspended at a concentration of between 10×10^6 and 30×10^6 cells/ml in 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0. The cells were vortexed for 15 sec and kept on ice for 15 min after which time they were vortexed again. At least 95% of this preparation contained nuclei as checked by phase contrast microscopy. NaCl (1M) was added to the lysate to a final concentration of 0.5M and

the sample mixed by inversion. After 30 min on ice the sample was centrifuged at 10,000 g for 20 min at 4°C in an 8 x 50 rotor in a MSE Hi Speed centrifuge. The supernatant fraction was removed and stored on ice. Alternatively, the nuclei were collected by low speed centrifugation (450 g for 3 min) and the supernatant fraction kept on ice. The nuclei were then washed three times in RSB and resuspended at a concentration of between 10×10^6 to 50×10^6 nuclei/ml in RSB after which an equal volume of 1M NaCl was added. After 30 min on ice the sample was centrifuged for 20 min at 10,000 g at 4°C in an 8 x 50 rotor in a MSE Hi Speed centrifuge. The supernatant fraction was removed and stored on ice.

2.4.2 Preparation of viral DNA

MVM DNA and ØX174 DNA was prepared from purified virus by a method which has been demonstrated to cause minimal strand breakage (Koczot et al, 1973). Purified virus was digested with micrococcal nuclease at 20 µg/ml, after adding $CaCl_2$ to a final concentration of 5 mM, at 37°C for 30 min. Virus samples were then made 0.3N with respect to NaOH and incubated for 20 min at room temperature. For unlabelled samples the disrupted virus was layered in 2 ml amounts onto 34 ml linear alkaline sucrose density gradients (5 to 20% (w/v) in 0.3M NaOH, 0.7M NaCl, 0.01M EDTA) formed in polyallomer centrifuge tubes. Centrifugation was in a Beckman SW27 rotor for 24 hr at 82,000 g at 4°C. The gradient was harvested, from the bottom, through a Gilford spectrophotometer equipped with a flow cell and the DNA peak was pooled and desalted using a Sephadex G25 column. For labelled samples the disrupted virus was layered in 0.2 ml amounts onto 4.8 ml linear alkaline sucrose gradients (5 to 20% (w/v) in 0.3M NaOH, 0.7M

NaCl, 0.01M EDTA containing 0.15% Sarkosyl) formed in Polyallomer tubes and centrifuged in a Beckman SW50 at 215,000 g (average) for 5 hr at 4°C. The gradients were harvested by direct puncture and 10 drop fractions collected. An aliquot was taken to measure acid precipitable radioactivity and the DNA containing peak was pooled and desalted using a Sephadex G25 column.

The concentration of DNA was estimated by measuring the extinction at 260 nm, assuming an E260 nm of 1 corresponds to 35 μg/ml (Bourguignon et al, 1976).

2.4.3 Preparation of BHK-21/C13 DNA (Gallimore et al, 1974)

BHK-21/C13 cells were seeded at a density of 20 x 10^6 cells per 80 oz roller bottle and harvested, by scraping into BSS, three to four days later by which time confluence had been reached. If the DNA was to be radioactively labelled, the medium was removed 20 hours after seeding and replaced with 100 ml of ETC 10 containing 50 μCi of ^3H thymidine and harvested as before. Whole cells were washed once in PBS and resuspended in 5 ml of 0.1M EDTA, 0.01M Tris-HCl, 0.01M NaCl pH 7.9. SDS was added to a final concentration of 0.5% (w/v), and the cells were mixed rapidly. The resulting stiff gel was sonicated to reduce the viscosity. Predigested pronase was added to a final concentration of 1 mg/ml and dithiothreitol to 0.001M. After digestion for 3 hr at 37°C, the mixture was extracted twice with an equal volume of phenol, saturated with 0.1M tris-HCl, pH 8.0, and once with chloroform/isoamylalcohol (24:1, v/v). The aqueous phase was adjusted to 0.1M sodium acetate, and nucleic acids were precipitated overnight at -20°C after the addition of 2 vol of ethanol. The precipitate was collected by centrifugation (10,000 g, 20 min, 8 x 50 MSE HiSpeed at

-10°C) and dissolved in 0.01M tris-HCl, 0.001M EDTA pH 7.9 and incubated with 50 µg/ml pancreatic ribonuclease (previously boiled for 10 min) at 37°C for 1 hr. Predigested pronase was then added to 50 µg/ml and incubation continued for 3 hr. The mixture was extracted twice with phenol and once with chloroform/isoamyl alcohol and precipitated with ethanol as described above. The DNA was redissolved in 0.01M tris-HCl, 0.01M NaCl, 0.001M EDTA pH 7.4, and desalted into the same buffer using a Sephadex G-25 column. The DNA prepared by this method had a mean sedimentation coefficient of 9S under denaturing conditions, and an E_{260}/E_{280} ratio of approximately 1.9. DNA concentration was measured by absorbance, assuming 1 E_{260} unit to correspond to a concentration of 50 µg/ml.

2.4.4 Centrifugation Techniques

a) Velocity Sedimentation

5 to 20% (w/v) linear sucrose gradients were prepared by layering 4 solutions containing equal volumes of 5%, 10%, 15% and 20% sucrose solutions in pollyallomer centrifuge tubes and left to diffuse overnight on ice. The neutral sucrose gradients contained either 0.01M tris-HCl, 0.01M EDTA, 0.2M NaCl pH 7.9 or 0.01M tris-HCl, 0.01M EDTA, 0.5M NaCl pH 7.0. The alkaline sucrose gradients contained 0.3M NaOH, 0.7M NaCl, 0.15% Sarkosyl and were also 5 to 20% (w/v) linear sucrose.

The volumes of gradients and sample were as follows:-

	Gradient vol (ml)	Sample vol (ml)
Beckman SW50.1	4.8	0.2
Beckman SW56	3.6	0.2
Beckman SW40	11.0	0.4
Beckman SW27	34.0	2.0

The conditions under which the velocity sedimentation runs were carried out are detailed in the legend to each figure. The gradients were fractionated by either direct puncture or upward displacement with a dense sucrose solution. The fractions were either collected onto Whatman 3MM discs directly or an aliquot spotted onto the discs for measurement of TCA precipitable radioactivity. Total radioactivity was measured by harvesting directly into scintillation vials, making the volume up to 1 ml with H_2O and adding 10 ml of triton/toluene. An aliquot from each fraction when harvested preparatively could also be assayed for total radioactivity.

b) Equilibrium CsCl

The DNA was mixed with a CsCl solution in 0.05M tris-HCl, 0.002M EDTA pH 7.4 to give an initial density of 1.70 g/cm^3. Centrifugation was carried out in a 3 ml volume in a Beckman SW50.1 rotor at 80,000 g (average) for 60 hr at 20°C. For virus samples the initial density was 1.43 g/cm^3 and centrifugation was in a 3 ml volume in a Beckman SW50.1 rotor at 80,000 g(average) for 40 hr at 20°C. All caesium chloride densities were measured by refractive index from the relationship. $\rho^{25} = 10.2402\,\eta^{25°C} - 12.648$ (Bruner and Vinograd, 1965).

All gradients were harvested by direct puncture and radioactivity was determined in the manner as for sucrose gradients.

c) Equilibrium NaI

The DNA was mixed with a NaI solution containing 0.01M tris-HCl, 0.01M EDTA pH 7.4, saturated with sodium sulphite, to give an initial density of 1.545 g/cm^3. The density was measured by refractive index from the relationship. $\rho^{25°C} = 5.3521\,\eta^{25°C} - 6.1348$ (Strayer, 1979). Centrifugation was in 5 ml amounts for 60 hr at 120,000 g in the Beckman Ti50 rotor at either 4°C or 20°C.

The gradients were harvested by direct puncture and collected into scintillation vials. A drop of β-mercaptoethanol was added and the volume was brought up to 1 ml with water and 10 ml of triton/toluene scintillation fluid was added.

2.4.5 Agarose gel electrophoresis (Hayward and Smith, 1972; McDonell et al, 1977)

a) Neutral

Agarose was dissolved at a concentration of either 1% (w/v) or 1.5% (w/v) by boiling in gel buffer (0.036M tris-HCl, 0.030M Na_2HPO_4, 0.001M EDTA pH 7.7) in a stoppered bottle. If tube gels were used (130 mm in length, 6 mm in internal diameter), the bottom ends were sealed with dialysis tubing and the hot gel solution poured into them. Once set, the dialysis tubing was punctured several times with a needle and a small portion of the gel extruded from the top by gently blowing with a rubber bulb at the opposite end. The top of the gel was then cut with a scalpel such that a flat edge was prepared. The gel was then pushed back into the gel tube by gently sucking, at the opposite end, with a rubber bulb. The electrophoresis buffer was prepared as a 10X stock solution and diluted immediately before use. The tube gels were placed in the electrophoresis tank and the gel buffer poured into the chambers. The sample (50 µl to 150 µl) was made between 5 to 10% (w/v) in sucrose and 0.025% (w/v) bromophenol blue and applied to the top of the gel by underlayering. Electrophoresis was for 3 hr at 100V for 1.5% agarose gels and 5 hr at 50V for 1.0% agarose gels. For slab gels (100 mm x 100 mm x 4 mm) the hot agarose was poured into the slab gel apparatus with dialysis tubing across the bottom to prevent leakage and a comb (with teeth 5 mm across)

was placed into the molten agarose. Once set, the comb was removed and sample application was as outlined before. Electrophoresis was for 6 hr at 60V for 1.5% agarose gels.

Radioactive bands were detected in tube gels by scintillation counting. The gels were sliced into 1 mm sections (Mickle Gel Slicer) and placed in insert scintillation vials. 0.2 ml of 0.3N NaOH was added and each vial was tightly capped and incubated at 60°C for 16 hr. The sample was then neutralised with 0.2 ml of 0.3N HCl and 4 ml of triton/toluene scintillator added. Radioactive bands in slab gels were detected by autoradiography. Gels were dried under vacuum and the X-ray film (Kodirex) was placed in direct contact with the gel for the required time at room temperature. The film was developed as described.

Unlabelled DNA bands were detected by staining with ethidium bromide (Sharp et al, 1973). Ethidium bromide (1 μg/ml in gel buffer) was added to the gels after electrophoresis and after approximately 60 min the fluorescent bands were visible by illumination with UV light. Photographs were taken using a Polaroid camera, equipped with a yellow filter, during UV illumination.

b) Alkaline

Agarose was dissolved in water by boiling, and a 1/10 volume of 10X alkaline gel buffer was added to the molten agarose. Tube gels and slab gels were prepared in an identical method to that described for neutral gels and sample application was as before. Electrophoresis for 1.5% agarose gels was for 20 hr at 50V. Before detection of radioactive bands either by slicing and counting or by autoradiography the gels were washed (3, 15 min washes) in 0.1M tris-HCl pH 7.5. If unlabelled DNA was to be detected approximately twice as much was applied and the gels washed in 0.1M tris-HCl, pH 7.5 before overnight staining in ethidium bromide (10 μg/ml in 0.1M tris-HCl pH 7.5).

2.4.6 Enzyme Incubations

A) S1 nuclease (Weigand et al, 1975)

The reaction mixture (0.3 ml) contained 0.03M sodium acetate, 0.1M NaCl, 0.002M $ZnSO_4$ pH 4.5 and 10 units of S1 nuclease. Incubation was at 37°C for various time periods. Extent of digestion was measured by the release of acid soluble radioactivity. Basically, 0.2 ml samples were taken from the reaction mixture and 0.05 ml of 6N PCA and 0.05 ml of carrier (100 µg DNA and 100 µg BSA per ml) was added. The sample was incubated on ice for 15 min and then centrifuged at 900 g for 15 min at 0°C. 0.20 ml of the supernatant fraction was taken for assay of acid soluble radioactivity. 0.05 ml of the original incubation mixture was taken for assay of total radioactivity. Radioactivity was measured by scintillation counting. The volume was made up to 1 ml with water, then 10 ml of triton/toluene scintillator was added and the sample counted.

B) DNase I

The reaction mixture (0.3 ml) contained 50 mM tris-HCl 0.01M $MgCl_2$, 0.001M $CaCl_2$, 0.1M NaCl pH 7.4 and 0.001 units of DNase I. Incubation was at 37°C for various times and percentage digestion was measured as before.

C) Micrococcal Nuclease

The reaction mixture (0.3 ml) contained 50 mM tris-HCl pH 7.4, 0.01M $CaCl_2$, 0.1M NaCl and 0.05 units of micrococcal nuclease. Incubation was at 37°C for various times and percentage digestion was measured as before.

D) ECoRI digestion

The reaction mixture (0.025 ml) contained 0.1M tris-HCl,

0.05M NaCl, 0.01M $MgCl_2$, 0.005M β-mercaptoethanol pH 7.5, 5 μg DNA and 5 units of ECoRI. Incubation was at 37°C overnight. Reaction was stopped by the addition of 0.005 ml of 0.1M EDTA and heated at 60°C for 10 min.

E) Hind III digestion

The reaction mixture contained 7 mM tris-HCl, 60 mM NaCl, 7 mM $MgCl_2$ pH 7.5, 5 μg DNA, 5 units of Hind III. Incubation was at 37°C overnight. Reaction was stopped by the addition of 0.005 ml of 0.1M EDTA and heated at 60°C for 10 min.

F) DNA polymerase

The reaction mixture contained 2 μg of MVM DNA, 10 units of DNA polymerase I (large fragment), 20 mM potassium phosphate buffer pH 7.0, 1 mM β-mercaptoethanol, 5 mM $MgCl_2$, 25% glycerol and appropriate concentrations of dNTP's and 10 μCi α-^{32}P TTP. Incubation was at 37°C for various times. Aliquots were taken for the measurement of acid precipitable radioactivity.

2.4.7 Hybridisation

DNA samples were made 0.3N NaOH and boiled for 10 min. This results in cleavage to yield molecules of approximately 9S, as measured by analytical ultracentrifugation under alkaline conditions. The fragments were then desalted into water, using a Sephadex G25 column, and lyophilised. They were resuspended in hybridisation buffer (0.01M tris-HCl, 0.001M EDTA, 0.5M NaCl, pH 7.5). An appropriate quantity of fragmented competing DNA was added and hybridisation in a 0.1 ml volume was carried out in sealed capillary tubes at 65°C for 72 hr. After the required time the hybridisation mixture was expelled into

test tubes using 200 µl of 3/2 x S1 buffer and 0.050 ml of S1 nuclease (10 units) added. The S1 sensitivity was measured as described before (section 2.4.6).

2.4.8 Electron Microscopy

A) Method for spreading double stranded DNA

All solutions for use in electron microscopy were filtered. Millipore HAWP for aqueous solutions and Millipore EWHP for ethanolic solutions. The basic technique is to spread the DNA solution over a hypophase and to adhere the DNA onto electron microscope grids (collodian coated) by gently touching the grid onto the surface of the film. The DNA spreading solution contains in a volume of 100 µl, 25 µl of 2M NH_4 Acetate, 4 mM EDTA pH 7.5, 50 µl of a DNA solution of 1-5 µg/ml in H_2O and 25 µl of 0.4 µg/ml cytochrome c. The hypophase is 0.25M NH_4 Acetate pH 7.5. After the grid is touched to the surface it is stained with uranyl acetate (5×10^{-3} Muramyl acetate in 50 mM HCl diluted 1:100 in 90% ethanol) for 20 sec, rinsed in 95% ethanol for 10-15 sec and put on filter paper to dry. The grids were then rotary shadowed with platinum palladium (1:4). The grids were viewed on a Philips 300 electron microscope. An internal standard was included to enable the molecular weights of the sample DNA to be calculated. The lengths of the molecules were measured by using a graphics tablet attached to a PDP11 computer.

B) Method for spreading single stranded DNA

Using the previous techniques single stranded DNA collapses upon itself by random re-annealing thus making the measurement of single stranded DNA lengths impossible. Formamide is included in

both the spreading solution and the hypophase in order to cause the melting out of the re-annealed region and thus the extension of the single stranded DNA. The spreading solution contained 0.5 μg/ml DNA, 0.02 to 0.1 mg/ml cytochrome c, 0.1M tris-HCl, 10 mM EDTA pH 8.5 in 40% formamide. The hypophase is 10% formamide, 10 mM tris-HCl, 1 mM EDTA (pH 8.5). Spreading and preparation of grids are as before. The DNA molecules were measured as before.

2.4.9 Autoradiography

Dried gels were marked for identification with ^{35}S-sulphate ink and then a piece of Kodirex X-ray film was secured on top. After an appropriate period of time the films were developed. The films were developed for 4-6 min in Kodak 0X-40 developer, washed briefly in water and fixed in Kodax FX-90. Processing was performed in a Kodak P3 X-ray film processing unit.

2.4.10 Measurement of radioactivity

Acid precipitable radioactivity was measured by spotting an aliquot of the sample or a fraction from a gradient onto Whatman 3MM discs. The discs were washed 3 times in 5% TCA by a batch technique, allowing between 5 to 10 ml for each disc. They were dried in ethanol and ether. The discs were then placed in scintillation vials and 0.5 ml of 1M hyamine hydroxide (in methanol) was added and the vials were capped and placed in a 60°C oven for 30 min. 5 ml of toluene PPO scintillator was added to each vial.

Samples for the measurement of acid soluble radioactivity have been previously outlined (section 2.4.6). Samples for the measurement of total radioactivity are prepared by making the volume of the aliquot up to 1 ml with water and adding 10 ml of triton/toluene scintillator.

Radioactivity was measured in a Searle liquid scintillation counter if only one label was present. The efficiency was 20% for ^{3}H samples prepared using hyamine hydroxide and toluene PPO, and 35% for ^{3}H samples prepared using triton/toluene. If dual label counting was required, e.g. $^{3}H/^{14}C$ or $^{3}H/^{35}S$, a Beckman scintillation counter was used. The spillover from the ^{3}H channel to the ^{14}C channel was negligible and the spillover from the ^{14}C channel to the ^{3}H channel is constant over a fairly wide range of efficiency. It is between 11% to 13% for all the samples prepared for scintillation counting by the methods outlined in this thesis. The spillover of either ^{3}H or ^{32}P into each other's channel was assumed to be negligible.

3. Results

3.1 Time course of Infection

The time course of infection of MVM in BHK-21/C13 cells was analysed in order to choose an appropriate time post-infection at which to study the events of DNA synthesis. This was achieved by labelling MVM-infected and mock-infected BHK-21/C13 cells with ^{3}H-thymidine at regular 2 hr intervals throughout the infectious cycle (Fig. 13). The radioactivity incorporated into viral DNA species was determined by using the selective extraction procedure of Hirt (1967) as modified for MVM (Tattersall et al, 1973). In this procedure the cells are lysed with SDS and digested with pronase. Then the cellular DNA is precipitated with high concentrations of NaCl while the viral DNA remains in the supernatant fraction. Thus, by using this method, one can measure the rate of viral DNA synthesis without having the problem of contaminating cellular DNA. Fig. 13 illustrates this analysis. There is little cellular contamination of the viral DNA fraction as judged by the presence of radioactivity in this fraction in the mock-infected cell extracts. Viral DNA synthesis increases steadily from 8 hpi to reach a peak at between 14 to 16 hpi. After this time there is a steady decrease in the rate of DNA synthesis. The following experiments which deal with Parvovirus DNA replication were performed at 16 hpi, the time just after the peak of viral DNA synthesis. Sixteen hpi was chosen because it was possible to obtain a high efficiency of incorporation of radioactivity into viral DNA species, at a time when infectious virus is being produced (Tattersall et al, 1973).

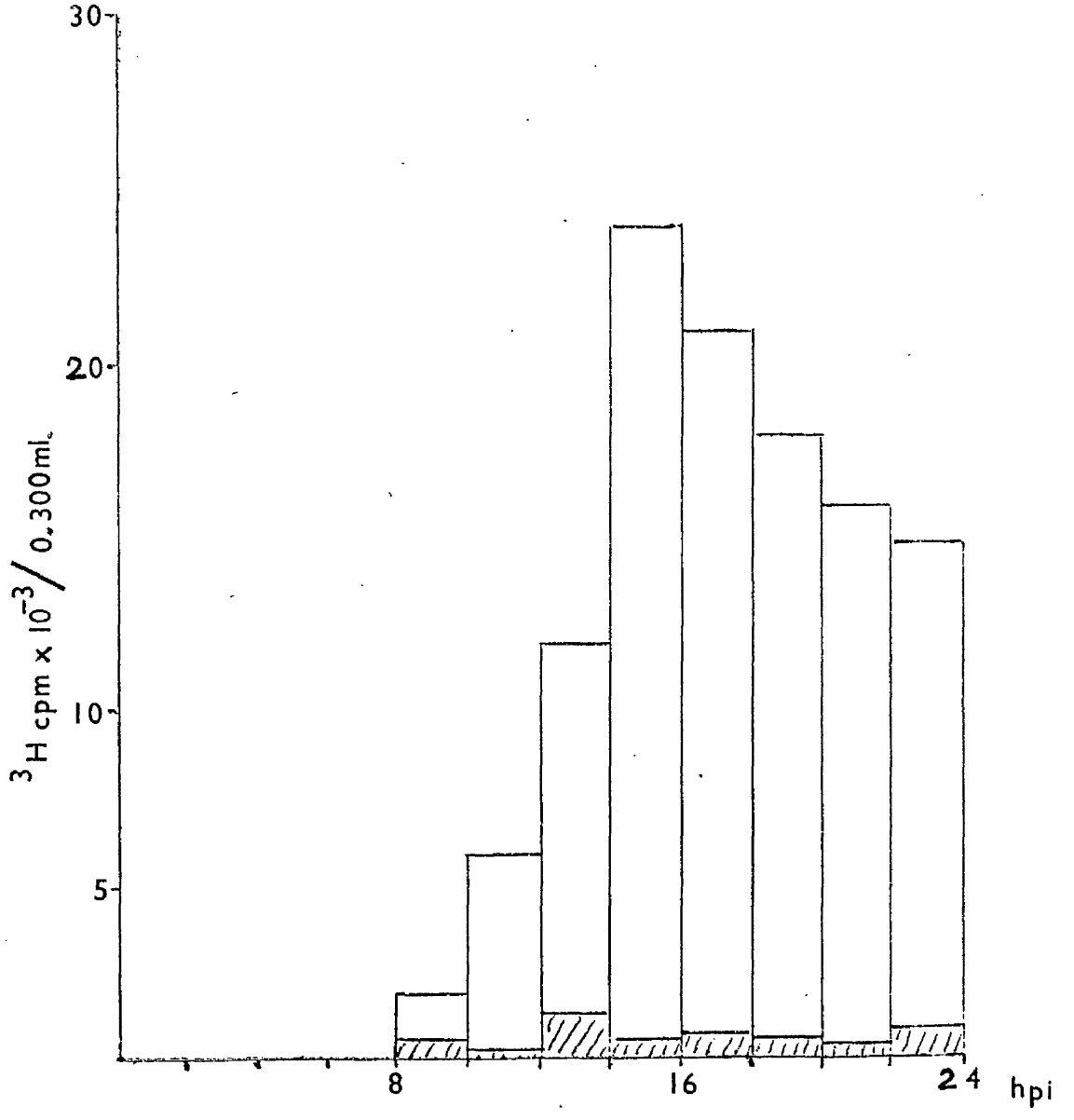
3.2 Nucleoprotein Complex Extraction Procedure

Sixteen hr after infection of either BHK-21/C13 cells or A9

Fig. 13

Rate of incorporation of ^{3}H-thymidine into low molecular weight DNA in MVM-infected and mock-infected BHK-21/C13 cells.

MVM-infected and mock-infected BHK-21/C13 cells were labelled with ^{3}H thymidine (5 μCi/ml) in regular 2 hr intervals up to 24 hpi and the low molecular weight DNA extracted by the Hirt/Pronase method. A 0.3 ml aliquot of the supernatant fraction was removed for the measurement of acid insoluble radioactivity as described in Materials and Methods. The clear areas represent the radioactivity present in a 0.3 ml sample of MVM-infected Hirt/Pronase supernatant fractions and the hatched areas represent the radioactivity present in a 0.3 ml sample of mock-infected Hirt/Pronase supernatant fractions.


30
20
10
5
^{3}H cpm x 10^{-3} / 0.300mL
8
16
24
hpi

cells with MVM, ^{3}H-thymidine was added for 2 hr. The cells were then treated with 0.25% Triton X-100 and a soluble extract was obtained. The extract was analysed on a neutral sucrose gradient and two peaks of radioactivity were observed (Fig. 14). Essentially no ^{3}H-thymidine labelled peaks were present in the mock-infected cells. The slower sedimenting peak was at the position of 21S SV40 form I DNA and the faster sedimenting peak was at the approximate position of virus. In A9 cells, the faster-sedimenting peak is present in greater amounts, whereas, in the BHK-21/C13 cells the opposite situation occurs. There is also a difference in the quantity of total radioactivity present between the two cell lines, infected with MVM. This may, however, be due to the efficiency of infection rather than a difference in the percentage of viral DNA which is extractable. On the assumption that the replication process of MVM is similar in both A9 cells and BHK-21/C13 cells then the disparity between the amounts of each viral DNA species, observed during sedimentation analysis, would be due to the triton extraction procedure. Clearly, the selectivity of extraction of the viral DNA species between the two cell lines in this protocol may prove to be advantageous but as a method of assessing the in vivo significance of nucleoprotein species it is open, initially, to ambiguous interpretation of results. It was decided, therefore, to devise a method of nucleoprotein complex extraction which was not dependent on the cell line.

3.2.1 Effect of NaCl concentration on the extraction of DNA from the nuclei of infected cells

In a first step to the derivation of a method for the quantitative extraction of viral nucleoprotein complexes from MVM

Fig. 14

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

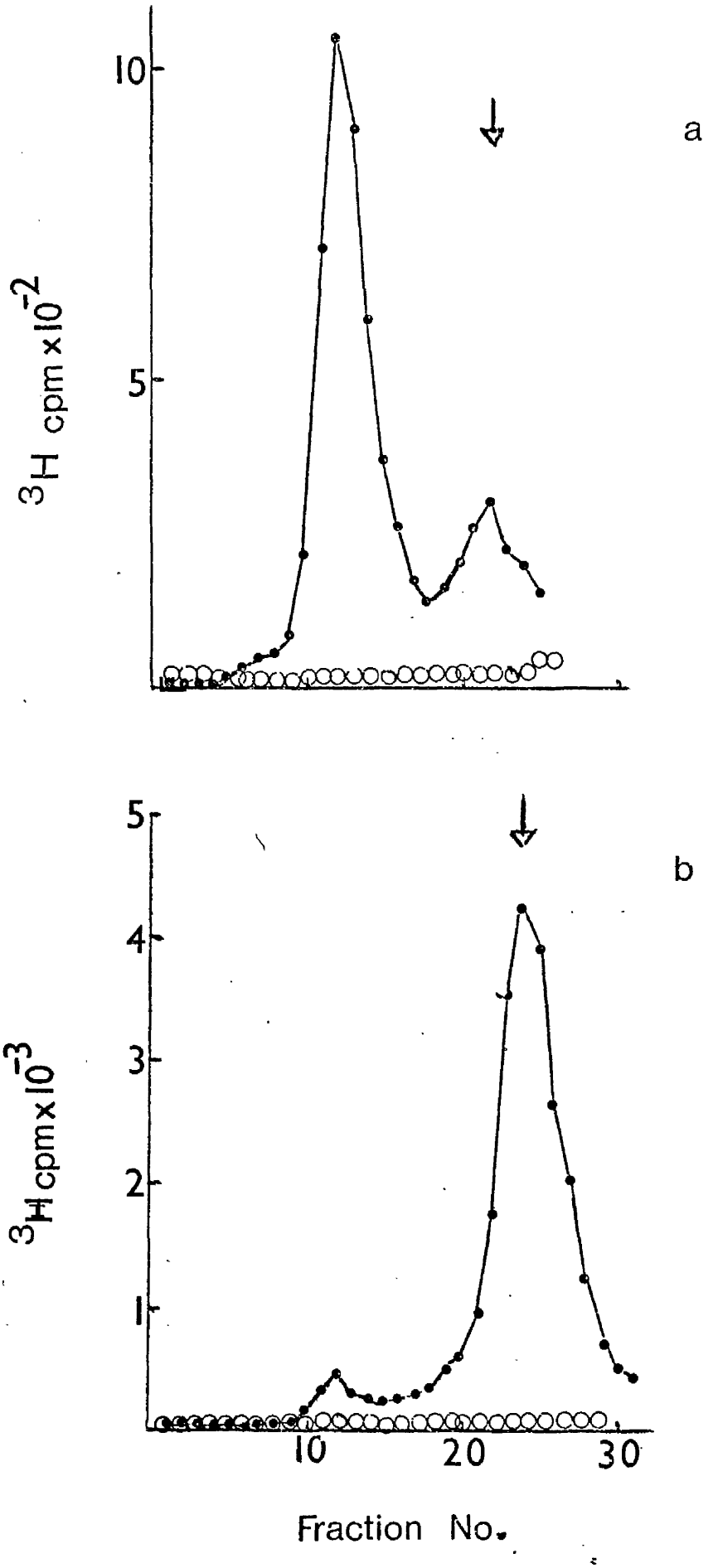
Infected A9 cells or BHK-21/C13 cells were labelled with ^{3}H-thymidine (10 μCi/ml) for 2 hr commencing at 16 hpi. The infected cells, on 90 mm plastic petri dishes, were lysed with 0.9 ml of 0.25% triton X-100 in 10 mM tris-HCl, 10 mM EDTA pH 7.9 and incubated at 20°C for 1 hr after which 0.1 ml of 2M NaCl was added and the cellular debris removed by centrifugation. 0.2 ml samples of the supernatant fractions (triton extracted DNA) were sedimented in 5-20% neutral sucrose gradients containing 0.2M NaCl for 90 min at 180,000 g (average) in a Beckman SW56 rotor at 4°C. The vertical arrow denotes the position of 21S ^{14}C-SV40 form I DNA.

(a) Infected A9 cells.

(b) Infected BHK-21/C13 cells.

●——● MVM infected.

○——○ mock infected.


10
5
^{3}H cpm x10^{-2}
a
5
4
3
2
1
^{3}H cpm x10^{-3}
b
10
20
30
Fraction No.

infected cells the effect of increasing concentrations of NaCl was studied. Nuclei were prepared from infected BHK-21/C13 cells using the non-ionic detergent, NP40. Hypotonic conditions were used, 10 mM tris-HCl, 10 mM EDTA pH 7.0, to minimise the leakage into the cytoplasmic fraction of nucleoprotein complexes during the preparation of the nuclei. The cells were then treated with NaCl to yield final concentrations of between 0 (no added NaCl) to 0.5M. As shown in Fig. 15 very little cellular DNA, as determined from mock infected cells, is released into the supernatant fraction at these NaCl concentrations. However, if the NaCl concentration is increased to above 0.5M there is an almost equivalent amount of radioactivity present in the supernatant fractions of both MVM infected and mock infected BHK-21/C13 cells. As the NaCl concentration is increased from 0 to 0.5M NaCl a greater quantity of labelled DNA is present in the MVM infected supernatant fraction. The morphology of the nuclei changes at these various NaCl concentrations. At 0.2M and 0.3M intact nuclei can be observed by phase contrast microscopy. However, at 0 (no added NaCl), 0.4M and 0.5M NaCl no nuclei can be observed and the nuclear suspension is very viscous suggesting that nuclear lysis has occurred and therefore chromatin has been released. The nucleoprotein species released at the five NaCl concentrations and also the cytoplasmic fraction, which contained between 10 to 20% of the levels of radioactivity present in the 0.5M nuclear extract, were analysed by neutral sucrose gradient velocity sedimentation (Fig. 16). This demonstrated that the cytoplasmic fraction contained the majority of the faster sedimenting species (designated CI) and that the effect of the increasing NaCl concentration was to increase the amount of the slower sedimenting species (designated CII) released into the super-

Fig. 15

The effect of NaCl concentration on the extraction of DNA from nuclei prepared from MVM-infected and mock-infected BHK-21/C13 cells.

Infected cultures of BHK-21/C13 cells were labelled with ^{3}H-thymidine (10 μCi/ml) for 2 hr commencing at 16 hpi. Nuclei were prepared from the infected cells by lysis with 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0 as described in Materials and Methods. The nuclei were resuspended in RSB and an equal volume of the appropriate NaCl concentration in RSB was added. After incubation on ice for 30 min, supernatant fractions were prepared by centrifugation of the samples at 10,000 g for 20 min. 0.050 ml samples were removed from the supernatant fractions for the determination of acid insoluble radioactivity.

●——● MVM infected BHK-21/C13 cells

●- - -● mock infected BHK-21/C13 cells

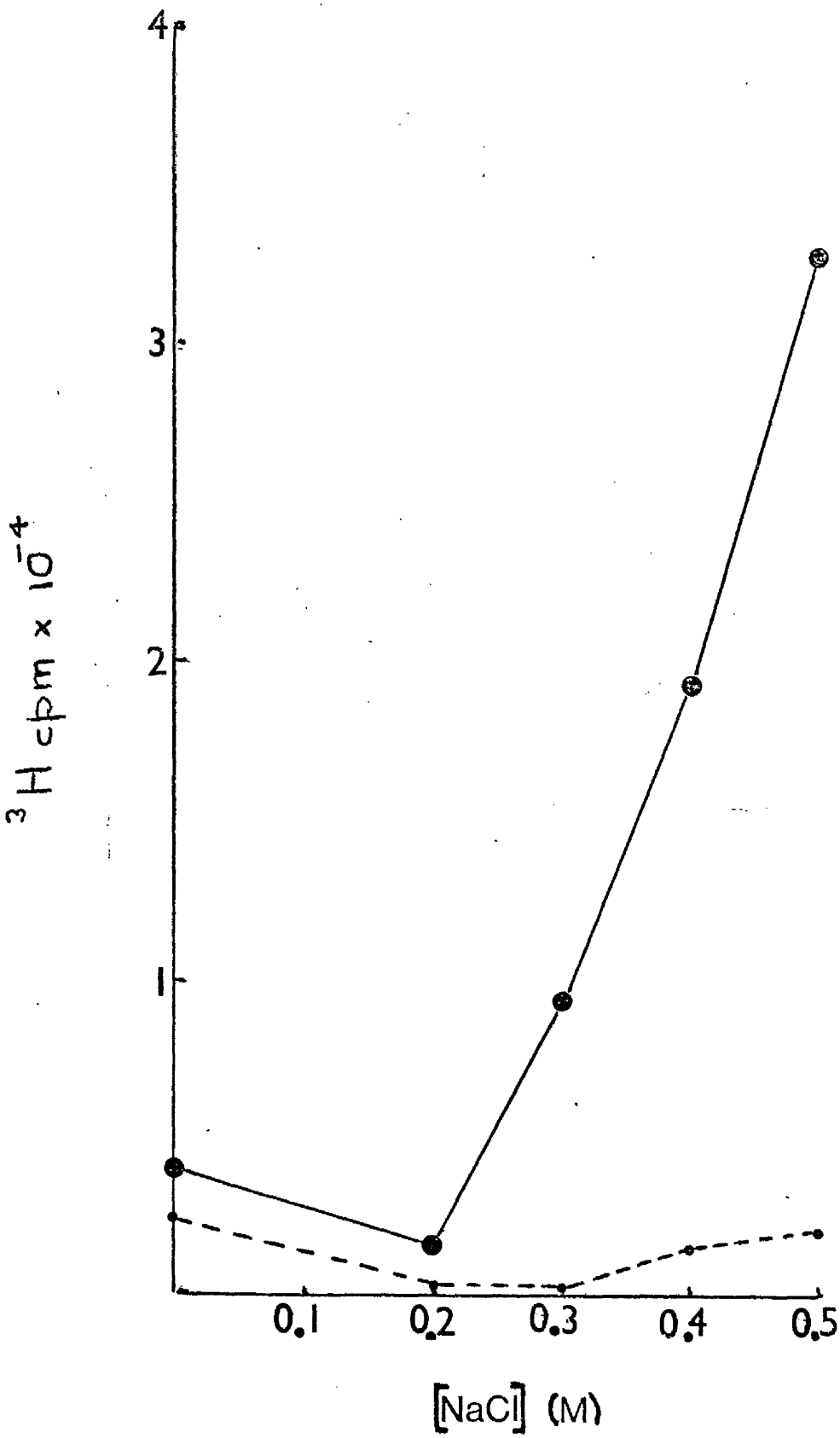

4
3
2
1
^3H cpm x 10^{-4}
0.1
0.2
0.3
0.4
0.5
[NaCl] (M)

Fig. 16

Sedimentation analysis of the DNA from NaCl extracts of MVM infected and mock infected BHK-21/Cl3 cells.

0.2 ml samples of NaCl extracted nuclei from MVM infected or mock infected BHK-21/Cl3 cells prepared as indicated in Fig. 15 and a 0.2 ml sample of the cytoplasmic fraction were made 0.5M with respect to NaCl and sedimented in 5-20% neutral sucrose gradients containing 0.5M NaCl for 90 min at 180,000 g (average) in a Beckman SW56 rotor at 4°C. Sedimentation is from right to left.

A cytoplasmic fraction

B no added NaCl nuclear extract

C 0.2M NaCl nuclear extract

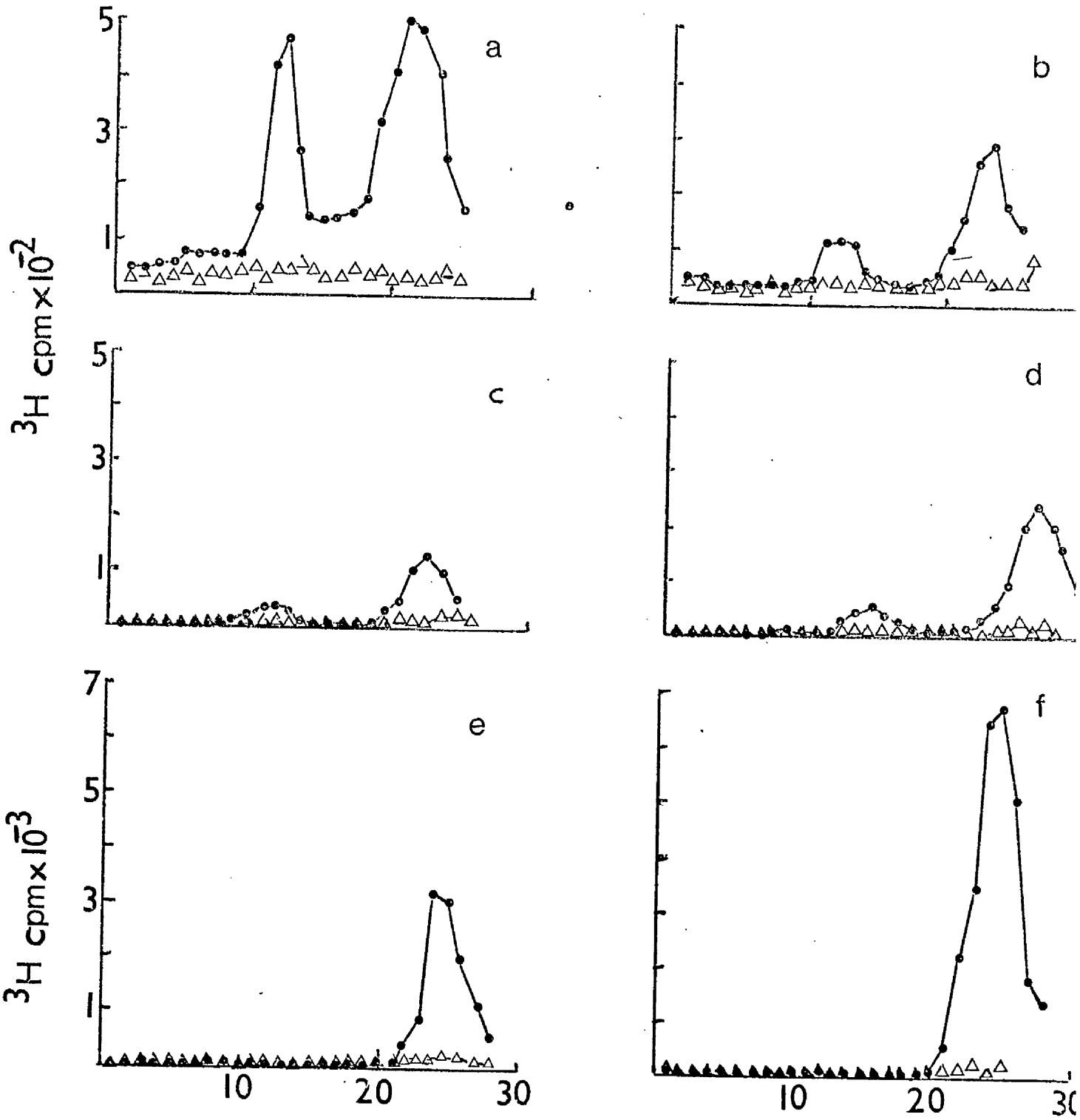
D 0.3M NaCl nuclear extract

E 0.4M NaCl nuclear extract

F 0.5M NaCl nuclear extract

●——● MVM infected

△——△ mock infected


a
b
c
d
e
f
5
3
1
7
10
20
30
^3H cpm$\times 10^{-2}$
^3H cpm$\times 10^{-3}$
Fraction No.

natant fraction. An essentially similar set of results were obtained with MVM infected A9 cells except that the percentage of DNA extracted at lower NaCl concentrations was less when compared to MVM infected BHK-21/C13 cells (Fig. 17). Sedimentation analysis of the A9 extracts on neutral sucrose gradients revealed minor differences between the cell lines (Fig. 18). In the case of MVM-infected A9 cells, the cytoplasmic fraction contained the majority of the CI species, however, since it was more difficult to prepare nuclei free of contaminating cytoplasmic debris, CI was present in the salt extractions of nuclei. Taken together these results would explain, at least in part, why the slower sedimenting species is more difficult to extract from MVM-infected A9 cells, with the triton X-100 method, than from MVM-infected BHK-21/C13 cells. Thus, the major labelled species in MVM-infected cells, whether A9 or BHK-21/C13, is CII (the slower sedimenting species). Also, because it was possible to obtain BHK-21/C13 nuclei essentially free of contaminating cytoplasmic debris, this cell line was chosen in which to perform the majority of the following experiments.

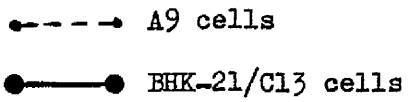
3.2.2 The effect of pH on the extraction of virus specific components from MVM-infected BHK-21/C13 cells

Unlike the previous experiments, nuclei were not separated from cytoplasm because there was the possibility of a differential effect of pH on the efficiency of extraction of nucleoprotein complexes therefore species may be extracted from, or retained in, a compartment of the cell at different pH values thus making interpretation difficult if the cell was divisioned before analyses. The infected cells were lysed, directly, in NP40, at different pH's, and the release

Fig. 17

Comparison of the effect of NaCl on the extraction of DNA from nuclei prepared from either MVM infected BHK-21/C13 cells or MVM infected A9 cells.

BHK-21/C13 cells and A9 cells were infected with MVM and labelled with ^{3}H thymidine (10 μCi/ml) for 2 hr commencing at 16 hpi. Nuclei were prepared and extracted with various NaCl concentrations as described in the legend to Fig. 15. 0.050 ml samples were removed from the supernatant fractions and acid insoluble radioactivity was measured. The results are expressed as a percentage of the acid insoluble radioactivity which was present in the 0.5M NaCl extract.



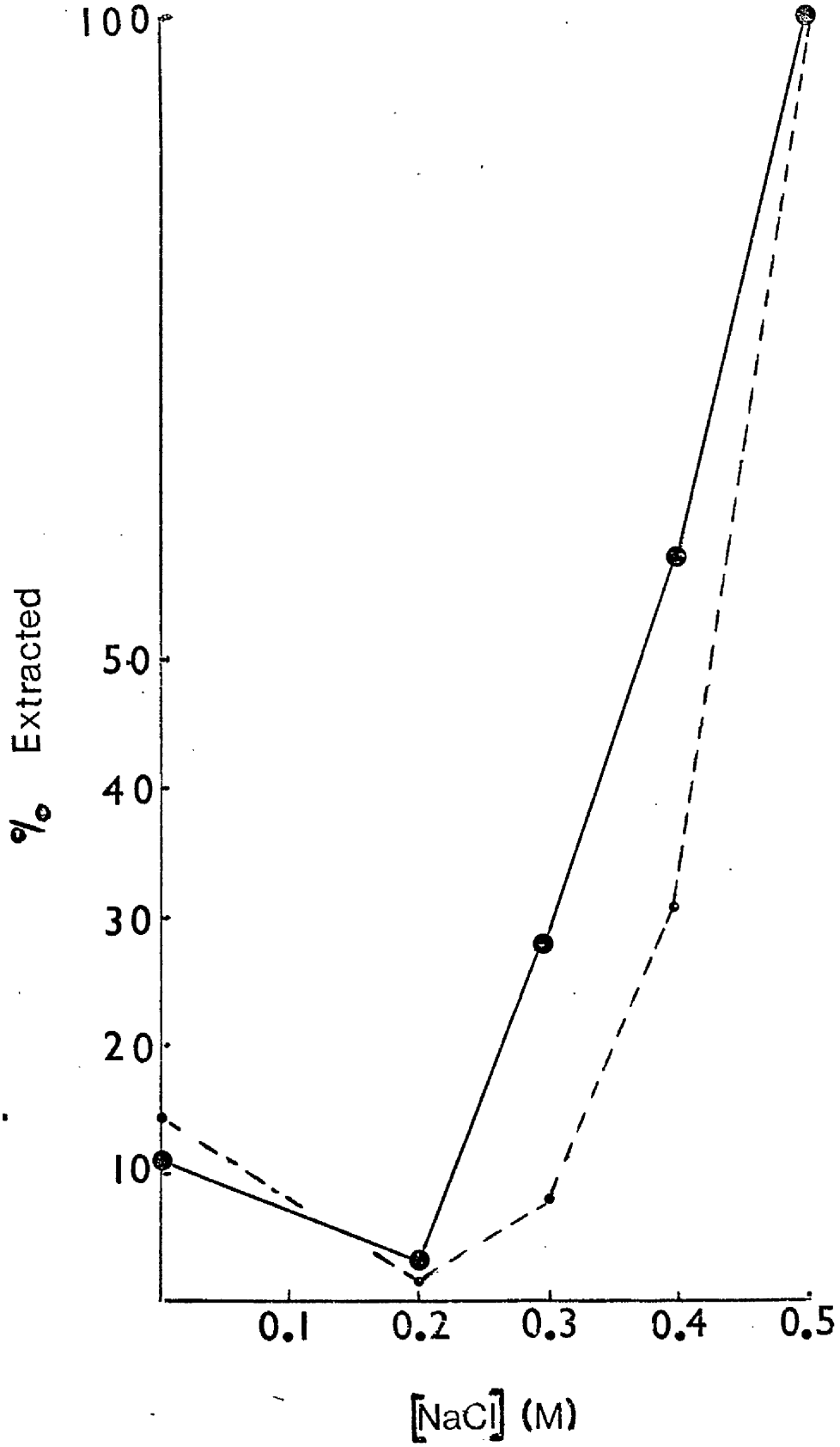

100
50
40
30
20
10
% Extracted
0.1
0.2
0.3
0.4
0.5
[NaCl] (M)

Fig. 18

Sedimentation analysis of the DNA from NaCl extracts of MVM-infected A9 cells.

0.2 ml samples of DNA extracted from nuclei of MVM-infected A9 cells, prepared as described in the legend to Fig. 17 and a 0.2 ml sample of the cytoplasmic fraction were sedimented under the conditions outlined in the legend to Fig. 16.

A cytoplasmic fraction

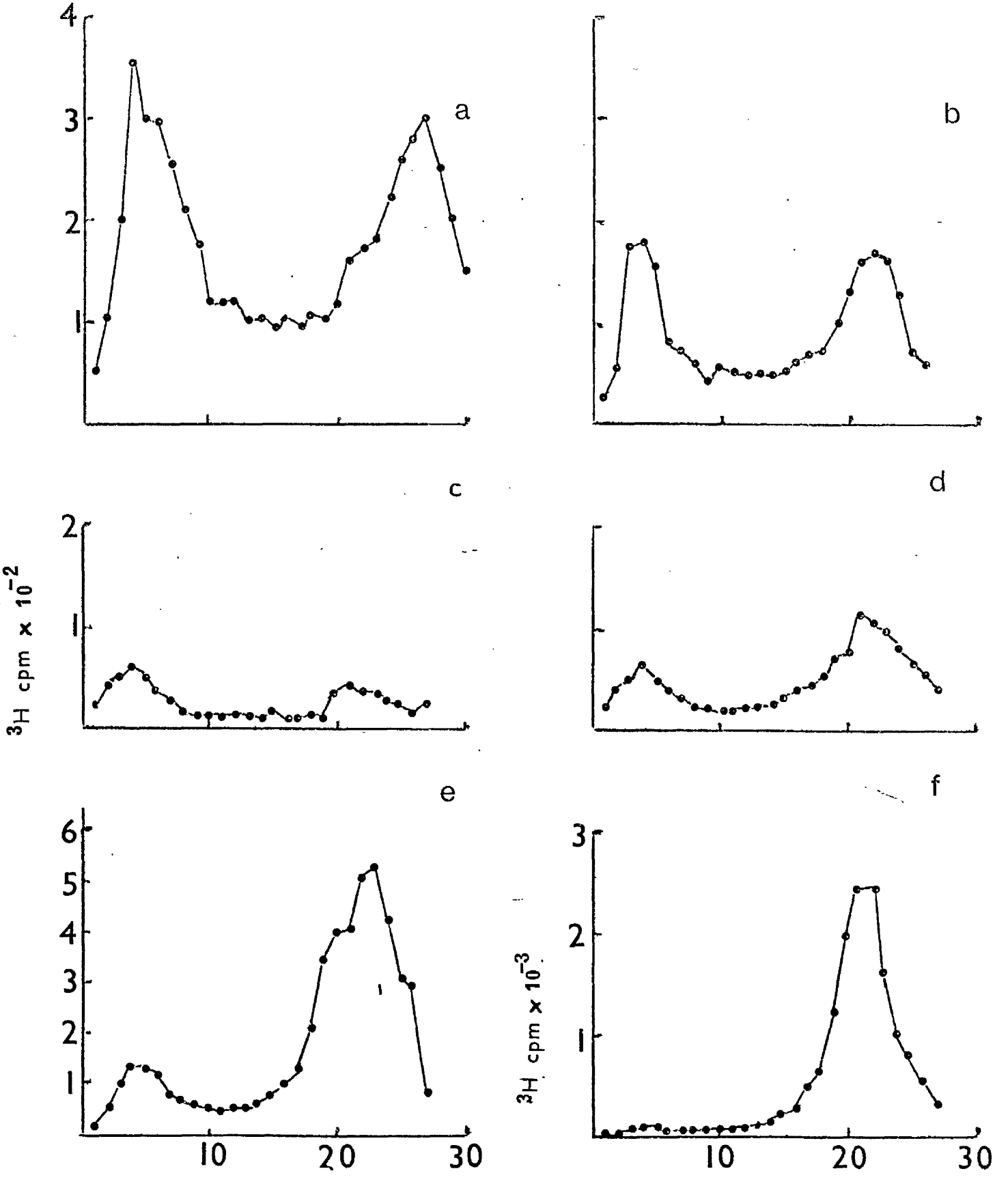
B no added NaCl, nuclear extract

C 0.2M NaCl nuclear extract

D 0.3M NaCl nuclear extract

E 0.4M NaCl nuclear extract

F 0.5M NaCl nuclear extract


a
b
c
d
e
f
^{3}H cpm x 10^{-2}
^{3}H cpm x 10^{-3}
Fraction No.

of nuclei was confirmed by phase contrast microscopy. NaCl was added, to the lysate, to a final concentration of 0.5M and the supernatant fraction, after centrifugation, analysed by velocity sedimentation in neutral sucrose gradients (Fig. 19). Below pH 7.0 there was cellular DNA contamination of the extracts as judged by the presence of significant amounts of rapidly sedimenting radioactivity in the mock infected cell extracts. As the pH is increased from 7.0 to 8.5 there is a decrease in the total amount of extractable radioactivity. The sucrose gradients were separated into three sections designated CI, intermediate and CII (Fig. 20). The major difference occurring between pH 7.0 to pH 8.5 is a significant decrease (40%) in the quantity of CII which is present, although there are also significant differences in the amounts of both CI and the intermediate fractions extracted between pH 7.5 and pH 8.5.

3.2.3 The effect of NP40 on the extraction of virus specific components from MVM-infected BHK-21/C13 cells

The non-ionic detergent, NP40, is an essential part of the extraction procedure. MVM-infected BHK-21/C13 cells were either treated with 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA, pH 7.0 or 10 mM tris-HCl, 10 mM EDTA pH 7.0 and allowed to stand on ice for 10 to 15 min. The NP40 treated samples contained nuclei whereas in the other sample the cells were swollen but few were broken, as judged by phase contrast microscopy. The samples were made 0.5M with respect to NaCl and the supernatant fractions analysed by velocity sedimentation on neutral sucrose gradients. Fig. 21 illustrates that the effect of NP40 is to extract both CI and CII. CI is not observed in the hypotonic buffer treated extracts and CII is considerably reduced in these extracts.

Fig. 19

Sedimentation analysis of the effect of pH on the extraction of DNA from MVM-infected and mock-infected BHK-21/Cl3 cells

Infected BHK-21/Cl3 cells were labelled with ^{3}H thymidine (10 μCi/ml) for 90 min commencing at 16 hpi. The cells were lysed in a solution of 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA at various pH's. NaCl was added to a final concentration of 0.5M and 0.2 ml samples of each supernatant fraction, after centrifugation at 10,000 *g* for 20 min, were analysed by sedimentation in 5-20% neutral sucrose gradients, containing 0.5M NaCl, for 90 min at 180,000 *g* (average) in a Beckman SW56 rotor at 4°C.

(a) Extraction at pH 8.5

(b) Extraction at pH 8.0

(c) Extraction at pH 7.5

(d) Extraction at pH 7.0

●——● MVM-infected

□——□ mock-infected

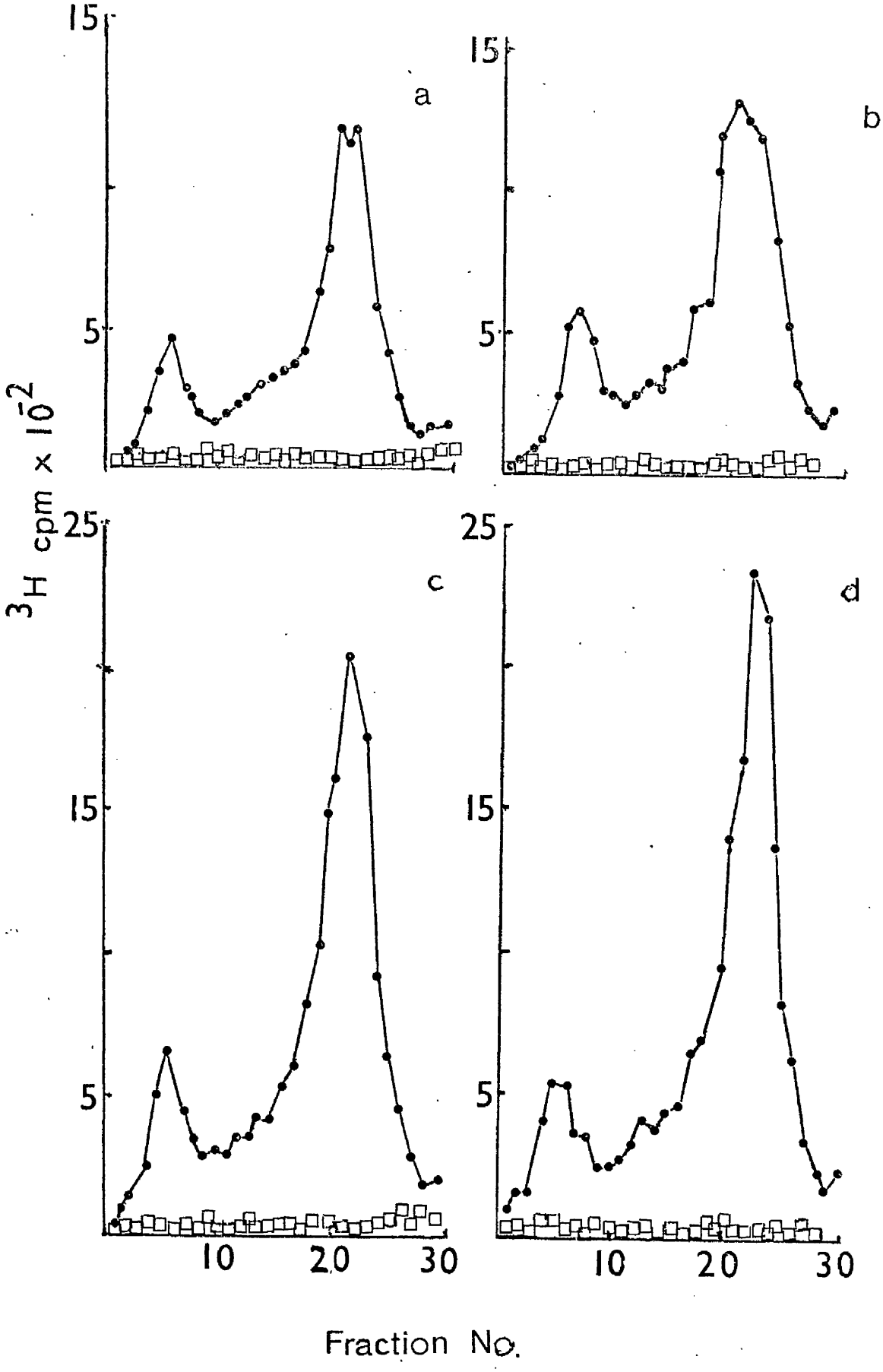

15
5
a
15
5
b
25
15
5
c
25
15
5
d
10
20
30
10
20
30
^{3}H cpm x 10^{-2}
Fraction No.

Fig. 20

The effect of pH on the extraction of DNA species from MVM infected BHK-21/C13 cells

The yields of "fast sedimenting" (designated CI) intermediate, and "slow sedimenting" (designated CII) DNA in the various pH extracts was estimated by summation of acid insoluble radioactivity present in fractions 4 to 9 (CI), fractions 10 to 18 (intermediate) and fractions 19 to 26 (CII) of neutral sucrose gradients, illustrated in Fig. 19. The total acid insoluble radioactivity in each region of the sucrose gradient at each pH is expressed relative to the amount present in the same region in the pH 7.0 extract.

A total sucrose gradient

B CI region

C intermediate region

D CII region

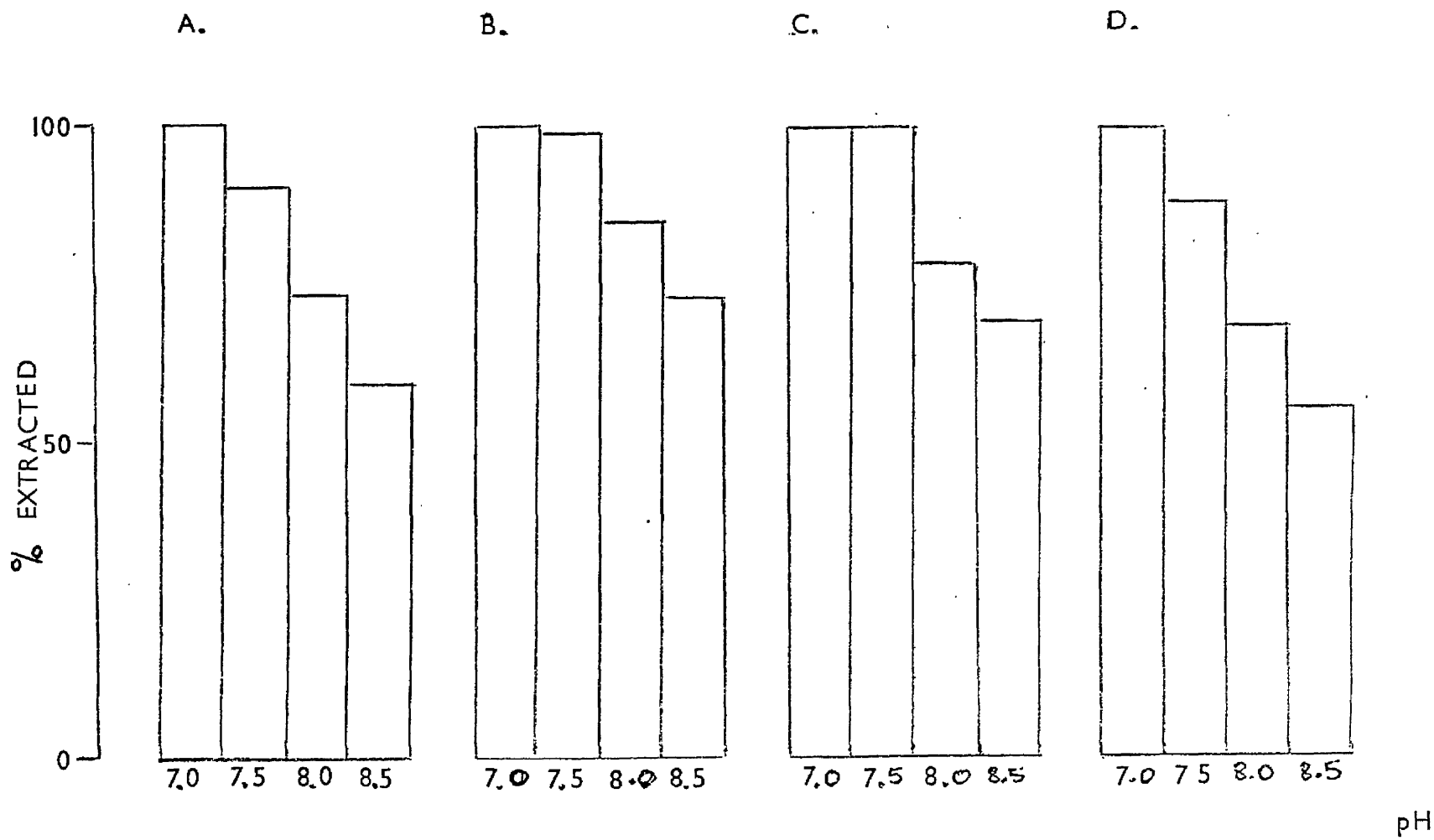
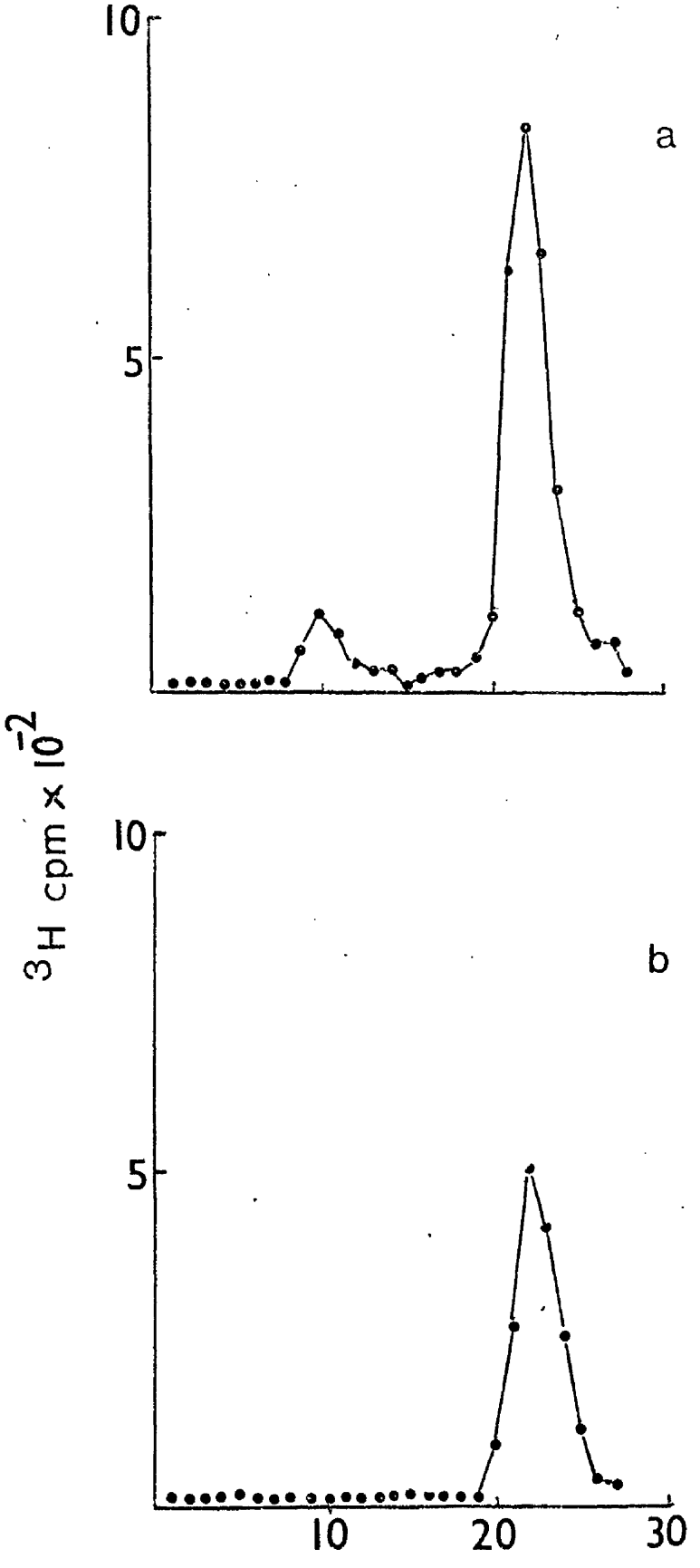

A.
B.
C.
D.
100
50
0
% EXTRACTED
7.0 7.5 8.0 8.5
7.0 7.5 8.0 8.5
7.0 7.5 8.0 8.5
7.0 7.5 8.0 8.5
pH

Fig. 21

Sedimentation analysis of the effect of NP40 on the extraction of DNA species from MVM infected BHK-21/C13 cells.

MVM-infected BHK-21/C13 cells were labelled with ^{3}H thymidine (10 μCi/ml) for 60 min commencing at 16 hpi. The infected cells were either treated with (a) 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0 or (b) 10 mM tris-HCl, 10 mM EDTA pH 7.0. After addition of NaCl to 0.5M 0.2 ml samples of the supernatant fraction, after centrifugation, were analysed by sedimentation in 5-20% linear sucrose gradients containing 0.5M NaCl. Centrifugation was as detailed in the legend to Fig. 16.


10
5
a
^{3}H cpm x 10^{-2}
10
5
b
10
20
30
Fraction No.

3.2.4 An estimation of the quantity of viral DNA within MVM-infected cells which is extracted by the NaCl-NP40 method

The maximum quantity of viral DNA containing species which can be extracted by this method designated the NaCl-NP40 method is at 0.5M NaCl with the 0.5% (v/v) NP40 solution at pH 7.0. The question remains, however, as to what percentage of the total viral DNA in the infected cell is extracted by these procedures. In order to estimate this quantity a method which utilises the direct lysis of infected cells without prior fractionation was used. The infected cells are either lysed with SDS under neutral conditions or SDS under alkaline conditions followed by centrifugation on sucrose gradients to separate the high molecular weight cellular DNA from the low molecular weight viral DNA. Thus, the amount of radioactivity in the low molecular weight region of the sucrose gradient of total cells was compared to the amount of radioactivity present in the same region of the sucrose gradient of DNA extracted by the NaCl-NP40 method. Table 6 indicates that 65% of the total viral DNA in the infected cell was extracted by the NaCl-NP40 method, since the alkaline lysis conditions are more likely to be representative of the total amount of viral DNA in the cell.

3.3 Isolation and characterisation of the viral nucleoprotein complexes from MVM-infected BHK-21/C13 cells

3.3.1 Slow sedimenting species, CII

The slower sedimenting nucleoprotein complex, designated CII, was purified from the high salt (0.5M) nuclear extract of MVM infected BHK-21/C13 cells by neutral sucrose gradient centrifugation. Essentially no radioactivity was present in the mock-infected extracts (Fig. 22B).

Table 6. Comparison of the amount of low molecular weight DNA extracted from MVM-infected BHK-21/C13 cells by different extraction procedures

	NaCl-NP40 method	Total cells
+ neutral SDS	65	62
‡ alkaline SDS	65	100

+MVM-infected BHK-21/C13 cells were labelled with ^{3}H thymidine (15 μCi/ml) between 16-18 hpi. The cells were resuspended at a concentration of 5 x 10^{5} cells/ml in SSC. 0.180 ml of this suspension was gently mixed with 0.010 ml of SDS and self digested pronase added to a final concentration of 2 mg/ml. After 1 hr at 37°C the lysate was poured onto a 15 to 30% neutral sucrose gradient. 0.180 ml of a NP40-NaCl extract of MVM infected BHK-21/C13 cells, labelled as above, was prepared as described in the legend to Fig. 19, diluted to give a final concentration of 0.15M NaCl and treated with 0.5% SDS and 2 mg/ml self digested pronase for 1 hr at 37°C and layered on a 15-30% neutral sucrose gradient. Centrifugation was for 3 hr at 215,000 g (average) at 25°C in a Beckman SW50.1 rotor. The acid insoluble radioactivity in the low molecular weight region of each gradient was totalled.

‡0.15 ml of the cell suspension, described above, was carefully pipetted into the upper layer, which consisted of 0.1 ml of 0.3M NaOH 0.001M EDTA 0.5% (w/v) SDS, of a 15 to 30% alkaline sucrose gradient. 0.15 ml of a NP40-NaCl extract of MVM infected BHK-21/C13 cells,

prepared as described above, diluted to give a final concentration of 0.15M NaCl was treated in an identical manner. After incubation in the dark, overnight at 25°C, centrifugation was for 3 hr at 215,000 g (average) at 25°C in a Beckman SW50.1 rotor. The acid insoluble radioactivity in the low molecular weight region of each gradient was totalled.

The results are expressed as the percentage of radioactivity present in the low molecular weight region of each gradient relative to that amount present, from an equal number of cells, in the low molecular weight region of total cells under alkaline-SDS conditions.

The ^{3}H-thymidine labelled material sediments heterogenously, with a leading edge towards the greater S values, therefore, fractions from two regions of the sucrose gradient were pooled and taken for further analysis. CII pool A constitutes the main band of the material and CII pool B constitutes the leading edge. These species were shown to consist of at least two separate entities by resedimentation on neutral sucrose gradients (Fig. 23). Thus the leading edge which is present on CII is due to the presence of material which sediments at a faster rate and is not due to an artifact of the sedimentation analysis. The sedimentation coefficient of CII pool A was determined relative to SV40 DNA, form I, which has a sedimentation coefficient of $21S^{o}_{20.w}$ (Fig. 24). Therefore, CII pool A sediments at a position slightly in front of SV40 DNA at approximately 22S. The sedimentation coefficient of CII pool B can now be calculated if one assumes a value of 22S for CII pool A. Thus, CII pool B sediments at approximately 30S (Fig. 23). These S values can be converted to molecular weight if one assumes that the relationship determined by Studier (1965) between linear double stranded DNA and molecular weight holds for nucleoprotein complexes. The necessary assumption is that the binding of protein to the DNA molecule does not radically affect the shape of the molecule. If this is the case then the molecular weight difference between 30S and 22S would correspond to approximately a doubling.

The 22S species, CII pool A, does not consist entirely of DNA but is instead in a DNA and protein complex. This was demonstrated by the change in sedimentation coefficient which occurred upon treatment of CII pool A with SDS and pronase (Fig. 25). CII pool A changes from a sedimentation coefficient of 22S to a sedimentation coefficient

Fig. 22

Preparative sedimentation analysis of the DNA extracted from MVM infected BHK-21/C13 cells by the NP40-NaCl method.

MVM-infected and mock-infected BHK-21/C13 cells were labelled with ^{3}H thymidine (25 μCi/ml) for 2 hr commencing at 16 hpi. The cells were lysed with 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0. The cytoplasmic fraction, isolated after low speed centrifugation, was made 0.5M with respect to NaCl, and sedimented in a 5-20% neutral sucrose gradient, containing 0.5M NaCl for 3 hr at 173,000 g (average) at 4°C in a Beckman SW40 rotor. After harvesting a 0.050 ml sample was taken from each fraction and acid insoluble radioactivity measured. The fractions indicated were pooled and dialysed against 50 mM tris-HCl, 5 mM EDTA pH 7.5 (a). The nuclei were washed three times in RSB then resuspended in RSB at a concentration between 10×10^{6} to 50×10^{6} nuclei/ml and an equal volume of 1.0 M NaCl was added. The supernatant fraction, after centrifugation for 20 min at 10,000 g at 4°C, was sedimented in a 5-20% neutral sucrose gradient, containing 0.5M NaCl for 16 hr at 90,000 g (average) at 4°C in a Beckman SW40 rotor. The fractions indicated were pooled and dialysed against 50 mM tris-HCl, 5 mM EDTA pH 7.5 (b). The pooled fractions are designated

1. CI
2. CII pool A
3. CII pool B.

●——● MVM-infected.

●- - -● mock-infected.

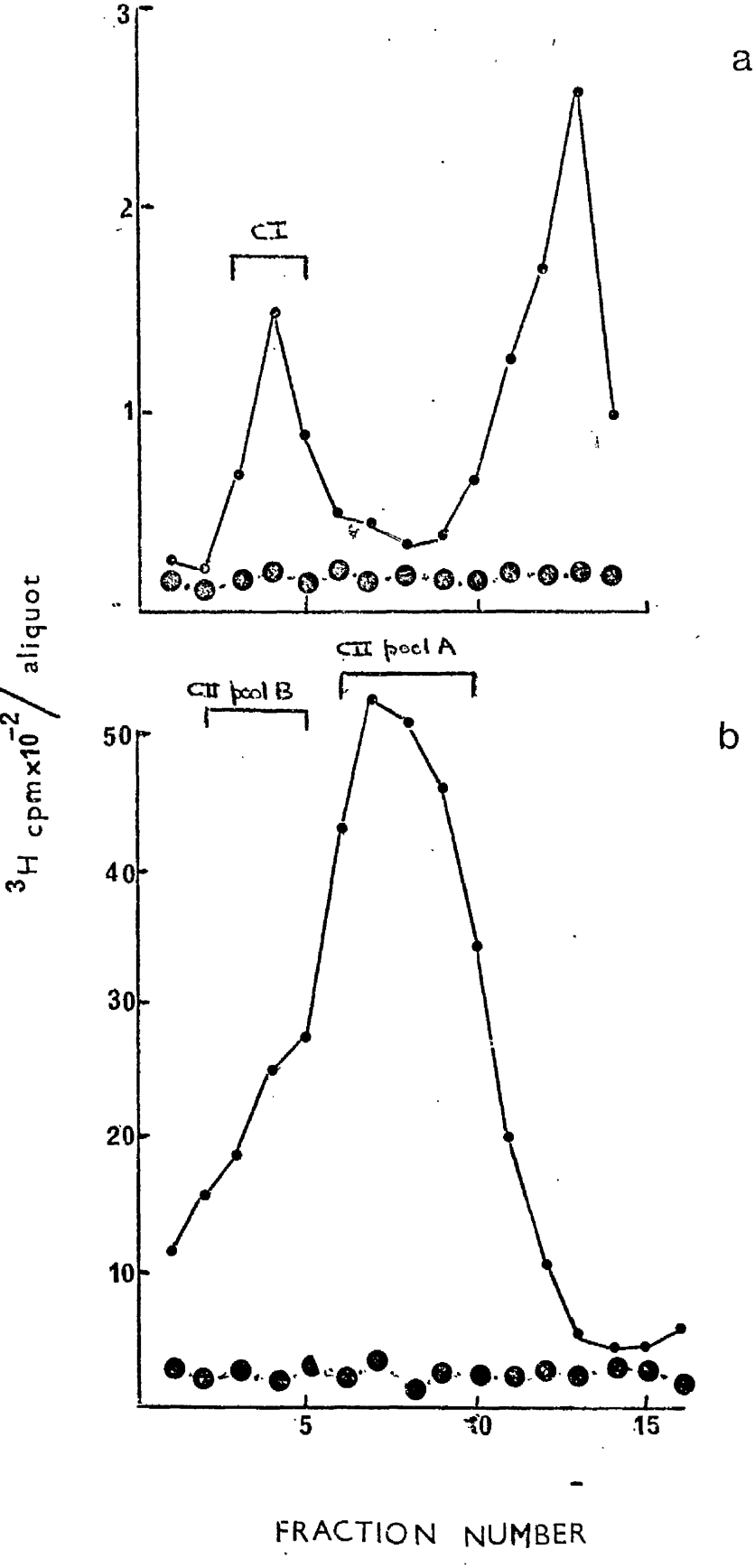

a
b
CI
CII pool A
CII pool B
3
2
1
50
40
30
20
10
5
10
15
^{3}H cpm×10^{-2} / aliquot
FRACTION NUMBER

Fig. 23

Sedimentation analysis of CII pool A and CII pool B.

^{3}H thymidine labelled NP40-NaCl extract from MVM-infected BHK-21/C13 cells, labelled from 16-18 hpi, was sedimented in a neutral sucrose gradient and the fractions in the CII pool A and CII pool B regions pooled as shown in Fig. 22B. A 0.2 ml sample of CII pool A (A) and CII pool B (B) was sedimented in a 5-20% sucrose gradient, containing 0.5M NaCl in a Beckman SW56 rotor at 233,000 g (average) for 3 hr at $4^{\circ}C$.

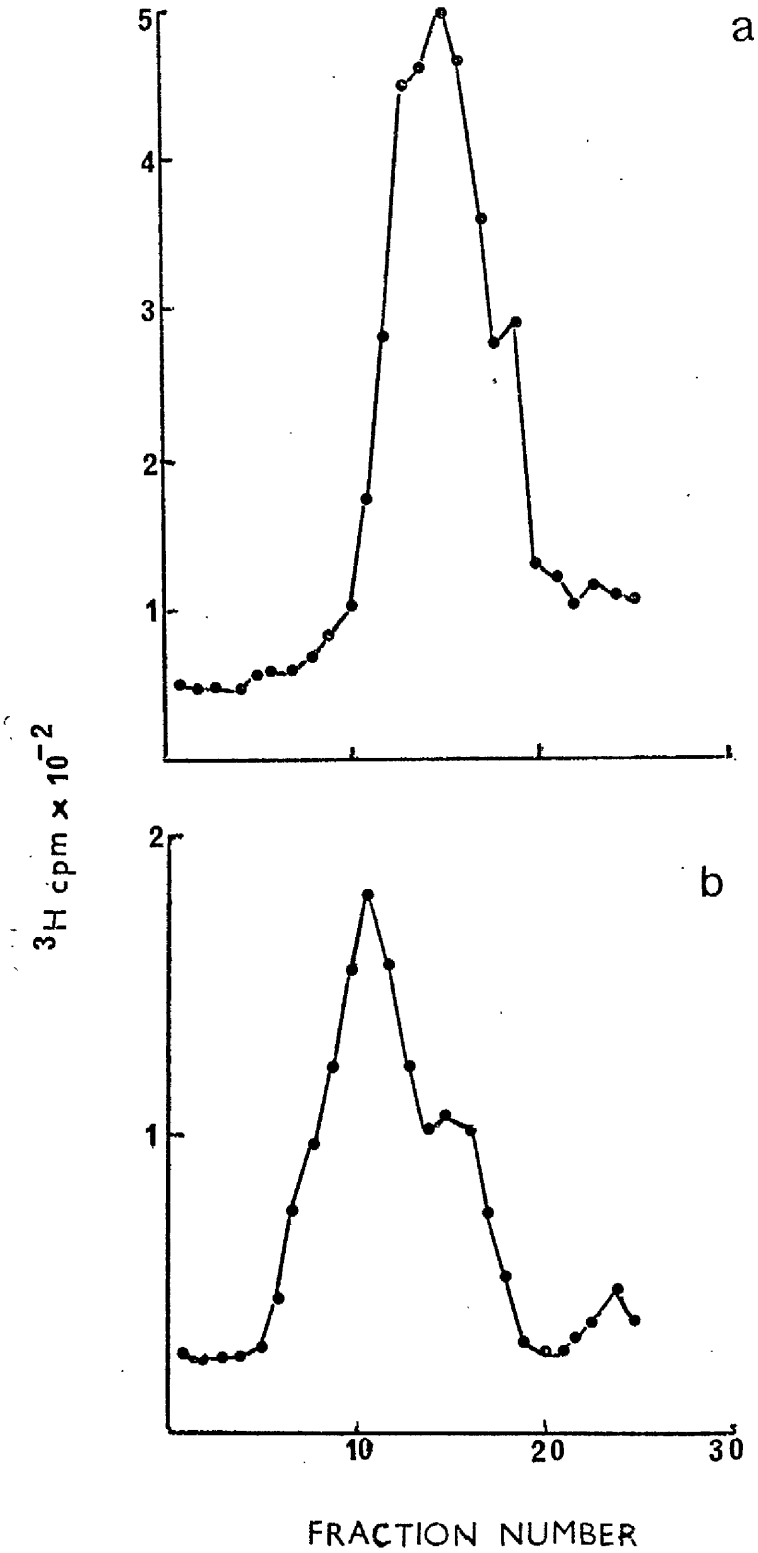
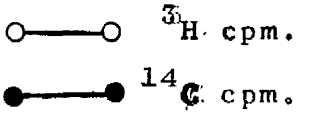
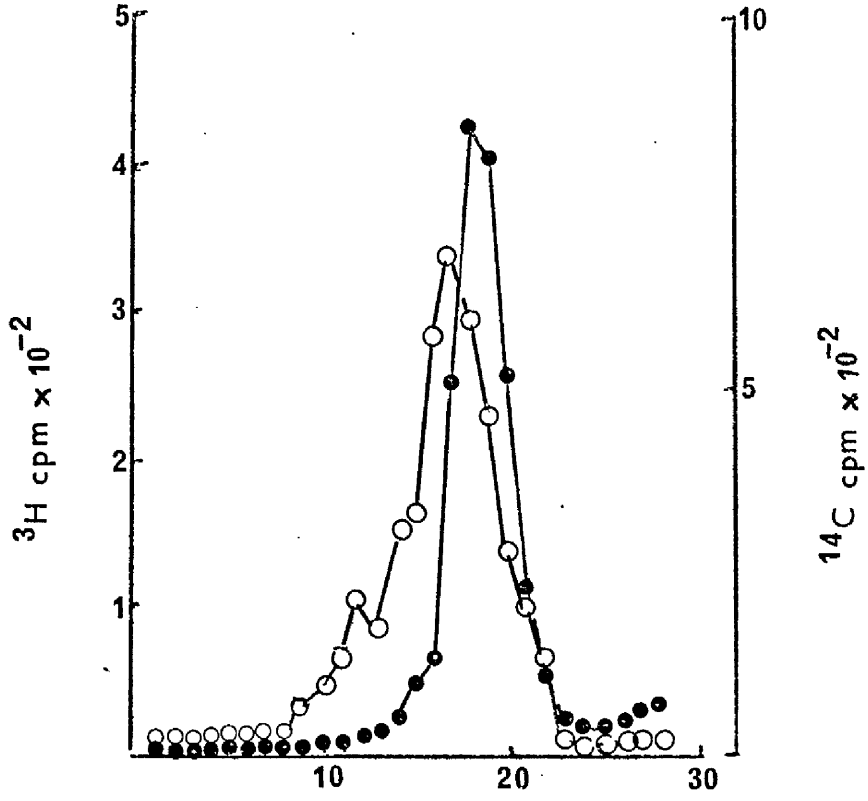

a
b
5
4
3
2
1
2
1
10
20
30
^{3}H cpm x 10^{-2}
FRACTION NUMBER

Fig. 24

Determination of the sedimentation coefficient of CII pool A.

A 0.2M sample of CII pool A, isolated as described in the legend to Fig. 22B, was mixed with ^{14}C-SV40 form I DNA and sedimented in a 5-20% neutral sucrose gradient, containing 0.5M NaCl for 3 hr at 233,000 g (average) in a Beckman SW56 rotor.




5
4
3
2
1
10
5
10
20
30
^{3}H cpm x 10^{-2}
^{14}C cpm x 10^{-2}
FRACTION NUMBER

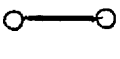
of 15S. There is also a change in the shape of the band. It changes from a broad symmetrical peak to a sharp symmetrical peak, indicating that the broad distribution is caused by the binding of protein to the DNA. This distribution could be as a result of differential protein binding to the DNA species resulting in either a difference in the amount of protein bound to each molecule of DNA or a difference in the shape of the molecule caused by the protein molecules binding to different areas of the DNA molecule, or both. Further indications that CII pool A consists of DNA-protein species was indicated by digestion studies with DNase I. DNA extracted from CII pool A by SDS and pronase treatment followed by phenol extraction was digested at an approximately similar rate by DNase I but the final amount digested from CII DNA was 74% whereas the final quantity digested from CII pool A was 64% (Fig. 26). Care was taken to ensure that an identical quantity of radioactivity was added to each reaction. These results indicate that the DNA present in CII pool A is protected from digestion by DNase I due to the presence of moieties which are removed by treatment with agents which destroy protein-DNA interactions. Taken together these results demonstrate that CII pool A is a nucleoprotein complex.

A possibility exists that the association of protein with CII DNA is a consequence of the extraction procedure and is not a reflection of the *in vivo* status of the CII DNA. Evidence that this is probably not the case is presented in Fig. 27. Purified ^{3}H labelled CII DNA is added to the NP40 lysate of ^{14}C thymidine labelled MVM-infected BHK-21/C13 cells and NaCl is added to a final concentration of 0.5M. The supernatant fraction, after centrifugation, was analysed by neutral sucrose gradient sedimentation (Fig. 27). As one

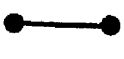
Fig. 25

The effect of SDS and pronase on CII pool A as determined by sedimentation analysis.

A 0.2 ml sample of CII pool A, isolated as described in the legend to Fig. 22, was treated with a final concentration of 0.5% SDS and 100 μg/ml self-digested pronase for 1 hr at 37°C. The sample was sedimented in a 5-20% sucrose gradient containing 0.5M NaCl in a Beckman SW56 rotor at 233,000 g (average) for 3 hr at 4°C. An untreated 0.2 ml sample of CII pool A was run in a parallel gradient.



untreated



SDS and pronase treated.

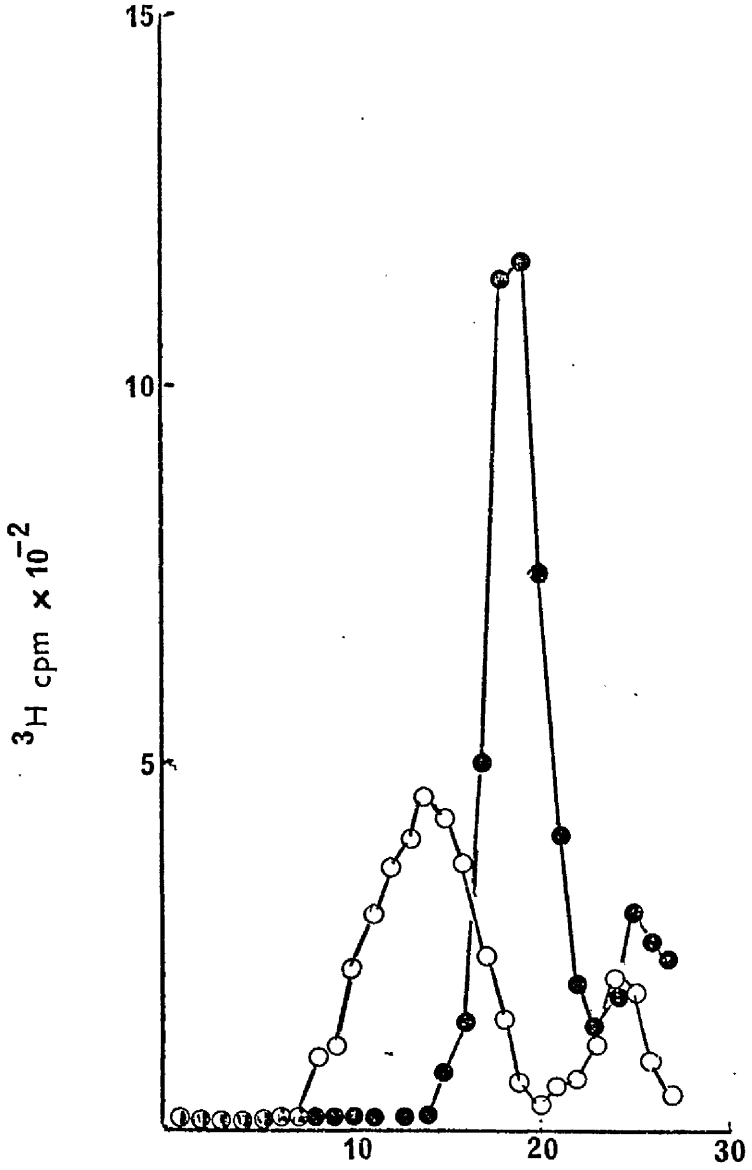

15
10
5
^{3}H cpm x 10^{-2}
10
20
30
FRACTION NUMBER

Fig. 26

Digestion of CII pool A with DNase I

CII pool A, isolated as described in the legend to Fig. 22, was digested with 0.001 units of DNase I under the conditions described in Materials and Methods. At various times an aliquot of the reaction mixture was removed and the acid soluble radioactivity measured. I_n a parallel reaction DNA prepared from CII pool A was digested under identical conditions.

●——● DNA prepared from CII pool A

●– – –● CII pool A.

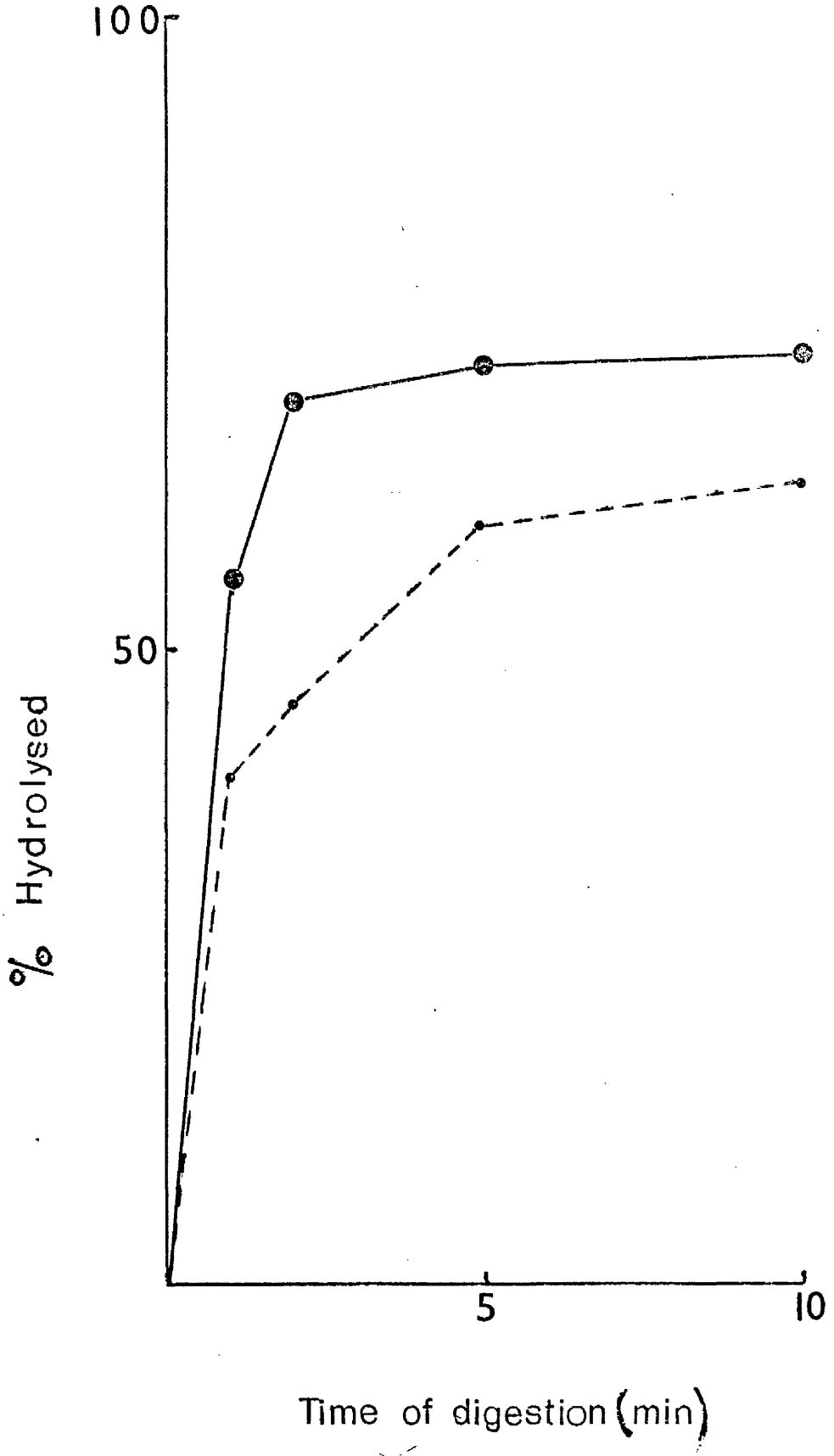

100
50
% Hydrolysed
5
10
Time of digestion (min)

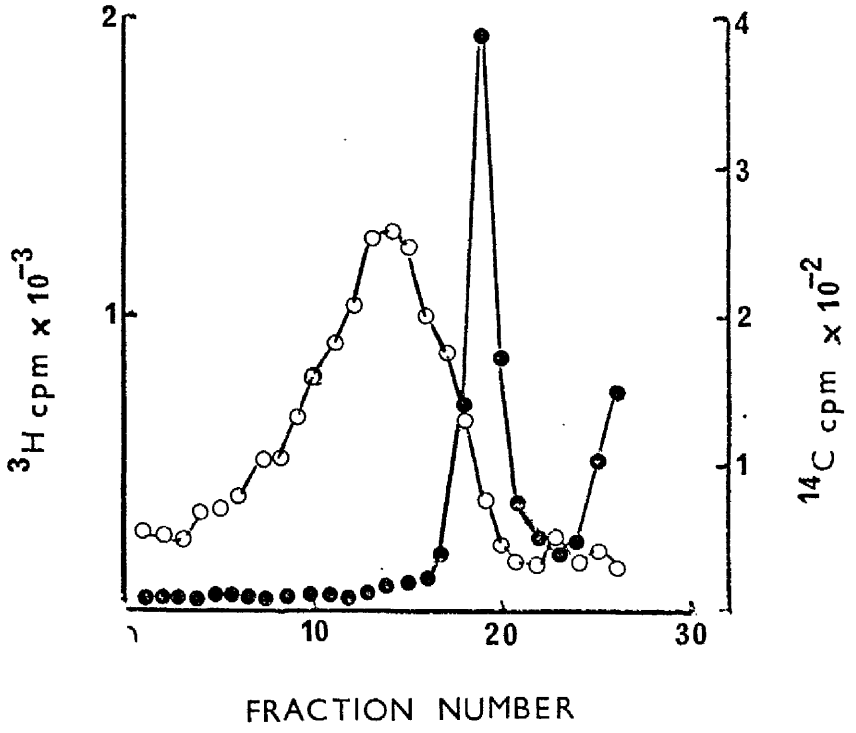
Fig. 27

Sedimentation analysis of DNA purified from CII pool A after addition to MVM infected BHK-21/C13 cell lysate and extracted by the NP40-NaCl method.

MVM infected BHK-21/C13 cells were labelled with ^{14}C-thymidine (10 µCi/ml) between 14-18 hpi. The cells were lysed with 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0 and an aliquot of ^{3}H DNA, purified from CII pool A by treatment with SDS and pronase and phenol extraction followed by ethanol precipitation, was added. After 10 to 15 min on ice, an equal volume of 1M NaCl was added and a 0.2 ml sample of the supernatant fraction, after centrifugation, was sedimented in a 5 to 20% neutral sucrose gradient, containing 0.5M NaCl, for 3 hr at 233,000 g (average) at 4°C in a Beckman SW56 rotor.

●—● ^{3}H cpm

○—○ ^{14}C cpm.


2
1
4
3
2
1
10
20
30
^{3}H cpm x 10^{-3}
^{14}C cpm x 10^{-2}
FRACTION NUMBER

can see the ^{3}H radioactivity does not co-sediment with the ^{14}C radioactivity. The ^{14}C radioactivity is incorporated into CII species *in vivo* whereas the ^{3}H label is in CII pool A DNA added during the extraction procedure, thus suggesting that the DNA-protein complex (CII) is not formed during the extraction procedure and may represent, in part, the structure which is present in the infected cell.

3.3.2. Characterisation of the DNA extracted from nucleoprotein complex CII

DNA was purified from nucleoprotein complex CII by digestion with SDS and pronase. For buoyant density analysis, hybridisation or digestion with enzymes the DNA was further purified by extraction with phenol and ethanol precipitation. The DNA was then redissolved and digested with heat treated Pancreatic RNase followed by further treatment with SDS and pronase. Phenol extraction, ethanol precipitation and (after the pellet has been redissolved) desalting, using a Sephadex G25 column completes the purification protocol. This procedure was used to remove RNA and any remaining SDS or pronase from the DNA. Clearly, these molecules could interfere with nuclease digestion studies or hybridisation reactions.

In order to determine the molecular weight of the DNA agarose gel electrophoresis, under both neutral and alkaline conditions, was undertaken. This technique was used due to the accuracy of the measurement of molecular weight when compared to sedimentation analysis on sucrose gradients. The reasons for this are twofold. Firstly, in sedimentation velocity experiments the difference in S value between a molecule of 3×10^{6} daltons and 6×10^{6} daltons is 4S, i.e. 15S

compared to 19S. Thus, small differences in S value correspond to large differences in molecular weight. Secondly, the availability of restriction endonuclease fragments of known molecular weight enables the construction of accurate calibration curves for agarose gel electrophoresis. This is not of such benefit in velocity sedimentation analysis since small differences in molecular weight would not be reflected in measureable changes in sedimentation coefficients.

Fig. 28 is an example of the construction of calibration curves for both neutral and alkaline agarose gel electrophoresis. The markers used are restriction endonuclease fragments of bacteriophage Lambda DNA which span the range from 1975 base pairs to 23787 base pairs. The molecular weights are given in the legend to Fig. 28. To give a calibration curve these data are most commonly plotted as log M versus mobility (mobility is expressed as the distance migrated). This curve is linear over part, though not all, of its range (Fig. 28B). The extent of the linearity is dependent on a number of factors, e.g. percentage of the agarose gel, voltage applied during electrophoresis and ionic strength of the electrophoresis buffer (Helling et al, 1974; Bearden, 1979). However, there is no convincing theoretical reason as to why the plot of log M versus mobility should give a straight line. As illustrated in Fig. 28 one can see that a calibration curve, though not linear over its entire range, can be constructed for both neutral and alkaline agarose gels and that molecular weights can be accurately measured. One should mention at this point that slight differences in the mobility of DNA molecules will occur from run to run, especially under alkaline conditions, therefore it is essential to run markers with each electrophoresis experiment. The markers on the alkaline gels do not band as sharply as they do on the neutral

Fig. 28

Construction of a calibration curve for the determination of the molecular weight of DNA by agarose gel electrophoresis.

Bacteriophage Lambda DNA was digested with the restriction endonucleases ECoRI and Hind III, in separate reaction mixtures, under the conditions described in Materials and Methods. 1 µg of digested Lambda DNA was electrophoresed on a 1.5% neutral agarose gel for 3 hr at 100V. The gel was stained with ethidium bromide and photographed under UV illumination. The mobility (distance migrated) was measured directly from the gel.

A. Photograph of stained gel.

   1. Lambda DNA digested with ECoRI.
   2. Lambda DNA digested with Hind III.

B. Plot of log M (M expressed in kilobase-pairs) vs mobility. 2 µg of Lambda DNA, digested with ECoRI and 0.1 µg of MVM DNA were separately electrophoresed on a 1.5% alkaline agarose gel for 20 hr at 40V and stained with ethidium bromide as described in the Methods section.

C. Photograph of stained gel.

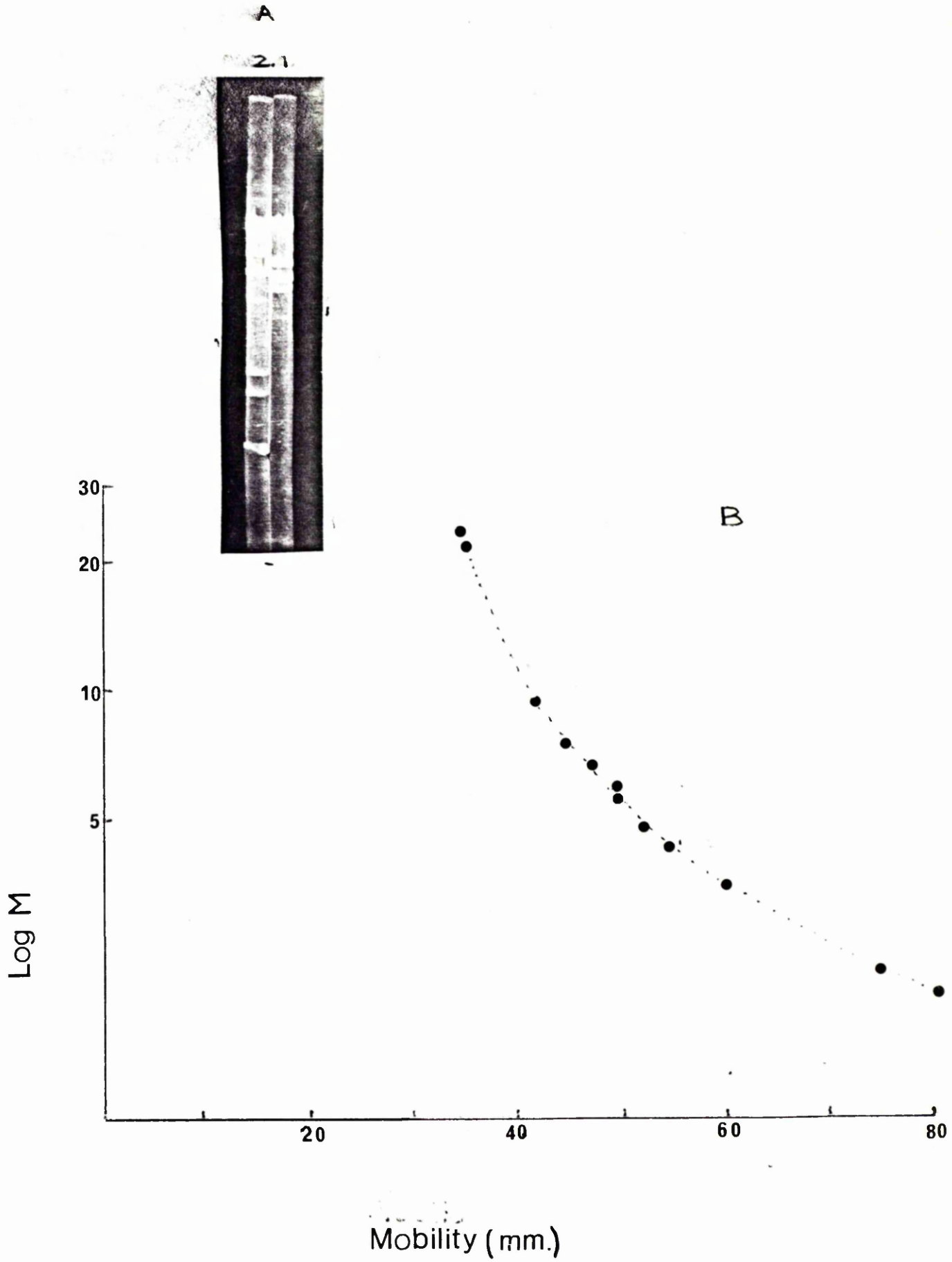
   1. Lambda DNA digested with ECoRI.
   2. MVM DNA.

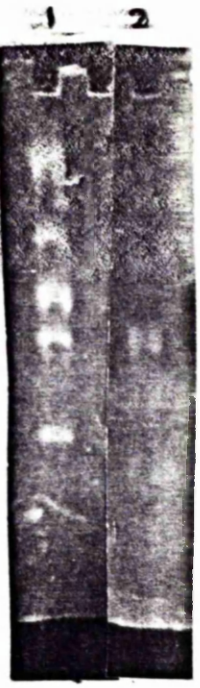
D. Plot of log M (expressed in kilobases) versus mobility.

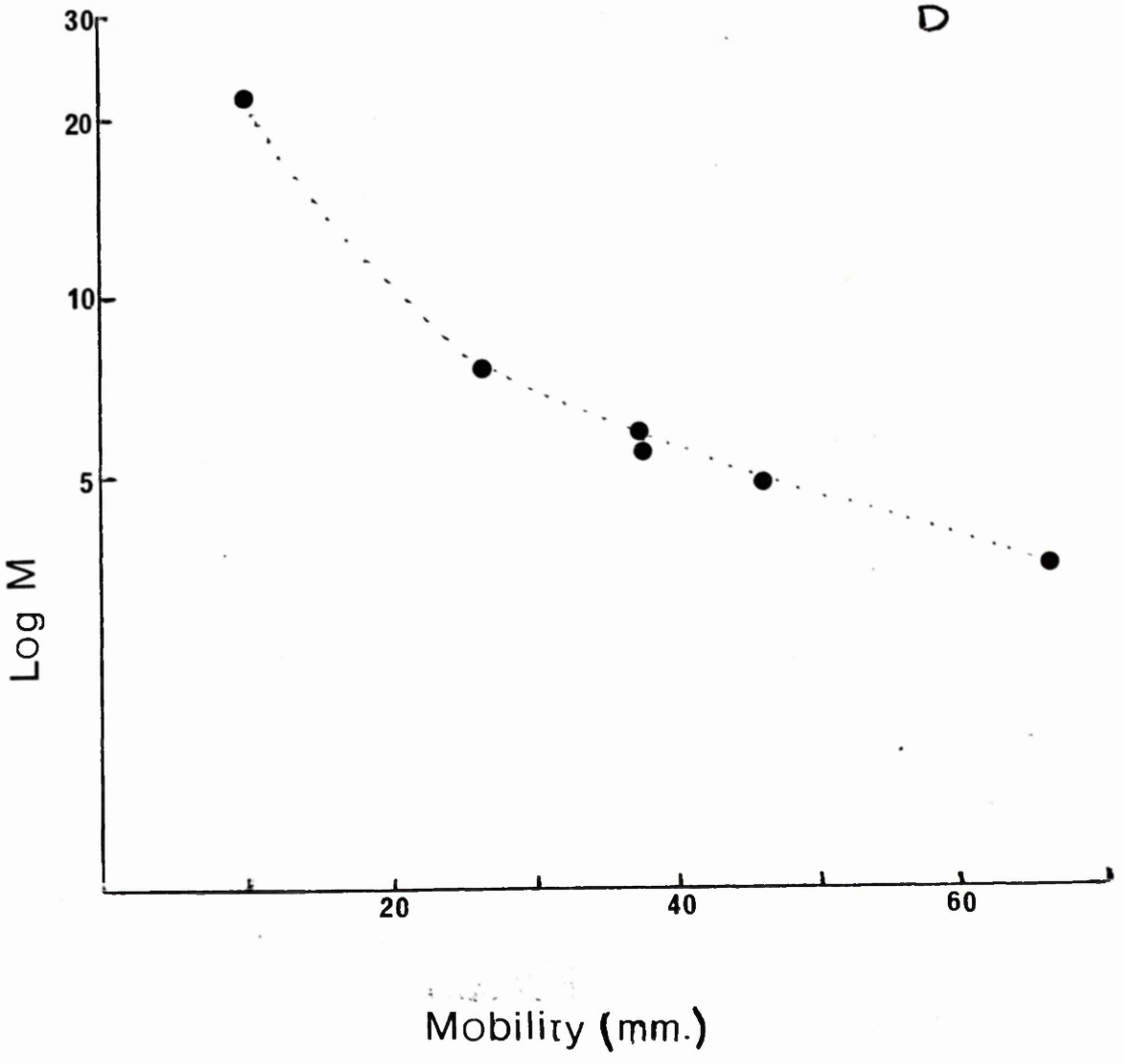
EcoRI Lam digest				Hind III Lam digest	
neutral agarose		alkaline agarose		neutral agarose	
M (base pairs)	mobility (mm)	M (base pairs)	mobility mm	M (base pairs)	mobility mm
21845	35	21845	11.2	23785	34.3
7525	44.8	7525	26.6	9466	42
5939	49.7	5939	37.8	6619	47.6
5534	49.7	5534	37.8	4256	54.6
4807	52.5	4807	46.2	2266	74.9
3496	60.2	3496	66.4	1975	80.2
				567	ND+
				631	ND+

+ ND not determined

Adapted from Cory and Adams (1977)


A
2.1
B
30
20
10
5
Log M
20
40
60
80
Mobility (mm.)


1
2


D
30
20
10
5
Log M
20
40
60
Mobility (mm.)

gels. This phenomenon has been observed previously (McDonell et al, 1977), although the reason for it is unknown.

The DNA components of CII pool A and CII pool B, as shown in Fig. 22B, were analysed by neutral agarose gel electrophoresis (Fig. 29). CII pool A consists of one major species of DNA. This species has a molecular weight equivalent to 4800 base pairs. There is also a minor band with a molecular weight twice that of the major band. CII pool B shows a more complex pattern. The major band again has a molecular weight equivalent to 4800 base pairs but also has two other distinctive bands at twice and four times the molecular weight of the major band. On alkaline agarose gels (Fig. 30) the pattern is very similar for both CII pool A and CII pool B. There are two peaks, corresponding to 4700 bases and 9300 bases with a considerable quantity of heterogenous fragments present both between the two peaks and in front of the faster moving peak. In CII pool B there is a greater quantity of the slower migrating peak relative to the faster migrating peak when compared to CII pool A. The ratios are 3.4:1 and 5.8:1 for CII pool B and CII pool A, respectively (ratio of faster migrating peak to slower migrating peak). Thus, CII pool B contains larger molecular weight DNA species under both alkaline and neutral conditions. In neutral conditions the species are up to four times the molecular weight of the monomer species, but under alkaline conditions the species are only up to twice the molecular weight of the monomer species.

The determination of the molecular weights of these DNA molecules, under neutral conditions, is based on the assumption that they are completely double stranded. Buoyant density analysis of

Fig. 29

Neutral agarose gel electrophoresis of DNA extracted from CII pool A and CII pool B.

0.10 ml samples of CII pool A and CII pool B prepared as indicated in the legend to Fig. 22 were incubated with final concentrations of 0.5% (w/v) SDS and 100 μg/ml pronase at 37°C for 1 hr. The samples were then made 0.025% in Bromo-Phenol Blue (BPB) and 5% in sucrose and electrophoresed on 1.5% neutral agarose tube gels for 3 hr at 100V. The gels were then sliced into 1 mm sections and prepared for liquid scintillation counting as described in Materials and Methods. Migration is from left to right.

(a) CII pool A.

(b) CII pool B.

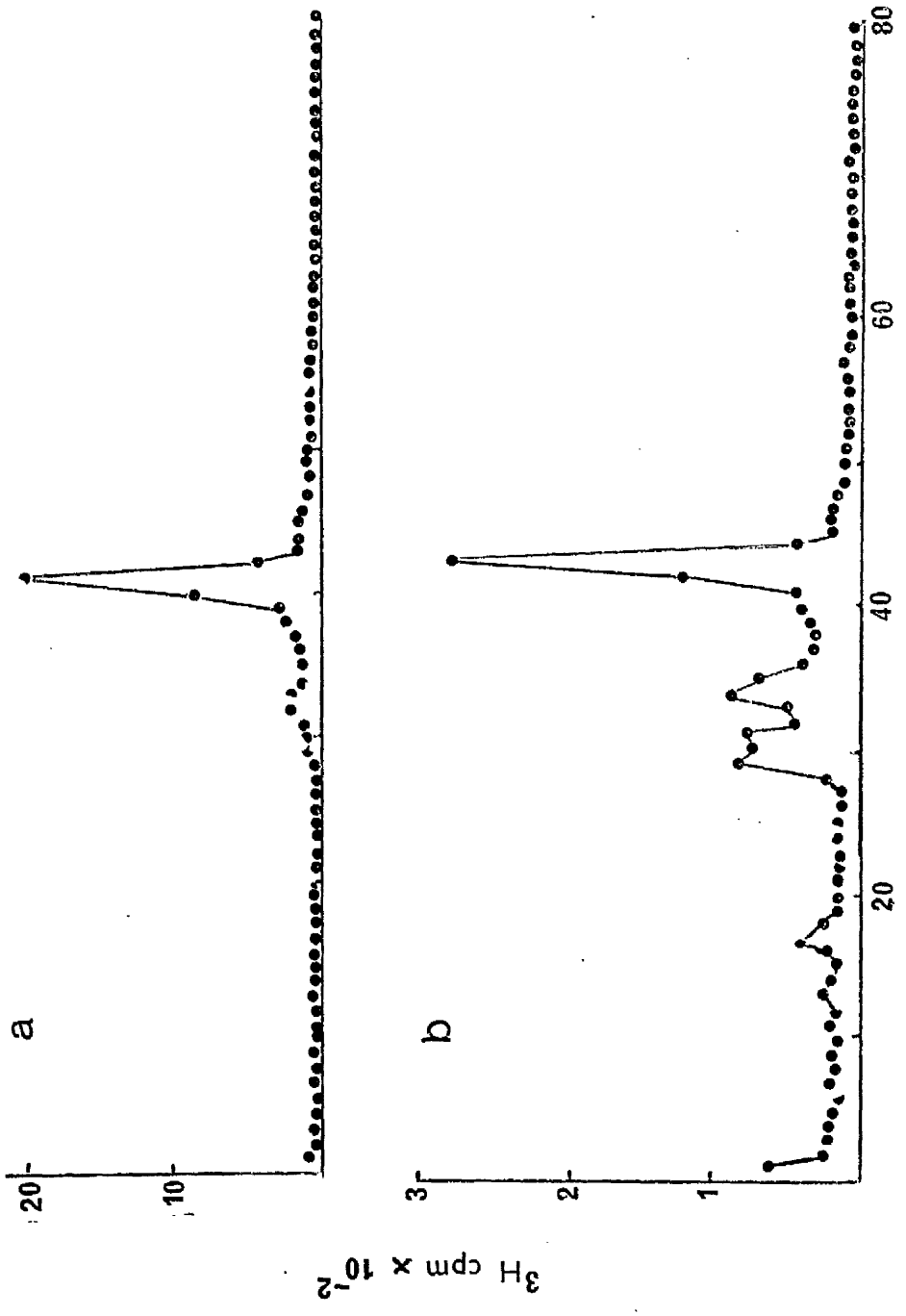

a
b
20
10
3
2
1
20
40
60
80
Fraction No.
^3H cpm x 10^{-2}

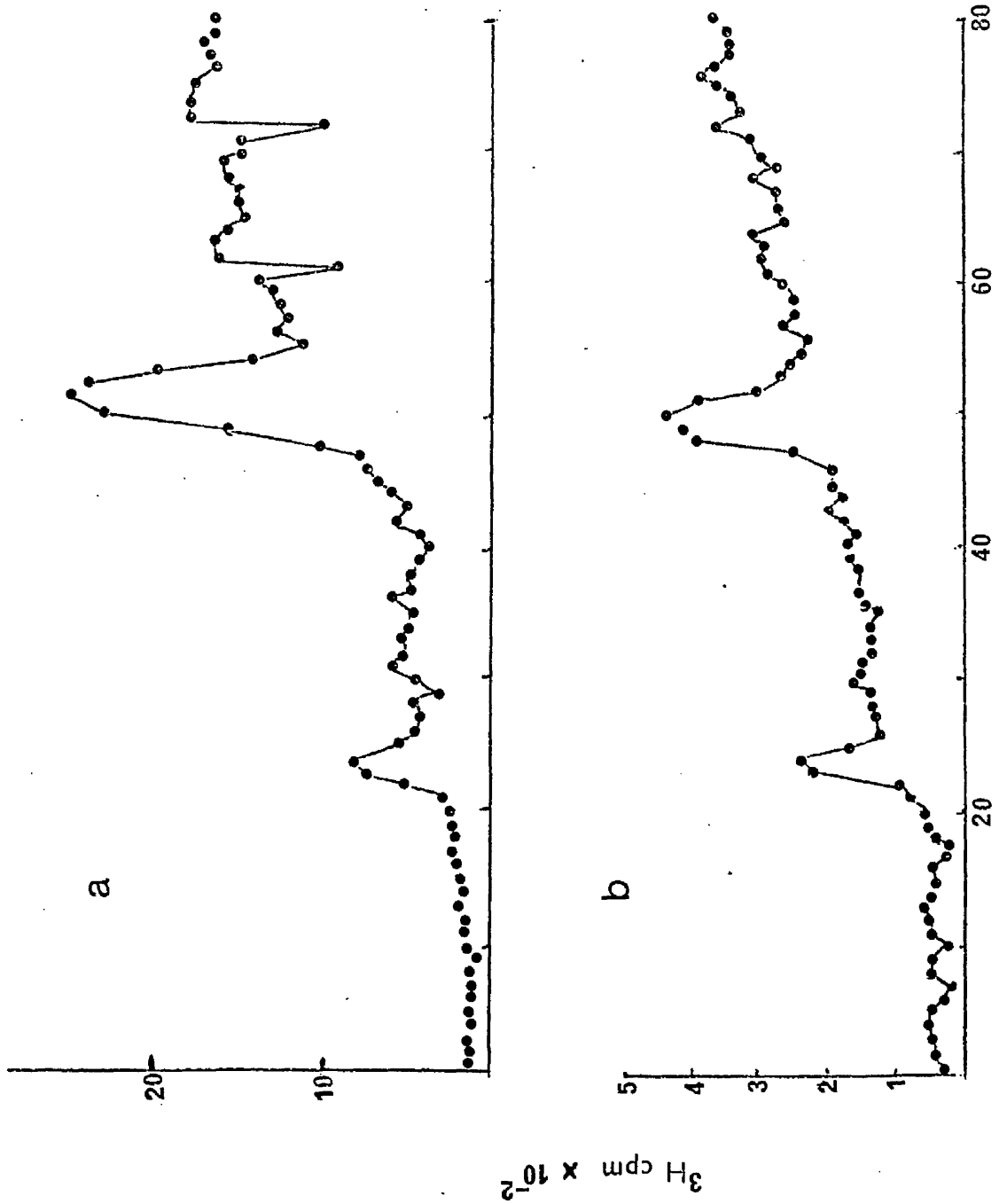
Fig. 30

Alkaline agarose gel electrophoresis of DNA extracted from CII pool A and CII pool B.

0.15 ml samples of CII pool A and CII pool B were deproteinised as described in the legend to Fig. 29. Electrophoresis on 1.5% alkaline agarose gels was for 16 hr at 50V. The gels were sliced and counted as described in the legend to Fig. 29. Migration is from left to right.

(a) CII pool A.

(b) CII pool B.


a
b
20
10
5
4
3
2
1
20
40
60
80
Fraction No.
3H cpm x 10-2

these molecules on NaI density gradients was thus attempted in order to answer this question. NaI was chosen because there is a large separation between double stranded and single stranded DNA molecules on these gradients (Birnie, 1972). DNA when centrifuged to equilibrium in high salt gradients bands at a position in the concentration gradient at which the density of the macromolecule equals the density of the solution. In this case the density of the macromolecule refers to the density of the molecular unit including water and the salt. Thus, differences in the hydration of macromolecules will result in different buoyant densities and thus a separation between double and single stranded DNA is possible. Single stranded DNA bands at a density (1.57 g/cm^3) higher than double stranded DNA (1.52 g/cm^3). Fig. 31 shows that both CII pool A and CII pool B consist of double stranded DNA with no detectable single stranded regions. Confirmation of this result was obtained by digestion with the single strand specific nuclease, S1. Fig. 32 shows that the nuclease S1 preparation used in these experiments is essentially single strand specific, in that 9% of native BHK-21/C13 DNA is digested, whereas 95% of the heat denatured BHK-21/C13 DNA is digested. Both CII pool A and CII pool B are digested to similar extents with nuclease S1, i.e. between 8 and 10% which indicates that these DNA species are indeed double stranded (Table 7). There is a significant difference between CII pool A and CII pool B in the percentage of material digested with nuclease S1 after heat denaturation (Table 7). CII pool B has much more material which is capable of rapid renaturation than does CII pool A. This is indicative of hairpin-like structures.

The DNA-containing species CII is probably viral DNA since it is not present in mock infected cells. However one cannot on this

Fig. 31

Buoyant density analysis on sodium iodide gradients of DNA purified from CII pool A and CII pool B.

DNA was purified from CII pool A and CII pool B by treatment with 0.5% SDS and 100 μg/ml self digested pronase at 37°C for 1 hr followed by phenol extraction and ethanol precipitation. The DNA was redissolved and treated with 50 μg/ml heat treated Pancreatic RNase at 37°C for 30 min. The DNA was then mixed with a NaI solution, saturated with sodium sulphite, to give an initial density of 1.545 g/cm^3, as measured by refractive index. 5 ml amounts were centrifuged to equilibrium at 120,000 g for 60 hr at 20°C in a Beckman Ti50 rotor. The gradients were fractionated by direct puncture and the total radioactivity measured. The arrow indicates the position of ^{32}P labelled MVM virion DNA run in each gradient as an internal marker. The density of double stranded DNA is 1.52 g/cm^3 and that of single stranded DNA 1.57 g/cm^3.

(a) DNA purified from CII pool A

(b) DNA purified from CII pool B.

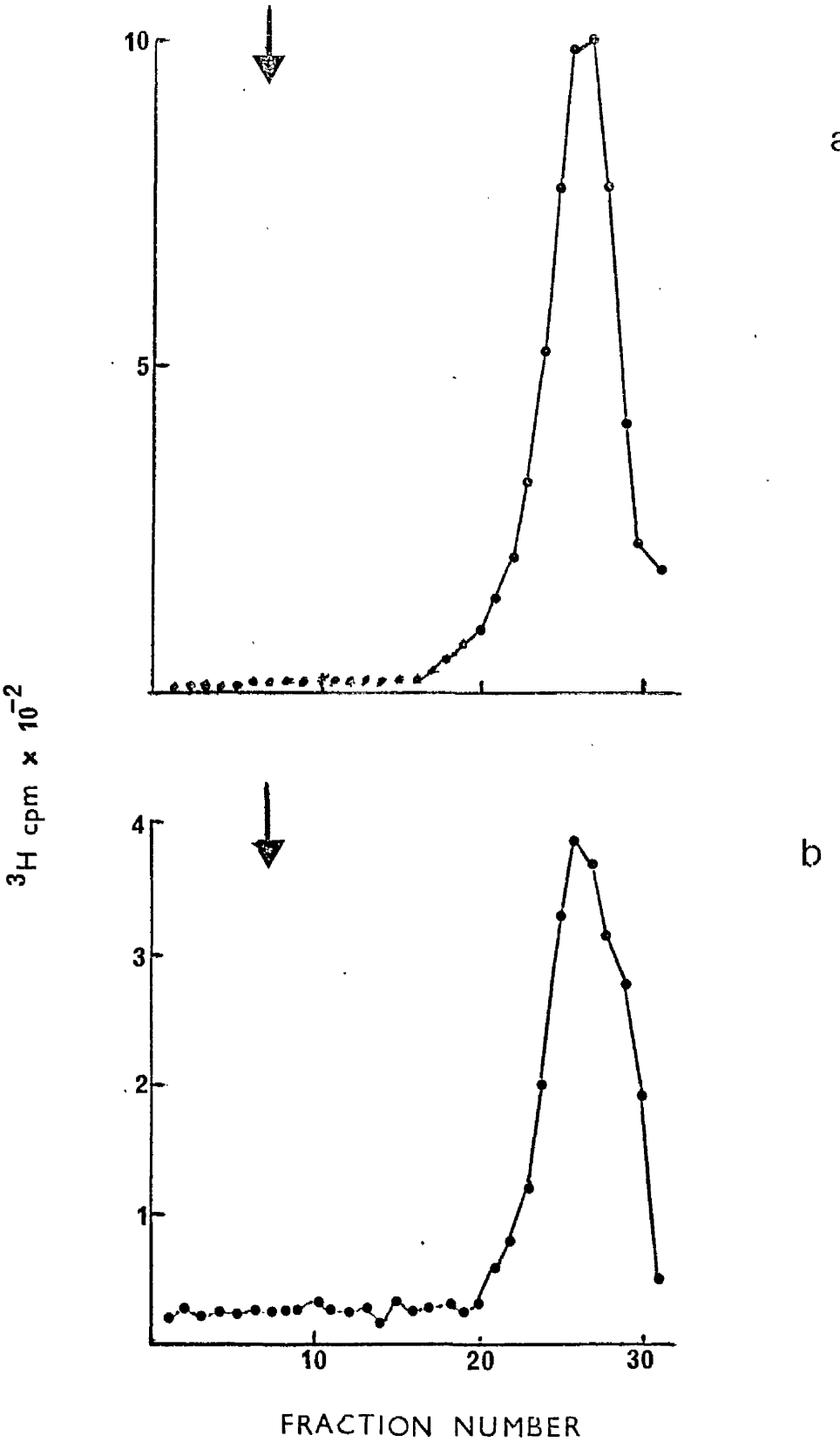
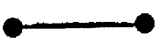

a
b
10
5
4
3
2
1
10
20
30
^{3}H cpm x 10^{-2}
FRACTION NUMBER

Fig. 32

S1 nuclease digestion of BHK-21/C13 DNA.

0.3 ml samples of BHK-21/C13 DNA, prepared as described in Materials and Methods, were incubated with 10 units of S1 nuclease at 37°C for various times. The reaction was stopped by the addition of EDTA and the acid soluble radioactivity measured. BHK-21/C13 DNA was heat denatured by boiling for 5 min followed by quench cooling on ice. Each time point is an average of two independent measurements.

native BHK-21/C13 DNA



heat denatured BHK-21/C13 DNA.

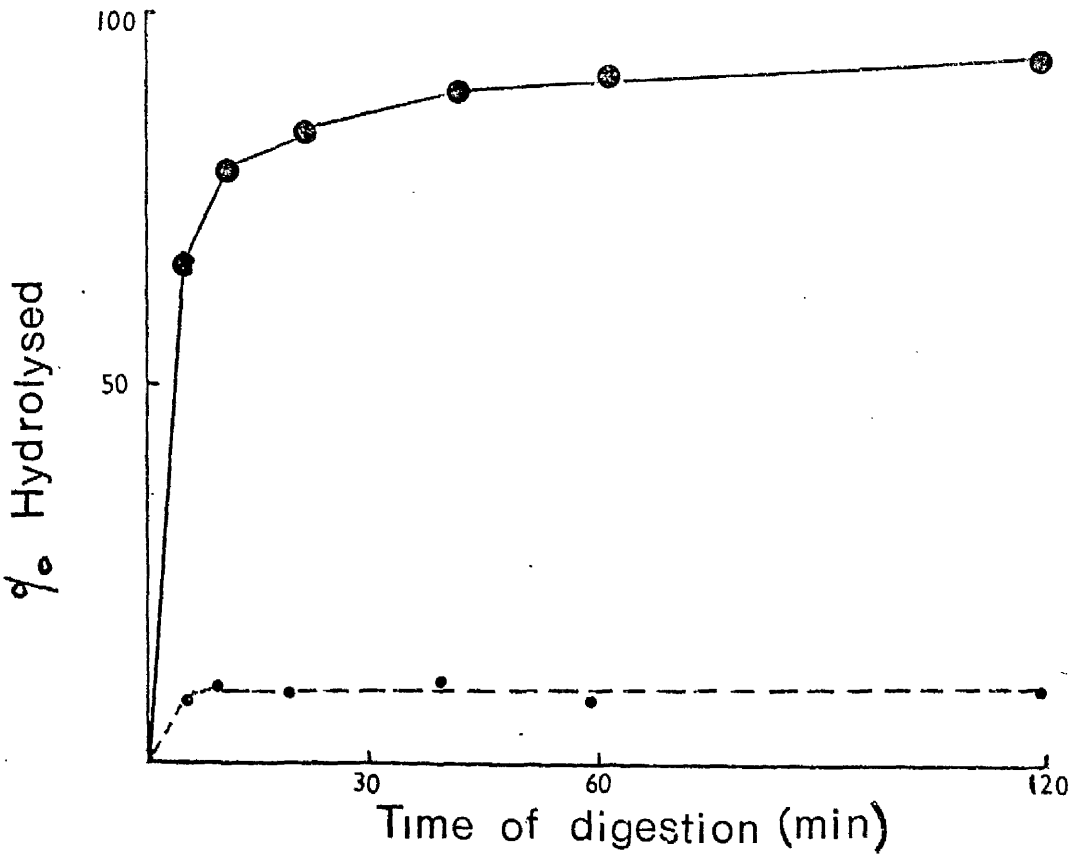

100
50
% Hydrolysed
30
60
120
Time of digestion (min)

Table 7. S1 nuclease digestion of CII DNA species

DNA	% Acid soluble
native CII pool A	7.8 ± 2.0
denatured CII pool A	71.2 ± 0.3
native CII pool B	9.9 ± 1.0
denatured CII pool B	57.1 ± 1.2

DNA from CII pool A and CII pool B was prepared as described in the legend to Fig. 31.

The DNA, either heat denatured or native, was digested with S1 nuclease for 2 hr at 37°C under identical conditions to those described in Fig. 32. The percentage acid soluble material was measured. The results are expressed as the mean ± 1 standard deviation and there were 5 independent measurements.

basis rule out the possibility that the virus causes specific degradation of host cell DNA. Hybridisation analysis does confirm the results obtained with mock infected cells, i.e. CII contains viral DNA. Radioactively labelled CII pool A DNA, which is double stranded, was hybridised with a vast excess of MVM viral DNA. The unlabelled viral DNA will therefore compete out any labelled viral DNA which is present in the duplex, thus the maximum quantity of labelled DNA which can be displaced is 50%. Table 8 shows the result of the hybridisation experiments. 15% of CII pool A DNA hybridised with zero order kinetics. This is a much lower value than obtained in Table 7 but is because the DNA in this instance was sheared to approximately 1000 bases in length before the hybridisation reaction was commenced. After 72 hr, 92% of CII pool A has hybridised, therefore, 77% of CII pool A DNA is available for competition hybridisation. Between 6 to 7% of this DNA can be displaced from the hybrid, presumably nonspecifically, by either BHK-21/C13 DNA or SV40 DNA. MVM viral DNA will displace between 35 to 37% of CII pool A DNA. Therefore, assuming a duplex structure with both strands labelled, at least 70 to 74% of the 77% available for displacement is viral. If the 35 to 37% which was displaced by MVM DNA could consist of between 6 to 7% nonspecifically displaced, only 56 to 60% of the maximum possible 77% is viral DNA assuming both strands to be equally labelled. In either case the majority of CII pool A DNA is of viral origin.

3.3.3 Analysis of the proteins associated with CII pool A

CII pool A was labelled with ^{35}S-L-methionine between 10 to 18 hpi. A long labelling time was required because it proved to be

Table 8. Displacement hybridisation analysis of CII pool A DNA

Competing DNA (μg)	Time of hybridisation (h)	% Hybrid	% Displaced
0	0	15	-
0	72	92	0
SV40 (5)	72	86	6
BHK-21/C13 (10)	72	85	7
MVM (1)	72	55	37
MVM (5)	72	57	35

DNA purified from CII pool A, as described in the legend to Fig. 31, and the competing DNA were sheared to approximately $95^{\circ}_{20\text{-}w}$ (measured under alkaline conditions) and hybridised (0.1 ml volume) at 65°C in 0.01M tris-HCl, 0.001M EDTA, 0.5M NaCl, pH 7.5 for 72 hr. The percentage hybrid was calculated by measuring the fraction single stranded using S1 nuclease. The conditions for S1 nuclease digestion are given in the legend to Table 7. The same amount of CII pool A DNA (approximately 1000 cpm) was used for each hybridisation reaction. Each point is an average of two independent measurements.

extremely difficult to incorporate radioactivity into this species. Even experiments with ^{14}C-labelled protein hydrolysate did not yield an improvement on this result. As illustrated in Fig. 33 ^{35}S-L-methionine label could be incorporated into CII pool A, however, due to the extremely low levels of radioactivity it did not prove possible to analyse these proteins. The reasons for this poor incorporation are unknown. Experiments using haemagglutination activity as an assay for viral proteins revealed that CII pool A did not haemagglutinate Guinea Pig RBC's. This could mean that the viral protein responsible for haemagglutination activity is not present in CII or, alternatively, virion structure is required for haemagglutination. Data from the literature are inconclusive in this point in that one group suggests that one of the virion proteins is responsible for haemagglutination (Salzman and White, 1970) whereas other groups suggest that virion structure is required for the haemagglutination activity (Johnson and Hoggon, 1973; Salo and Mayor, 1977). Clearly, experiments using antibodies prepared against viral polypeptides would be a better method to demonstrate the existence of viral proteins in CII pool A.

3.3.4 Fast sedimenting species, CI

The sedimentation coefficient of the fast sedimenting nucleoprotein complex species, CI, was determined relative to the sedimentation coefficient of mature MVM virions. ^{32}P labelled MVM virus was added to the NP40 lysate of ^{3}H thymidine labelled MVM-infected BHK-21/C13 cells. NaCl was added to a final concentration of 0.5M and a sample of the supernatant fluid, after centrifugation, removed for sedimentation analysis on neutral sucrose gradients (Fig. 34). This analysis

Fig. 33

Sedimentation analysis of ^{35}S-L-methionine labelled CII.

MVM-infected BHK-21/C13 cells were labelled with ^{35}S-L-methionine (50 µCi/ml), as described in Methods section, between 10-18 hpi and with ^{3}H thymidine (1- µCi/ml) between 16-18 hpi. The cells were harvested and nuclei prepared by lysis of the cells with 0.5% (w/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0. A 0.5M NaCl nuclear extract was prepared and sedimented in a 5 to 20% linear sucrose gradient at 90,000 g (average) at 4°C for 16 hr in a Beckman SW40 rotor. The ^{3}H labelled peak fractions were pooled, as indicated in the legend to Fig. 22, and dialysed against 0.5M NaCl, 0.010M tris-HCl, 0.010M EDTA pH 7.0. A 0.2 ml sample of this material was then sedimented in a 5 to 20% linear sucrose gradient at 233,000 g (average) at 4°C for 3 hr in a Beckman SW56 rotor. The result of this analysis is illustrated.

●——● ^{3}H cpm

○——○ ^{35}S cpm

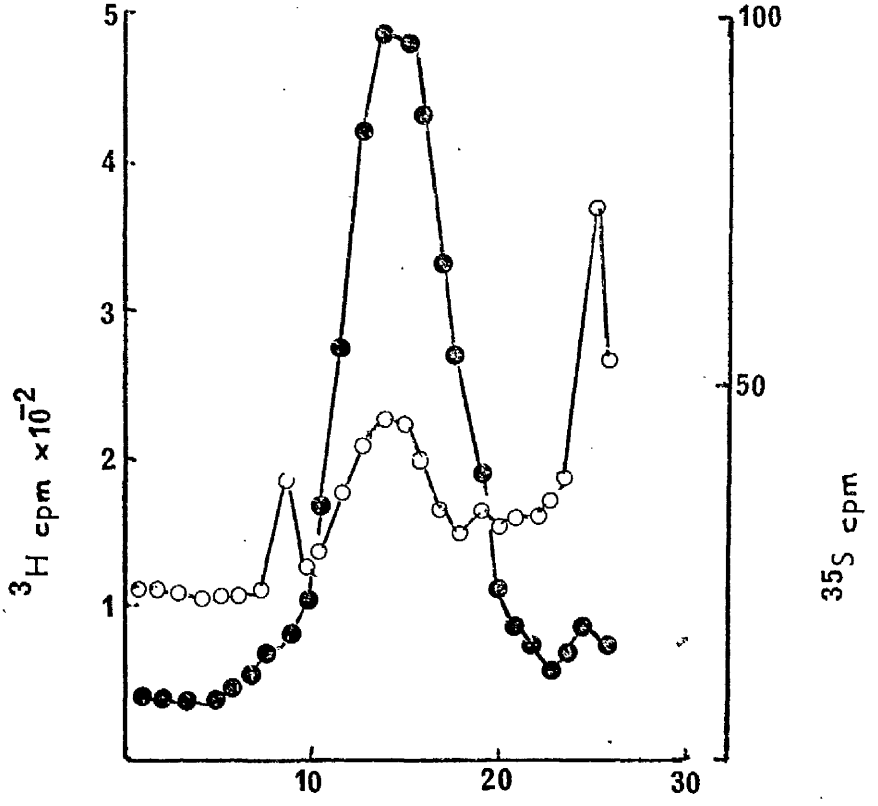
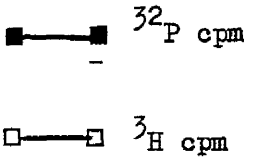
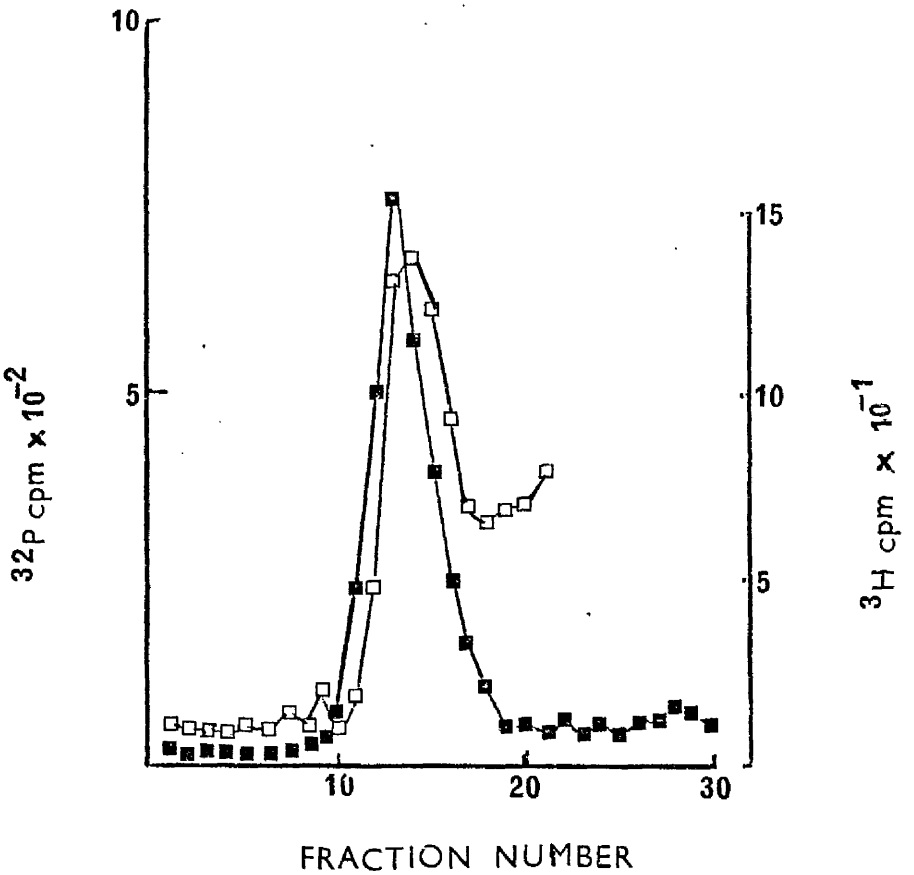

5
4
3
2
1
100
50
10
20
30
^{3}H cpm $\times 10^{-2}$
^{35}S cpm
FRACTION NUMBER

Fig. 34

Sedimentation analysis of CI.

MVM infected BHK-21/C13 cells were labelled for 90 min with ^{3}H thymidine (10 μCi/ml), commencing at 16 hpi. The cells were lysed with 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0 and an aliquot of ^{32}P labelled MVM virions added. After 10 to 15 min on ice, NaCl was added to a final concentration of 0.5M and a 0.2 ml sample of the supernatant fraction, after centrifugation, sedimented in a 5 to 20% sucrose gradient, containing 0.5M NaCl, for 90 min at 180,000 g (average) at 4°C in a Beckman SW56 rotor.




10
5
15
10
5
10
20
30
^{32}P cpm x 10^{-2}
^{3}H cpm x 10^{-1}
FRACTION NUMBER

demonstrates that the ^{3}H labelled material does not cosediment with the ^{32}P labelled MVM virus, however, it does not rule out the possibility that at least some of the CI particles have the same sedimentation coefficient as mature MVM virions. Since the virus marker was added to the NP40 lysate and thus is present during the extraction procedure it is unlikely that the lower sedimentation coefficient of the in vivo labelled species is due to degradation during the isolation protocol.

The nucleoprotein complex species, CI, was purified from the cytoplasmic fraction of MVM-infected BHK-21/Cl3 cells. The cells were lysed with NP40 and the nuclear and cytoplasmic fractions separated. The cytoplasmic fraction was then centrifuged to remove cellular debris and an equal volume of 1M NaCl added. The sample was then fractionated on neutral sucrose gradients, containing 0.5M NaCl, and the fractions were pooled as indicated and dialysed to remove NaCl and sucrose (Fig. 22A). These pooled fractions are designated CI. Essentially no radioactivity was present in the mock-infected extracts.

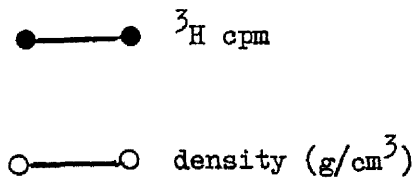
The buoyant density of CI in caesium chloride gradients was demonstrated to be greater than that of mature virus (Fig. 35). The density of the major peak was at, approximately, 1.44 g/cm^3, however, a portion of CI banded at much higher densities (fractions 1 to 5 in Fig. 35), but the nature of this fraction is unknown.

Mature virus is resistant to digestion with deoxyribonuclease (Vasquez and Brailovsky, 1965; Rose et al, 1966). CI is, however, sensitive to digestion with micrococcal nuclease (Table 9). There is still a considerable percentage (78%) which is resistant to digestion under these conditions, whilst the DNA prepared from CI is almost completely degraded to acid soluble products. Thus, CI is like virus but has some significantly different features.

Fig. 35

Buoyant density analysis in caesium chloride gradients of CI.

CI, purified as described in the legend to Fig. 22, was added to a solution of CsCl in 10 mM tris-HCl, 10 mM EDTA pH 7.5 to give an initial density of 1.44 g/cm^3. 3 ml amounts of this solution were centrifuged to equilibrium at 80,000 g (average) for 40 hr at 20°C in a Beckman SW50.1 rotor. The gradient was harvested by direct puncture and aliquots removed for the determination of acid precipitable radioactivity and density measurements. The arrow indicates the position of mature virions.



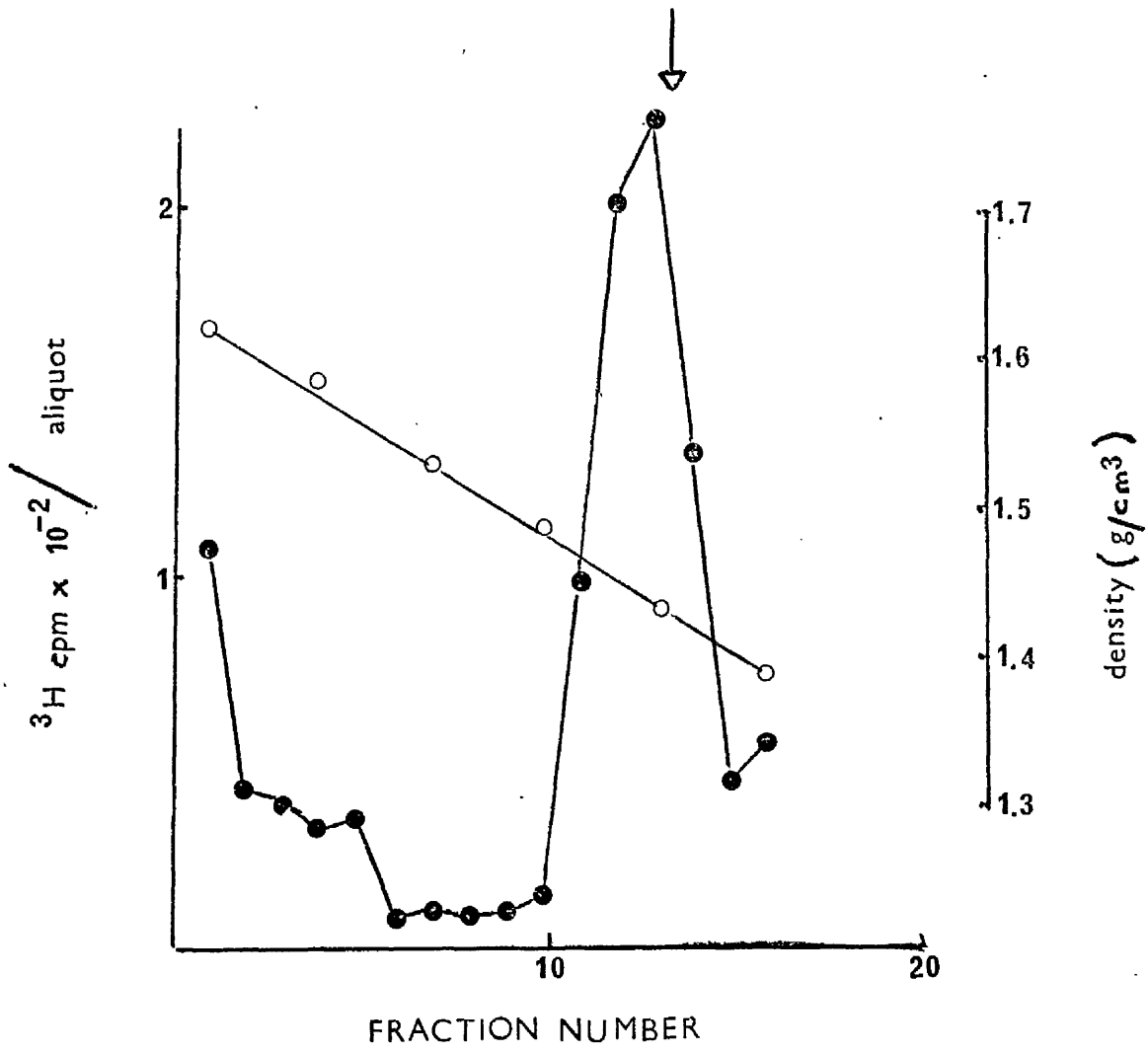

2
1
^{3}H cpm x 10^{-2}/ aliquot
1.7
1.6
1.5
1.4
1.3
density (g/cm^3)
10
20
FRACTION NUMBER

Table 9. Digestion of CI with micrococcal nuclease

	% Acid soluble	% Resistant to digestion
CI species	22.0 ± 1.8	78
CI DNA	93.6 ± 6.4	6.4

CI, prepared as described in the legend to Fig. 22, was incubated for 30 min at 37°C with 0.01 units of micrococcal nuclease as described in the Methods section. The percentage acid soluble radioactivity was measured. An equal amount of CI DNA purified from CI, as described in the legend to Fig. 37, was also digested under the identical conditions.

The results are expressed as the mean ± 1 standard deviation. 3 independent incubations were measured.

3.3.5 Characterisation of the DNA extracted from nucleoprotein complex, CI

CI DNA was purified from the pooled fractions, designated CI (Fig. 22A) by treatment with SDS and pronase. In analysis, apart from agarose gel electrophoresis, the DNA was further purified by phenol extraction and pancreatic ribonuclease treatment, as described for CII DNA.

Neutral agarose gel electrophoresis (Fig. 36A) demonstrates that CI consists of two species. The major species has a mobility which corresponds to that of single stranded viral DNA. The minor species could be defective genomes of MVM. A similar situation has been observed to occur for H-1 virion DNA when analysed by neutral agarose gel electrophoresis (Rhode, 1977a). Alkaline agarose gel electrophoresis (Fig. 36B) demonstrates that CI DNA can again be separated into two species. The major species has a mobility which corresponds to that of single stranded MVM DNA. The minor species is less obvious in this instance due perhaps to diffusensss of the alkaline gel. Thus, CI DNA would appear to correspond to MVM virion DNA.

Further evidence that this is the case has been obtained by demonstrating that CI DNA is single stranded. Buoyant density analysis on sodium iodide gradients has revealed that CI DNA bands at the same position as single stranded MVM DNA (Fig. 37). This indicates that the DNA is single stranded. Confirmation of this result was obtained by studies involving S1 nuclease. S1, which under these conditions is specific for single stranded DNA, digested 88.5% of CI DNA (Table10). The remaining 12% is not available for digestion even when CI DNA is heat denatured, thus suggesting that the double stranded content of

Fig. 36

Agarose gel electrophoresis of DNA purified from CI.

DNA was purified from CI, prepared as described in the legend to Fig. 22, by digestion with 0.5% (w/v) SDS and 100 μg/ml pronase at 37°C for 1 hr.

(a) A 0.10 ml sample of DNA from CI was electrophoresed on a 1.5% neutral agarose tube gel for 3 hr at 100V. The gel was sliced into 1 mm sections and prepared for liquid scintillation counting as described in Materials and Methods.

(b) A 0.10 ml sample of DNA purified from CI, as described above, was electrophoresed on a 1.5% alkaline agarose tube gel for 12 hr at 50V. The gel was sliced and counted as described above.

Migration is from left to right.

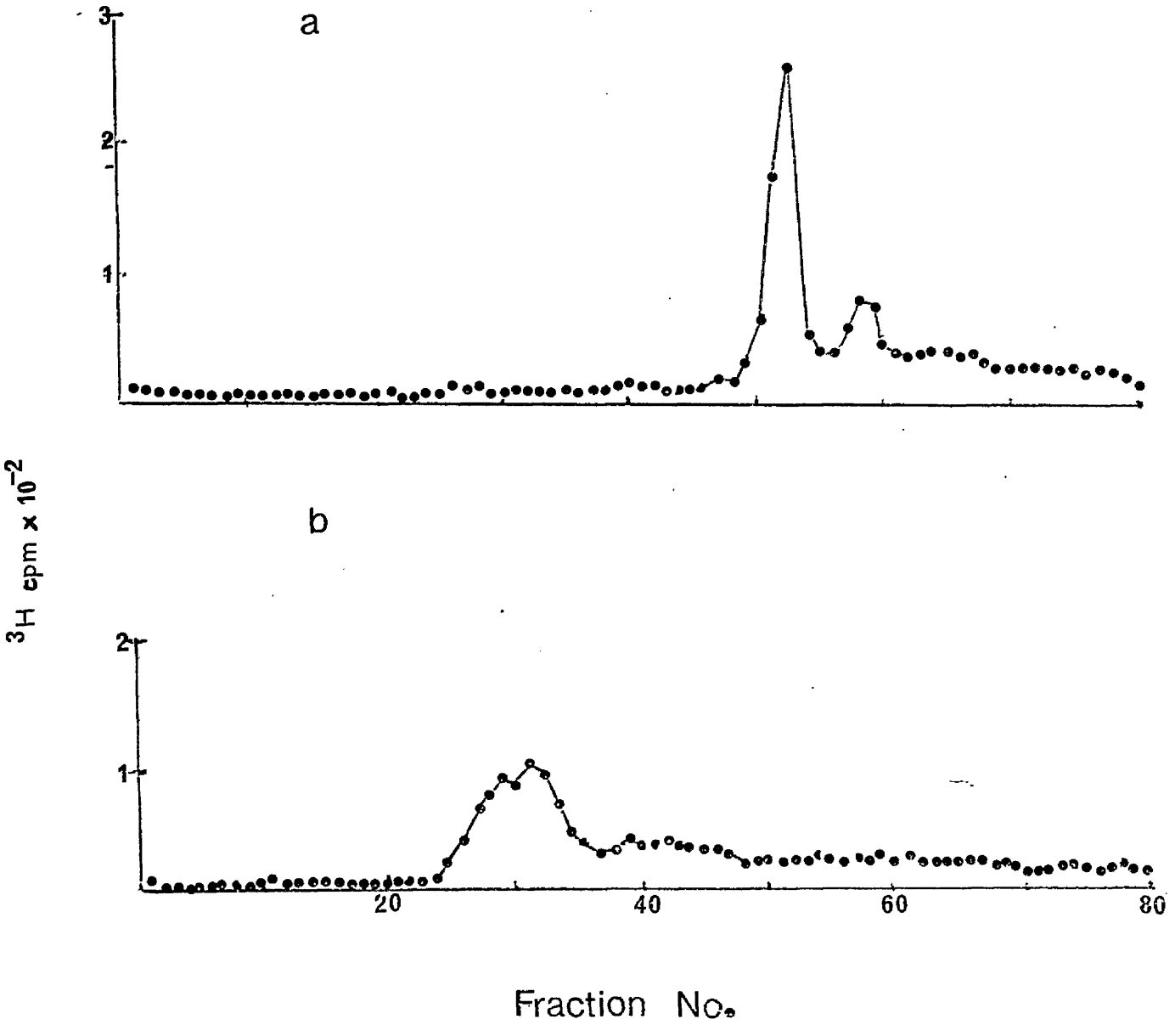

a
b
3
2
1
2
1
20
40
60
80
^3H cpm x 10^{-2}
Fraction No.

Fig. 37

Buoyant density analysis in sodium iodide gradients of DNA purified from CI.

DNA was purified from CI as described in the legend to Fig. 31. The DNA was mixed with a NaI solution, saturated with sodium sulphite, to give an initial density of 1.45 g/cm^3. 5 ml amounts were centrifuged to equilibrium as described in the legend to Fig. 31. The arrow indicates the position of ^{32}P labelled MVM virion DNA run as an internal marker.

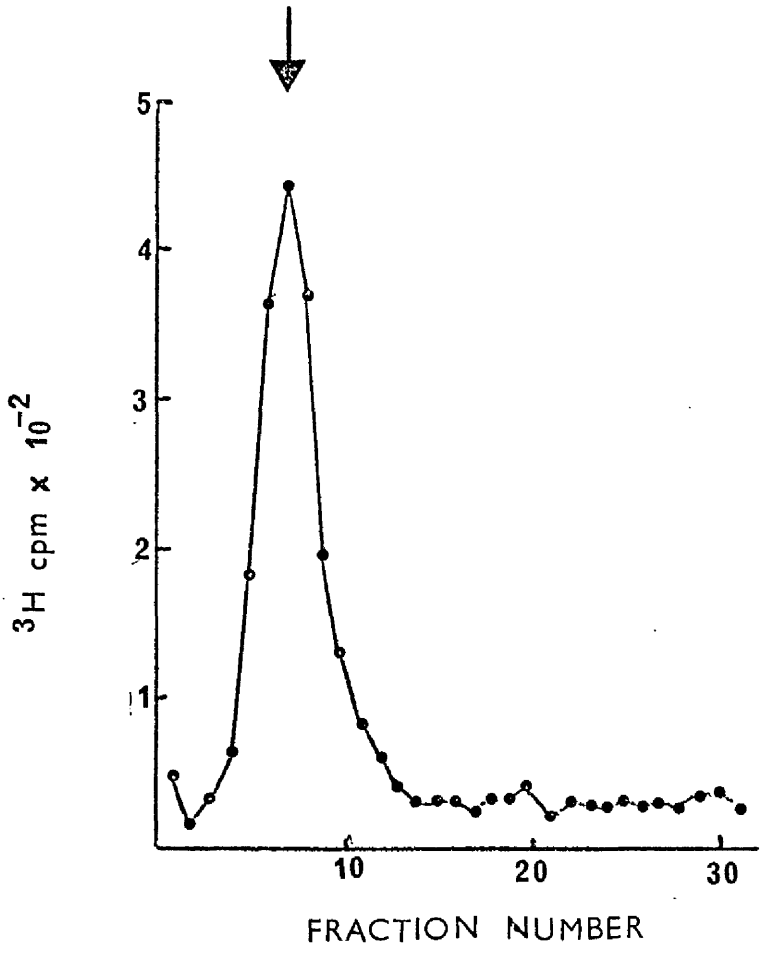

5
4
3
2
1
^{3}H cpm x 10^{-2}
10
20
30
FRACTION NUMBER

Table 10.

S1 nuclease sensitivity of purified CI DNA.

DNA	% Acid soluble
native CI	88.5 ± 0.6
denatured CI	88.4 ± 0.4

DNA purified from CI, as described in the legend to Fig. 37, either native or heat denatured was digested with S1 nuclease under the conditions described in the legend to Table 7. The results are expressed as the mean ± 1 standard deviation and there were 3 independent measurements.

this DNA can re-anneal with zero order kinetics. A similar amount of S1 resistant MVM virion DNA has been demonstrated before (Bourguignon et al, 1976). Therefore, CI DNA is single stranded and is the same molecular weight as MVM viral DNA.

These experiments indicate that CI DNA has very similar physicochemical properties as MVM virion DNA and is not present, at least in detectable amounts, in mock-infected cells. To definitely establish the viral nature of CI DNA one must perform hybridisation experiments, however, if CI DNA consists entirely of viral single strands then it will not hybridise with MVM viral DNA. Thus, a negative result could be interpretated in two ways. This problem can be surmounted by the observation that DNA polymerase I (large fragment) will synthesise a complementary strand using MVM viral stranded DNA as the template (Bourguignon et al, 1976), and therefore, a probe can be made for the necessary hybridisation experiments.

MVM DNA was incubated under the reaction conditions, described in methods section 2.4.6 with DNA polymerase I (large fragment) and thymidine-5' triphosphate was supplemented with 5 μCi α-^{32}P TTP in each reaction mixture. Fig. 38A shows the time course of incorporation of radioactivity into acid insoluble material. Thus the reaction is essentially complete after two hours of incubation. The products were analysed by neutral agarose gel electrophoresis (Fig. 38B). Thus, one can see that duplex MVM DNA is synthesised, under these conditions, from MVM viral DNA. The reaction conditions may be such that once a DNA molecule is initiated it is preferred to elongate that molecule rather than commence the initiation of new molecules. This would explain why there is an increase in the amount of incorporation between 1 and 2 hr yet there is not a significant

Fig. 38

Synthesis of duplex MVM DNA, from single stranded virion DNA, by DNA polymerase I (large fragment).

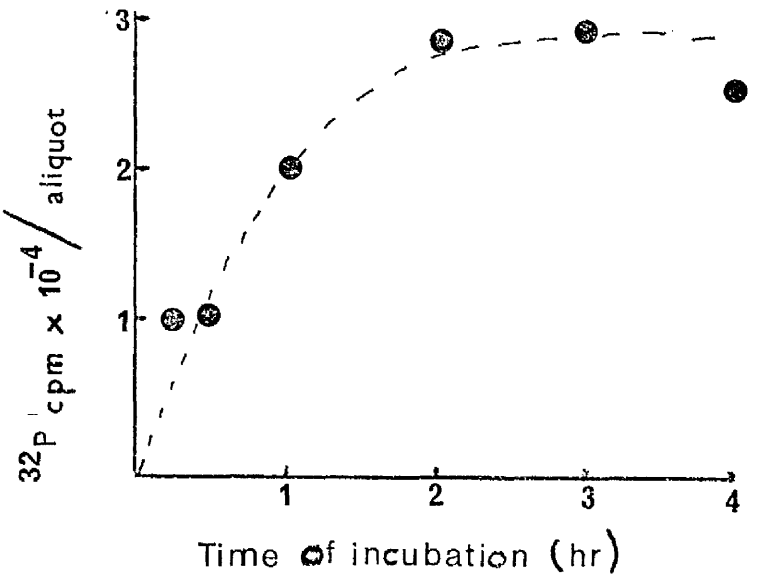
(a) Time course.

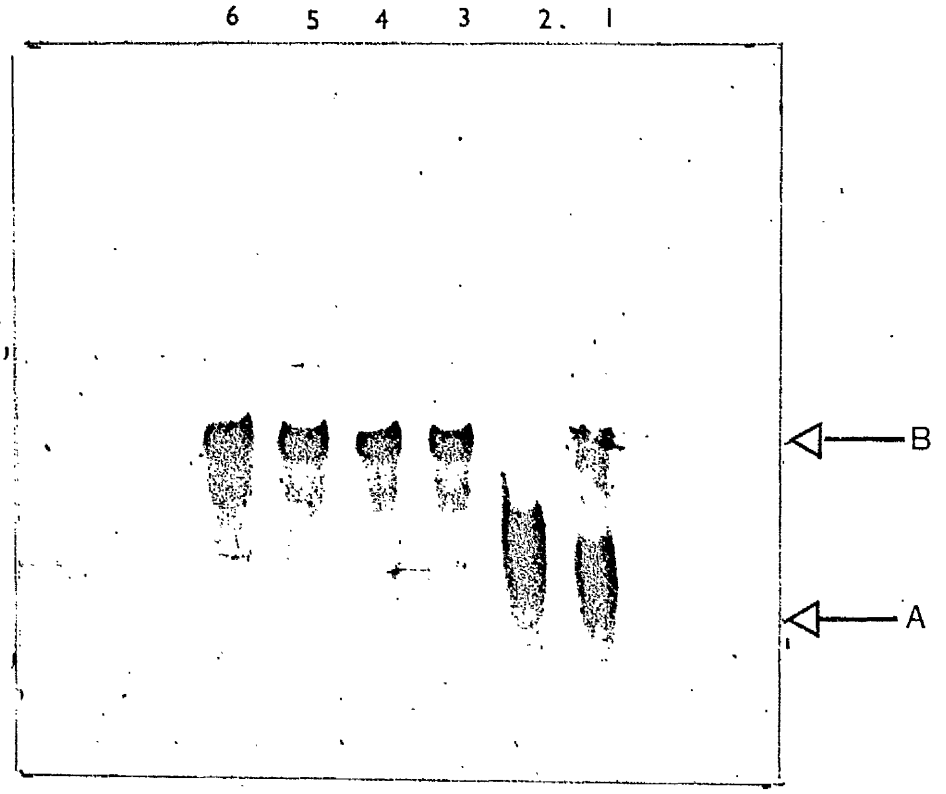
0.1 μg of MVM DNA was incubated with 0.4 units of DNA polymerase I (large fragment) under the conditions described in Materials and Methods. At various times the reaction was stopped by the addition of EDTA and the acid precipitable radioactivity measured.

(b) 1% Agarose Gel electrophoresis.

A sample (10,000 cpm) from each time point was applied to a 1% neutral agarose slab gel. Electrophoresis was for 5 hr at 50V. The gel was stained with ethidium bromide (to detect the position of the ECoRI digested Lamda DNA markers) and then dried and autoradiographed. Arrow A indicates the position of single stranded MVM DNA and arrow B indicates the expected position of MVM duplex DNA.

1. 15 min incubation
2. 30 min incubation.
3. 60 min incubation
4. 120 min incubation.
5. 180 min incubation.
6. 240 min incubation.


^{32}P cpm x 10^{-4}/ aliquot
3
2
1
1
2
3
4
Time of incubation (hr)


6 5 4 3 2 1
B
A

proportion of molecules intermediate between duplex MVM DNA and viral DNA at the 1 hr time point. This would also assume that DNA molecules were in excess over DNA polymerase I molecules. Thus, the optimum conditions for the synthesis of MVM duplex DNA were defined.

It was initially envisaged that after synthesis of MVM duplex, the hairpin region (formed due to the initiation event at the 3' end of viral DNA) would be cleaved with S1 nuclease and the strands separated by neutral agarose gel electrophoresis after alkaline denaturation of the sample. However, the hairpin region proved to be extremely resistant to treatment with S1 nuclease and even in the small proportion of molecules that were cleaved, the strand separation procedure was not convincing. Thus, this methodology was abandoned and instead it was decided to adopt a two stage experiment. In stage 1, CI DNA would be hybridised with an excess of MVM viral DNA and the percentage of CI DNA in the hybrid measured. In stage 2, CI DNA would be hybridised with duplex MVM DNA, synthesised *in vitro*, and the percentage of CI DNA in the hybrid measured. Therefore, by comparing the results from these two experiments it should prove possible to determine the viral nature of CI DNA. Table 11 illustrates the results from this type of experiment. Thus, CI DNA will hybridise almost completely to MVM duplex DNA, synthesised *in vitro*, but will not hybridise to viral MVM DNA. This indicates that CI DNA is hybridising to the complementary strand and is therefore viral DNA. Thus, CI DNA is single stranded viral DNA of the same molecular weight as MVM viral DNA extracted from mature virions.

3.4 Kinetic studies of the relationship between nucleoprotein complexes CI and CII

Table 11. Hybridisation analysis of CI DNA

Competing DNA (μg/)	Time of hybridisation (hr)	% Hybrid
0	0	12
0	72	15
MVM (0.5)	72	18
MVM (5)	72	19
MVM duplex (1.0)	72	79
MVM duplex (5)	72	81

^{3}H labelled DNA prepared from CI, as described in the legend to Fig. 37, and the unlabelled competing DNA were sheared to approximately $95^{o}_{20.w}$ (measured under alkaline conditions) and hybridised (0.1 ml volume) at 65°C in 0.01M tris-HCl, 0.001M EDTA, 0.5M NaCl, pH 7.5 for 72 hr. The percentage hybrid was calculated by measuring the fraction single stranded using S1 nuclease. The conditions for S1 nuclease digestion are given in the legend to Table 7. The same amount of CI DNA (approximately 500 cpm) was used for each hybridisation reaction. Each point is an average of two independent measurements.

All the experiments on the nucleoprotein complexes described previously were performed with DNA molecules which were labelled for periods of between 60 to 120 min. Thus these molecules probably represent products of the replication process and not replicative intermediates. In an attempt to identify replicative intermediates, shorter labelling times were employed. MVM infected BHK-21/Cl3 cells were labelled with ^{3}H-thymidine for between 5 to 30 min and nucleoprotein complexes were extracted by lysis of the cells with NP40 followed by treatment of the lysate with a final concentration of 0.5M NaCl. The supernatant fraction, after centrifugation, was analysed by neutral sucrose gradient velocity sedimentation. This analysis demonstrates that the radioactive precursor, i.e. ^{3}H thymidine is first incorporated into CII like species (Fig. 39), and after about 30 min of continuous labelling a small amount of CI is present. Thus, there is a time lag before the appearance of CI, suggesting that CI is a product rather than a percursor. Alternatively, these results could be interpretated as CI having a different replication rate from CII because no relationship has been demonstrated to exist between these nucleoprotein complexes. Pulse-chase experiments were initiated in order to differentiate between these alternatives. In this type of experiment a molecule is radioactively labelled for a short period of time and then the fate of this radioactive label is analysed by allowing replication to continue in the presence of a vast excess of unlabelled precursor. In a 20 min pulse the label is first incorporated into CII and then, during chase conditions, the radioactivity appears in the CI region of the sucrose gradient (Fig. 40). An important point to notice is that there is virtually no increase in radioactivity during the chase period. This

Fig. 39

Sedimentation analysis of rapidly labelled DNA extracted from MVM infected BHK-21/C13 cells by the NP40-NaCl method.

MVM infected BHK-21/C13 cells were labelled with ^{3}H thymidine (25 μCi/ml) for short times, commencing at 16 hpi. The cells were lysed with 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0 and after 10 to 15 min on ice, NaCl was added to a final concentration of 0.5M. 0.2 ml samples of the supernatant fraction, after centrifugation, were sedimented in 5-20% neutral sucrose gradients, containing 0.5M NaCl, for 90 min at 180,000 g (average), at 4°C in a Beckman SW56 rotor.

(a) 5 min label with ^{3}H thymidine.
(b) 10 min label with ^{3}H thymidine.
(c) 20 min label with ^{3}H thymidine
(d) 30 min label with ^{3}H thymidine.

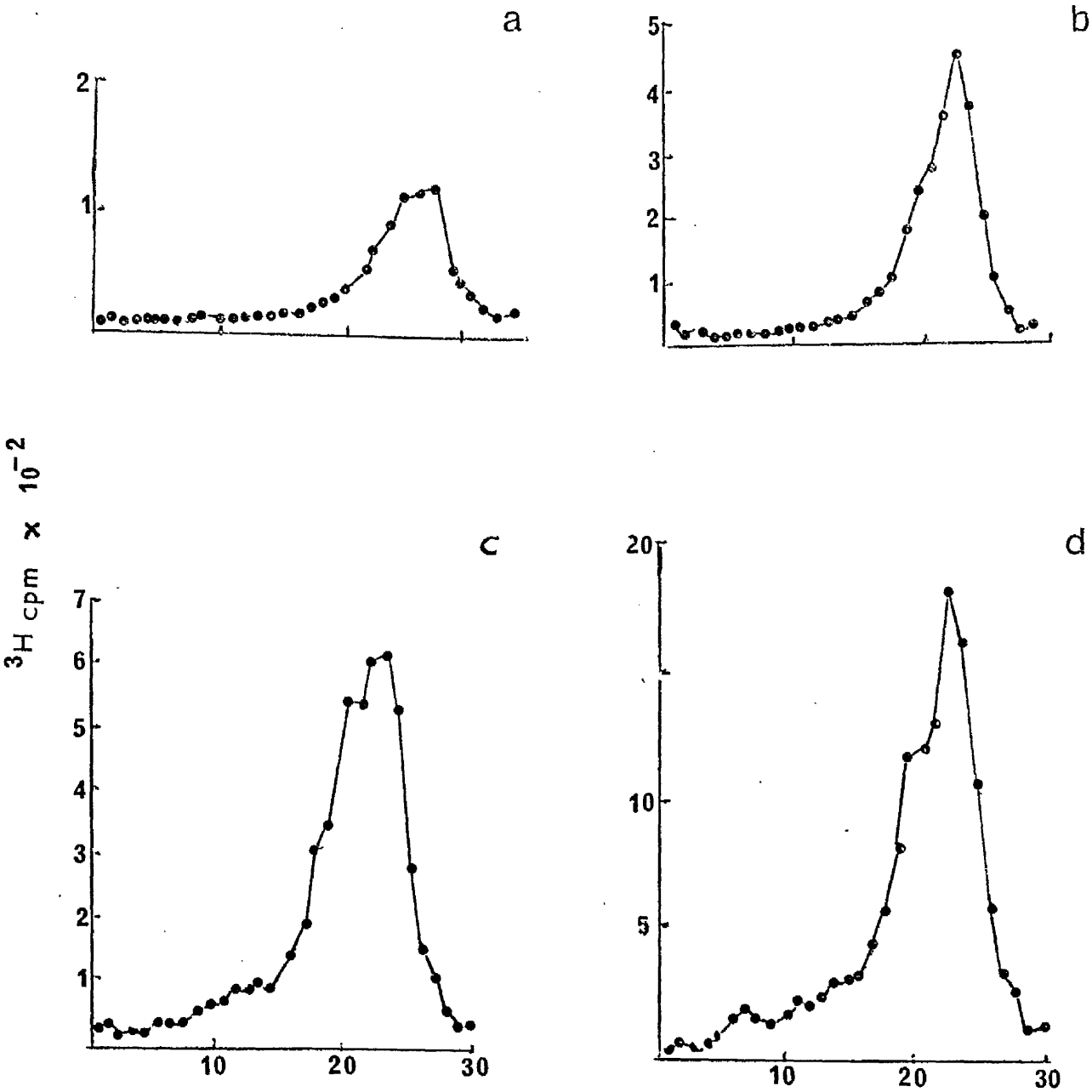

a
b
c
d
^{3}H cpm x 10^{-2}
Fraction No.

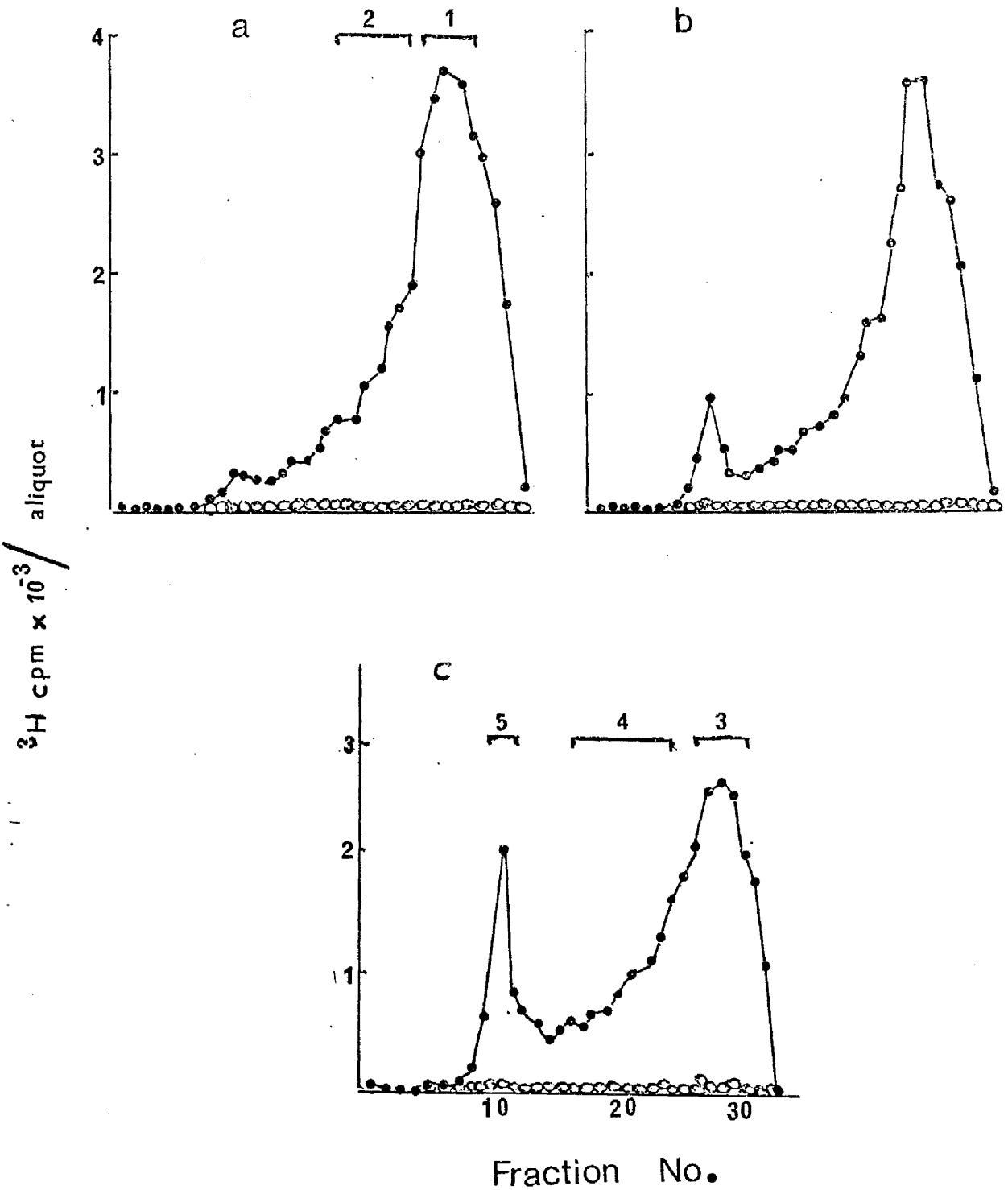
Fig. 40

Pulse chase kinetics - NP40-NaCl extraction of MVM-infected and mock-infected BHK-21/C13 cells.

MVM-infected and mock-infected BHK-21/C13 cells were labelled with ^{3}H thymidine (25 -Ci/ml) for 20 min at 16 hpi. The DNA was extracted by the NP40-NaCl method, described in the legend to Fig. 39 before (A) and after chasing for 15 min (b) and 60 min (c) in the presence of unlabelled thymidine (100 µg/ml) and 2' deoxycytidine (10 µg/ml). 0.2 ml of each sample was sedimented in 5-20% neutral sucrose gradients, containing 0.5M NaCl, for 90 min at 180,000 g (average) at $4^{o}C$. Fractions were pooled for further analysis as indicated on each figure.

●——● MVM infected

○——○ mock infected.


a
b
c
2
1
5
4
3
4
3
2
1
3
2
1
10
20
30
^{3}H cpm x 10^{-3} / aliquot
Fraction No.

is essential because the possibility of different replication rates could not be eliminated since radioactivity incorporated into a putative product could have occurred due to either de novo synthesis or formation from a precursor molecule. The sucrose gradient can be separated into three regions, CI, intermediate and CII, as described in the legend to Fig. 20. After the 20 min pulse 68% of the radioactivity within these three regions is present in CII, 27% is in the intermediate fraction, and 5% is in the CI region. After a 15 min chase, 64% is in CII, 26% intermediate fraction and 10% is in the CI region. After a 60 min chase, 50% is in the CII region, 27% is in the intermediate region and 22% is in the CI region. These results can be interpretated in two ways. Firstly, CII is a precursor to CI and the intermediate fraction is metabolically active but a dead end product or slowly forming an undetermined product. Secondly, CII is converted to the intermediate fraction which is then converted to CI. These pulse-chase experiments cannot distinguish between these possibilities.

DNA was purified from the various indicated fractions on the neutral sucrose gradient (Fig. 40) by treatment with SDS and pronase. This DNA was then analysed by neutral agarose gel electrophoresis (Fig. 41). 3 main peaks are present in pooled samples 1, 2, 3 and 4. These peaks correspond to 3×10^6, 6×10^6 and 12×10^6 daltons and are present in different amounts. In pooled samples 1 and 3 the ratios of the peaks are very similar. Pooled samples 2 and 4 also have very similar ratios of peaks. In none of these samples is DNA with the mobility of virion DNA present. However, in pooled sample 5, DNA with the mobility of single stranded viral DNA is present. Sodium iodide buoyant density gradients have confirmed

Fig. 41

Neutral agarose gel electrophoretic analysis of the DNA extracted from the fractions indicated in Fig. 39.

The fractions indicated in Fig. 39 were pooled and digested with final concentrations of 0.5% (w/v) SDS and 100 μg/ml pronase at 37°C for 1 hr. 0.15 ml samples were electrophoresed in 1.5% agarose gels for 3 hr at 100V. The gels were sliced into either 0.9 mm sections (A, B, C) or 1.0 mm sections (D and E) and the radioactivity determined by liquid scintillation counting as described in Materials and Methods. Migration is from left to right.

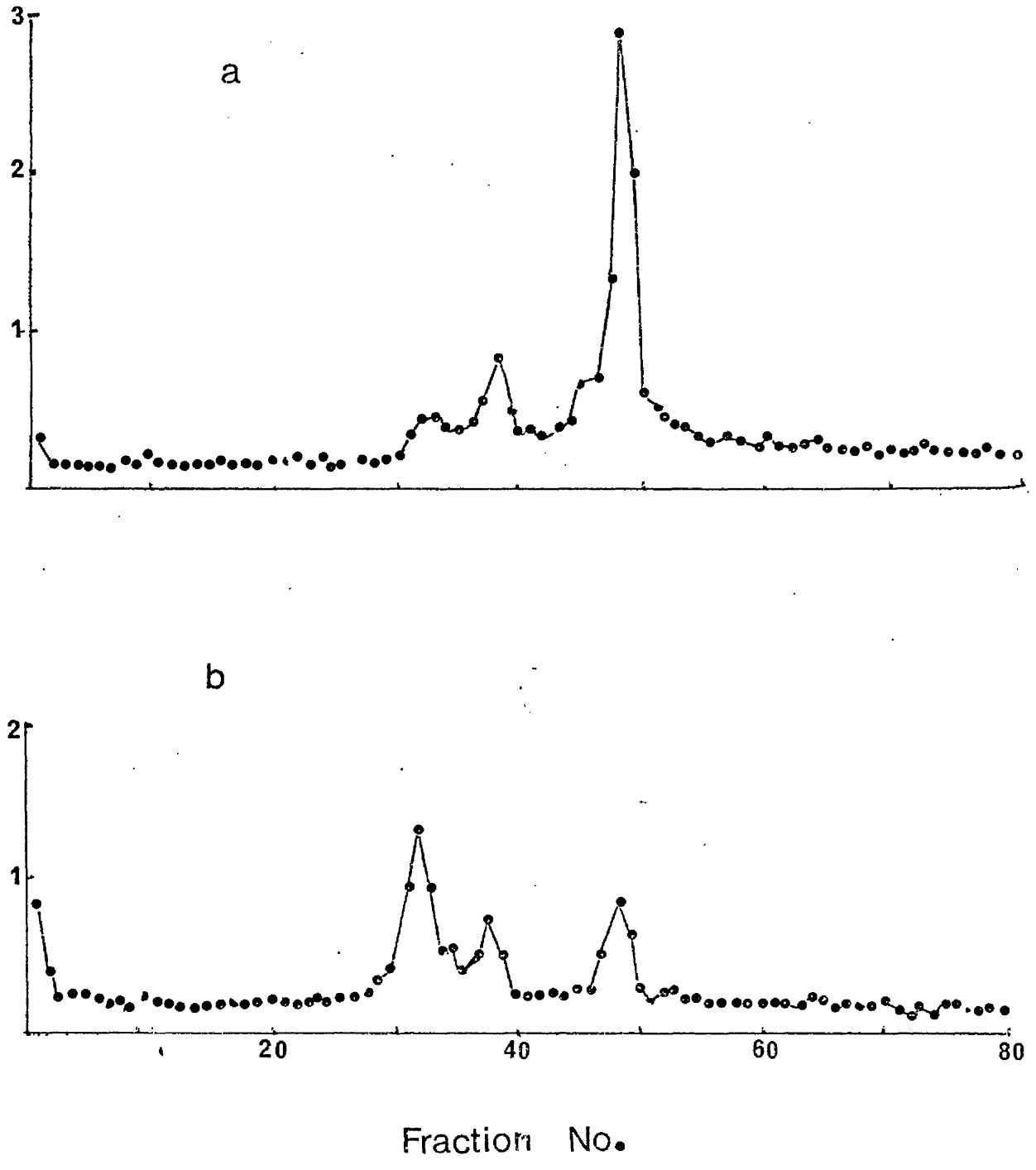
(a) pool I in Fig. 40

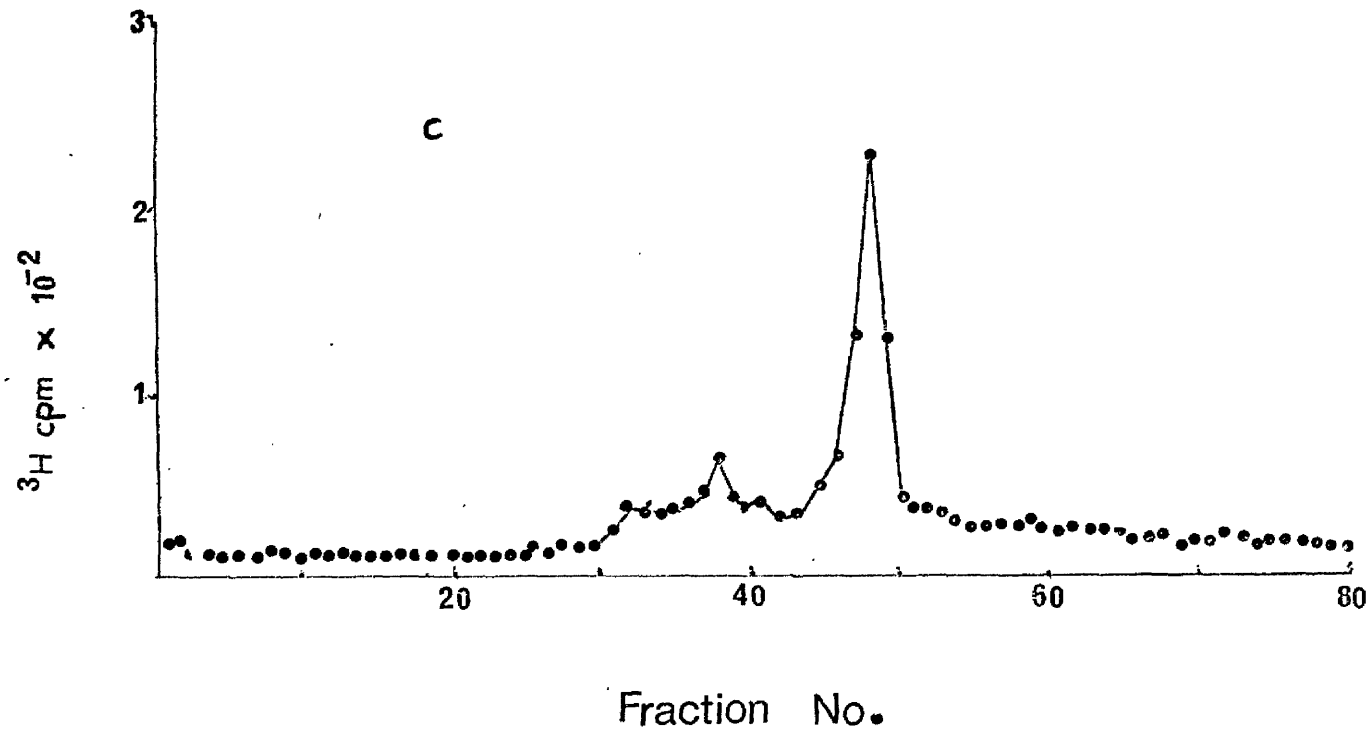
(b) pool 2 in Fig. 40

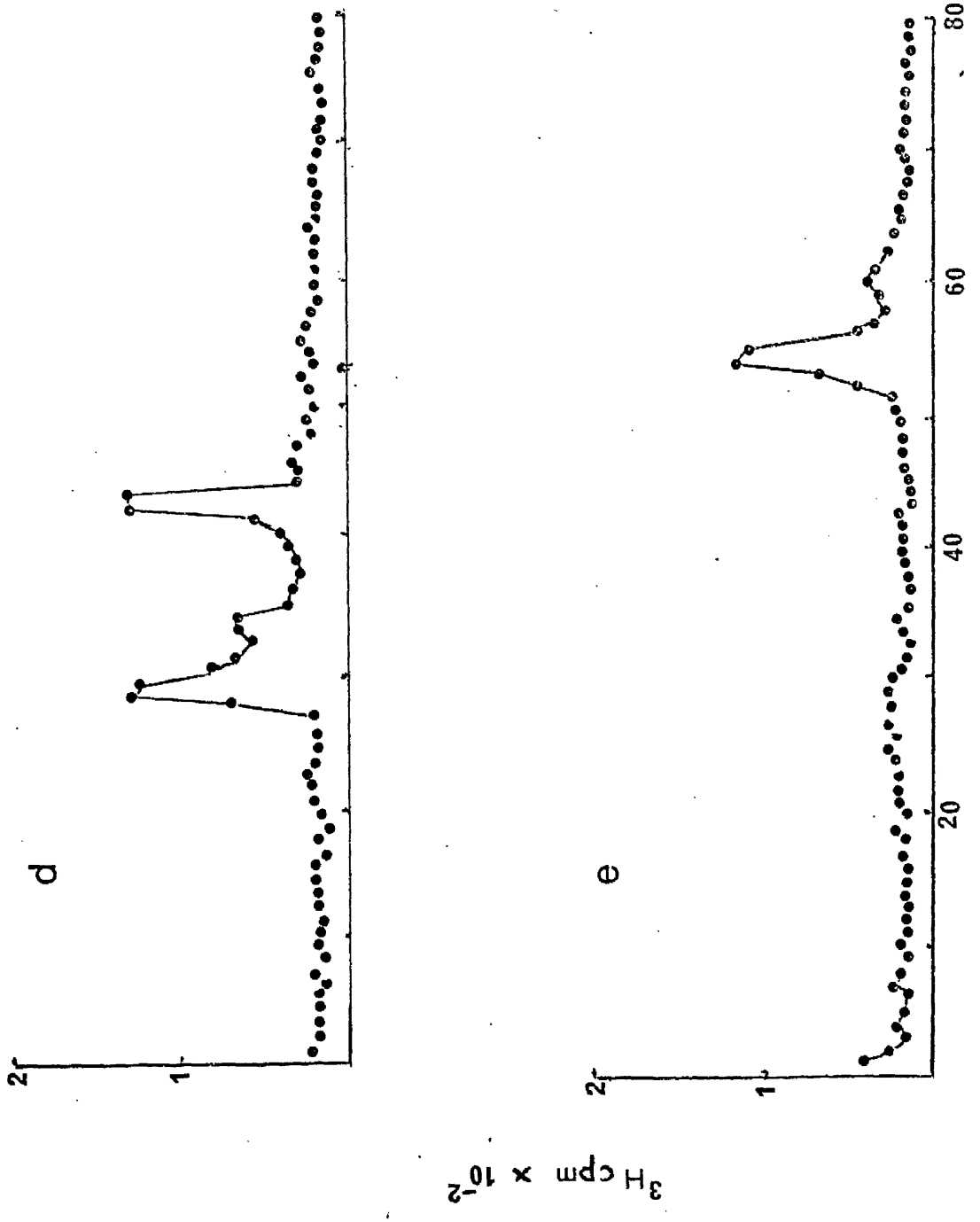
(c) pool 3 in Fig. 40

(d) pool 4 in Fig. 40

(e) pool 5 in Fig. 40.


a
b
3
2
1
2
1
20
40
60
80
^3H cpm x 10^{-2}
Fraction No.


C
3
2
1
^{3}H cpm x 10^{-2}
20
40
50
60
Fraction No.


d
e
2
1
2
1
20
40
60
80
Fraction No.
^3H cpm x 10^{-2}

that pools 1 to 4 consist of double stranded DNA and that pool 5 consists of single stranded DNA (data not shown). Thus MVM DNA replicates in the form of nucleoprotein complexes.

3.5 Electron microscopy

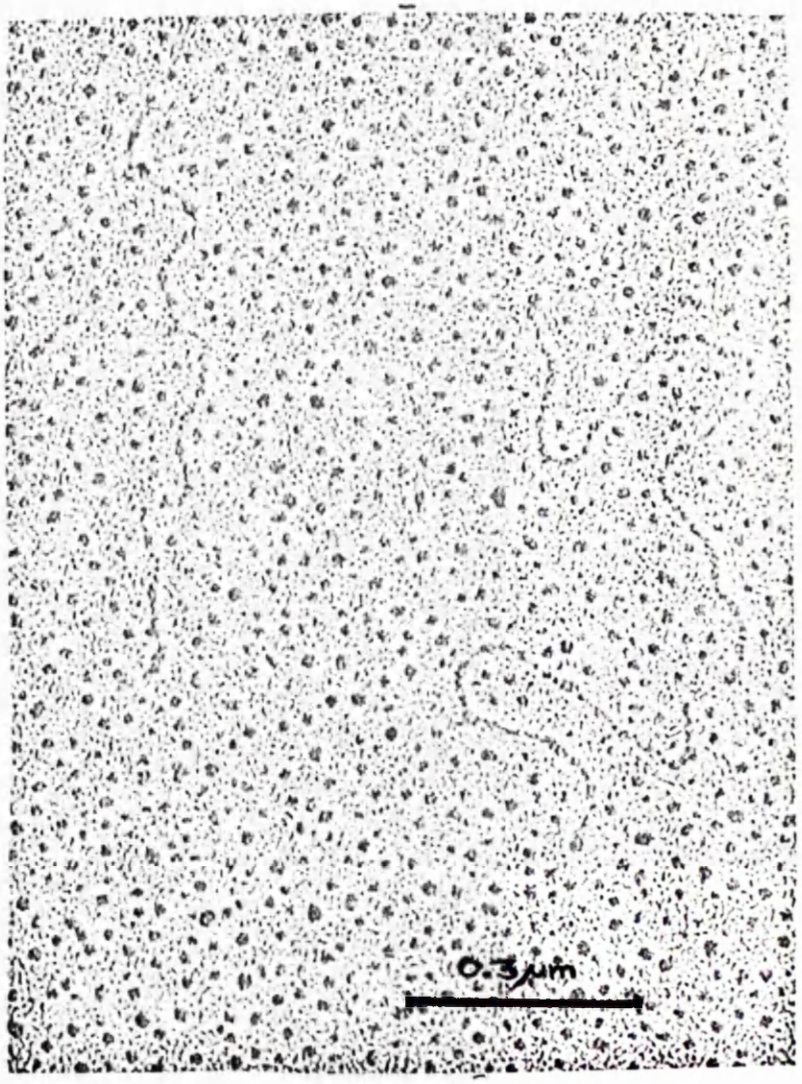
DNA was mounted for electron microscopy by one of two variations of the basic protein film technique (Kleinschmidt, 1968; Davis et al, 1971). These techniques take advantage of the observation that globular proteins, in solution, are capable of producing surface films on an aqueous solution. The DNA can be adsorbed to the protein by interaction with the basic side groups of amino acid residues. Thus, when a solution containing both the basic protein, usually cytochrome c, and the nucleic acid is spread onto an aqueous solution, termed the hypophase, a monolayer containing nucleic acid adsorbed to the protein film is observed. This monolayer is then itself adsorbed to a solid support (electron microscope grid), dehydrated and usually stained with uranylacetate and shadowed with a heavy metal (Platinum and/or Palladium) to improve the contrast. Therefore, the structure visualised in the electron microscope is a collapsed column of basic protein around the DNA. In the aqueous technique, double stranded DNA appears as a gently curved filament (wormlike tail). Single stranded DNA is condensed into bushes because of random base-base interactions. In the formamide technique, the DNA is mounted at a formamide and salt concentration such that double stranded DNA is stable, but the random base interactions in single stranded DNA are melted out and single stranded DNA also appears as a curved filament, which can be distinguished from double stranded DNA.

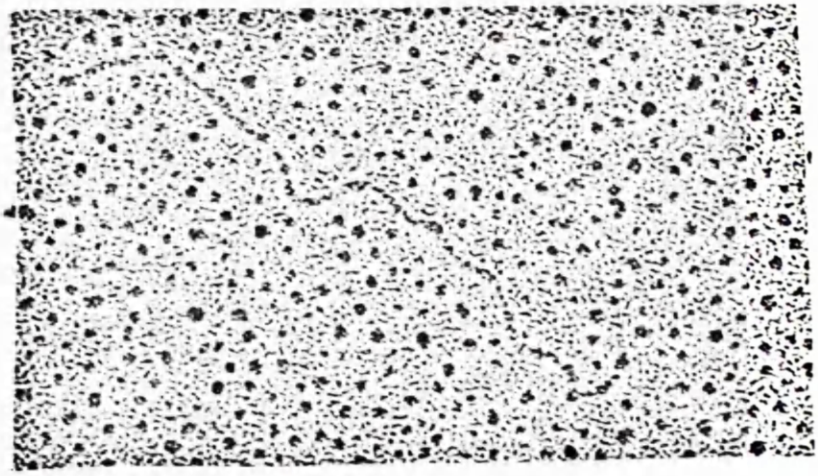
MVM virion DNA was mounted for electron microscopy using the formamide technique. Fig. 42 is a typical photograph of MVM single stranded DNA. As can be seen the DNA is linear and indeed no circular structures were observed even upon renaturation. This is consistent with the results of Bourguignon et al (1976) who also demonstrated that no circular structures of MVM DNA could be observed, thus implying that MVM DNA does not possess an inverted terminal repeat. The molecular weight of MVM DNA was calculated by comparing the length distributions of MVM DNA with those of an internal marker of known molecular weight. ØX174 was used as an internal marker for two reasons. Firstly, it is single stranded and circular and thus can be easily distinguished from MVM DNA which is linear. Secondly the molecular weight of ØX174 DNA is very accurately known because the sequence, and hence number, of the nucleotides have been determined (Sanger et al, 1977). In order to calculate the molecular weight of MVM DNA, the lengths of ØX174 DNA were measured and the mean $\pm$ 1 standard deviation was determined. This length was then assumed to correspond to 5375 bases and the appropriate conversions made. Thus ØX174 DNA had a molecular weight $\pm$ 1 standard deviation of 5375 $\pm$ 245 bases. The length of MVM was then measured, at the same magnification, and the molecular weight $\pm$ 1 standard deviation determined to be 4863 $\pm$ 426 bases. 108 molecules were measured. In the case of ØX174 DNA all the molecules that have been scored were included in the calculation of the molecular weight. For MVM DNA only the molecules in the range 4000 to 5800 bases were included in the calculations. This range consists of 73% of the measured molecules and was used because it was assumed that full length molecules would be those molecules which occurred within plus or minus 15% of the peak of

Fig. 42

Electron micrograph of MVM DNA.

Viral DNA was purified by direct lysis of MVM virions on alkaline sucrose gradients. The entire viral DNA band was desalted, using a Sephadex G25 column, into 10 mM tris-HCl, 1 mM EDTA pH 7.5 and mounted for electron microscopy by the formamide technique, as described in the Methods section.


0.3 µm



molecules occurring at 4800 to 5000 bases. Also, there is a marked difference between the histograms of ØX174 DNA and MVM DNA. ØX174 DNA has a much smaller size range of molecules, whereas MVM DNA has a proportion (27%) of smaller than unit length molecules. This may be because only intact ØX174 DNA molecules are scored since broken molecules would be included in the MVM DNA table. The data for MVM DNA (Fig. 43a) is a combination of two sets of results. The first set is MVM DNA mounted by itself and the second is MVM DNA plus ØX174 DNA. The length of the molecules was measured at the same magnification. There was essentially no difference in distribution between the two sets of data except that a few more molecules in the high molecular weight range of the histogram (5600 to 6000) were present in the sample which included MVM DNA and ØX174 DNA and thus may have possibly represented broken ØX174 DNA molecules. Thus this distribution is due to the presence of smaller than unit length MVM DNA molecules although the data is biased in favour of ØX174 DNA for the previously stated reason. These small molecules may represent defective genomes or may be a consequence of the DNA preparation method. However, since ØX174 DNA is prepared in an identical manner it is likely that these molecules do represent defective genomes. Similar distributions of MVM DNA have been observed previously (Bourguignon et al, 1976).

DNA was prepared from MVM-infected and mock-infected cells for electron microscopy by the methods previously described. MVM-infected or mock-infected BHK-21/Cl3 cells were harvested at 17 hpi and lysed with NP40. The cytoplasmic fraction was separated by low speed centrifugation and the nuclei washed three times with RSB. The nuclei were then lysed with 0.5M NaCl, final concentration, and the

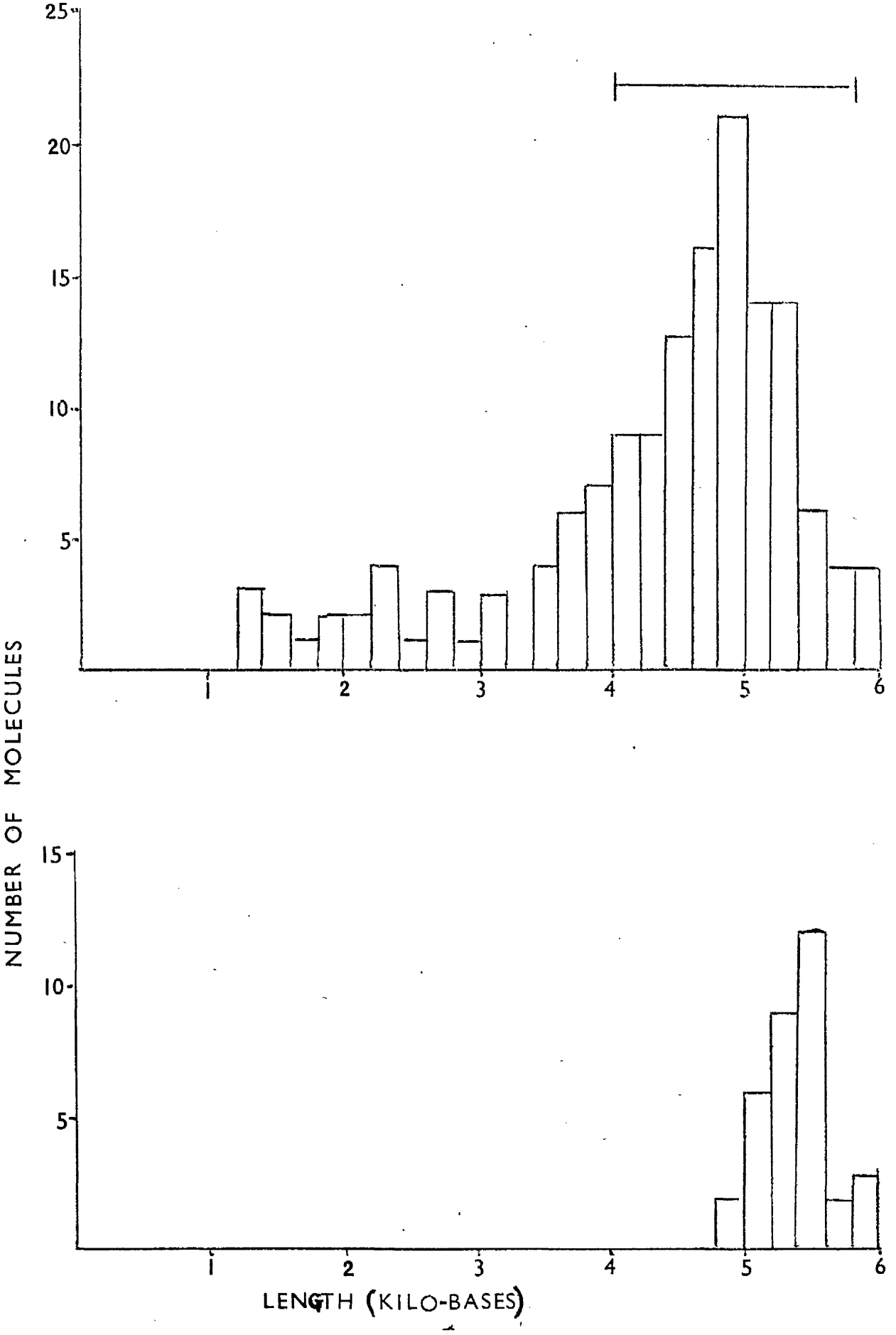
Fig. 43

Histograms of the length distributions of ØX174 DNA and MVM DNA mounted for electron microscopy by the formamide technique.

DNA was purified from MVM and ØX174 virions by alkaline lysis followed by sedimentation in 5 to 20% alkaline sucrose gradients. The DNA was prepared and mounted for electron microscopy as described in the legend to Fig. 42. Two preparations were analysed. One contained MVM DNA and ØX174 DNA, the other contained only MVM DNA. The length of ØX174 DNA was measured and the mean $\pm$ 1 standard deviation calculated. This mean was assumed to correspond to a length of 5375 bases. Under identical magnification the lengths of MVM DNA from each preparation were measured. Each length was converted to a molecular weight, in bases, by assuming that the mean length measured for ØX174 DNA corresponded to 5375 bases.

(a) MVM DNA. The bar indicates the range of lengths chosen to represent intact molecules.

(b) ØX174 DNA.


25
20
15
10
5
1
2
3
4
5
6
NUMBER OF MOLECULES
15
10
5
1
2
3
4
5
6
LENGTH (KILO-BASES)

supernatant fraction, after centrifugation, prepared. Both the cytoplasmic fraction and the nuclear supernatant fraction were deproteinised by treatment with SDS, pronase and phenol. The nucleic acids were precipitated with ethanol and after resuspension digested with heat treated pancreatic RNase. The samples were again deproteinised and ethanol precipitated. After resuspension the samples were extensively dialysed against 10 mM tris-HCl, 10 mM EDTA, pH 7.5. An aliquot from each sample was analysed by neutral agarose gel electrophoresis (Fig. 44). In the nuclear supernatant fraction from MVM infected BHK-21/C13 cells five bands are visible (track 4). The top band (highest molecular weight) probably represents cellular DNA contamination since it is present in the mock infected extract (track 1). The second band probably represents dimer and it is not present in mock infected extracts. The third band represents monomer RF DNA and the fourth band probably represents defective, shorter than unit length, DNA. The fifth band is at the position of single stranded virion DNA. Thus, the MVM infected nuclear extract contains 4 bands which are probably of viral origin since they are not present in mock infected cells. The MVM infected cytoplasmic extract (track 5) contains one major band at the position of MVM viral DNA and one diffuse band. The origin of the diffuse band is unknown though it may be of cellular origin since it is present in the mock infected extract (track 8).

The DNA prepared from the nuclear extract of MVM-infected BHK-21/C13 cells (track 5) was mounted for electron microscopy by the aqueous technique. Fig. 45 is a typical photograph of this preparation. The linear molecules are MVM DNA and the circular molecules are the plasmid pBR322 DNA which was added as an internal marker. No circular

Fig. 44

Agarose gel electrophoresis of DNA extracted from infected BHK-21/C13 cells by the NP40-NaCl method.

MVM-infected and mock-infected BHK-21/C13 cells were harvested at 17 hpi. The cells were lysed with 0.5% (v/v) NP40 in 10 mM tris-HCl, 10 mM EDTA pH 7.0. The cytoplasmic fraction was removed. The nuclei were treated with a final concentration of 0.5M NaCl and the supernatant fraction, after centrifugation at 10,000 g for 10 min at 4°C, prepared. Both cytoplasmic and nuclear fractions from MVM-infected and mock-infected cells were treated with 0.5% SDS and 2 mg/ml self-digested pronase for 3 hr at 37°C. The samples were phenol extracted and ethanol precipitated. They were redissolved in 50 mM tris-HCl, 1 mM EDTA pH 7.5 and incubated with 50 µg/ml, heat treated, pancreatic RNase for 1 hr at 37°C. The samples were again treated with SDS, pronase, phenol extracted and ethanol precipitated. They were redissolved in 10 mM tris-HCl, 1 mM EDTA pH 7.5 and extensively dialysed against the same buffer. An aliquot of each sample was electrophoresed on a 1.0% neutral agarose gel for 5 hr at 50V. Markers of Lambda DNA, digested with restriction endonuclease ECoRI, and MVM viral DNA were co-electrophoresed.

track 1, mock infected nuclear extract; track 2, MVM viral DNA; track 3, Lambda DNA, digested with ECoRI; track 4, MVM infected nuclear extract; track 5, MVM infected cytoplasmic extract; track 6, Lambda DNA digested with ECoRI; track 7, MVM viral DNA; track 8, mock infected cytoplasmic extract.

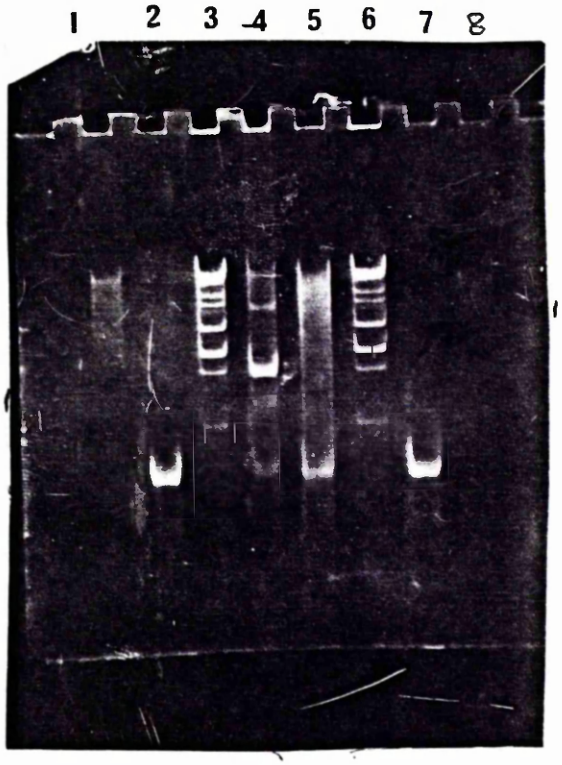
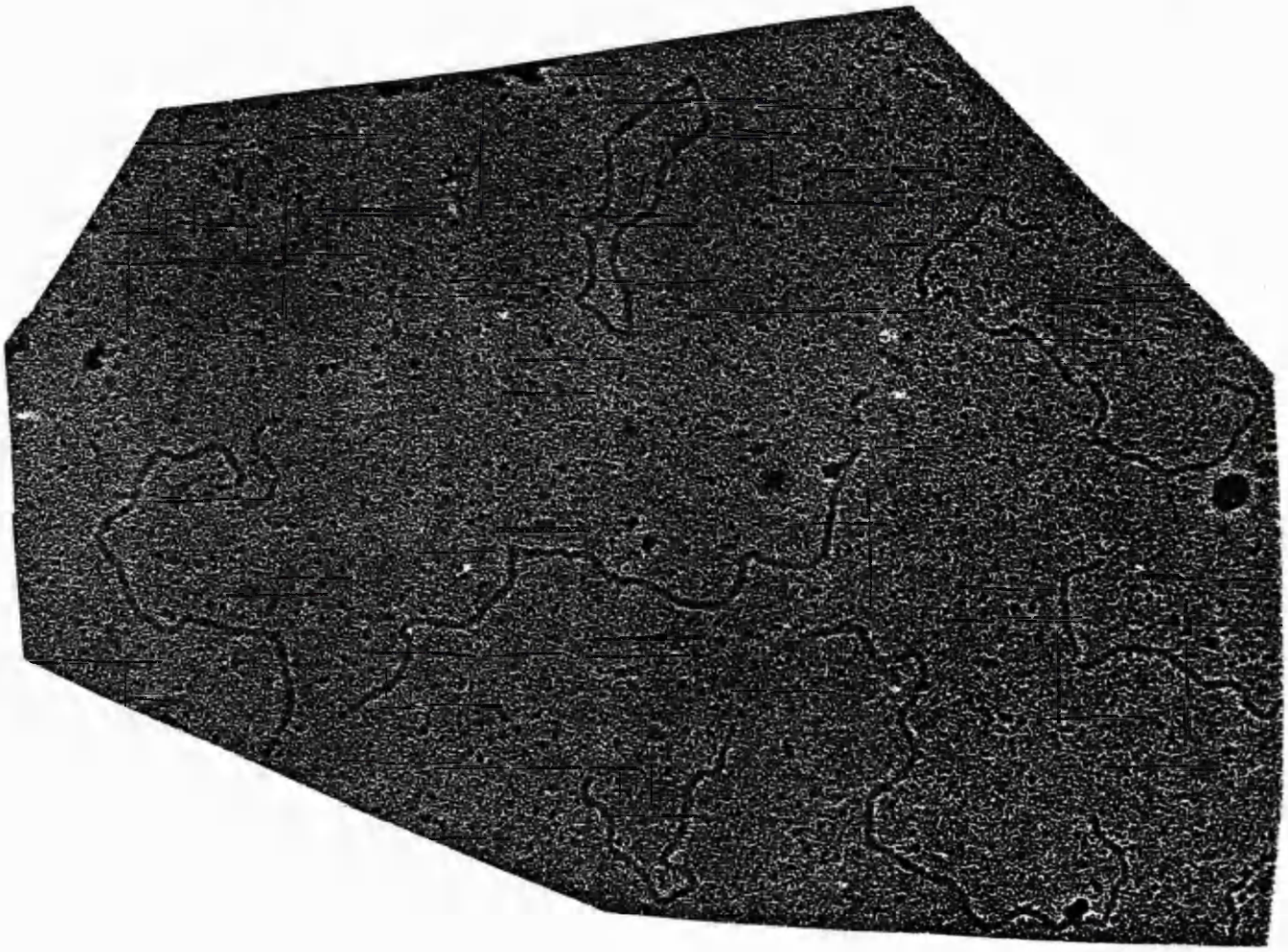
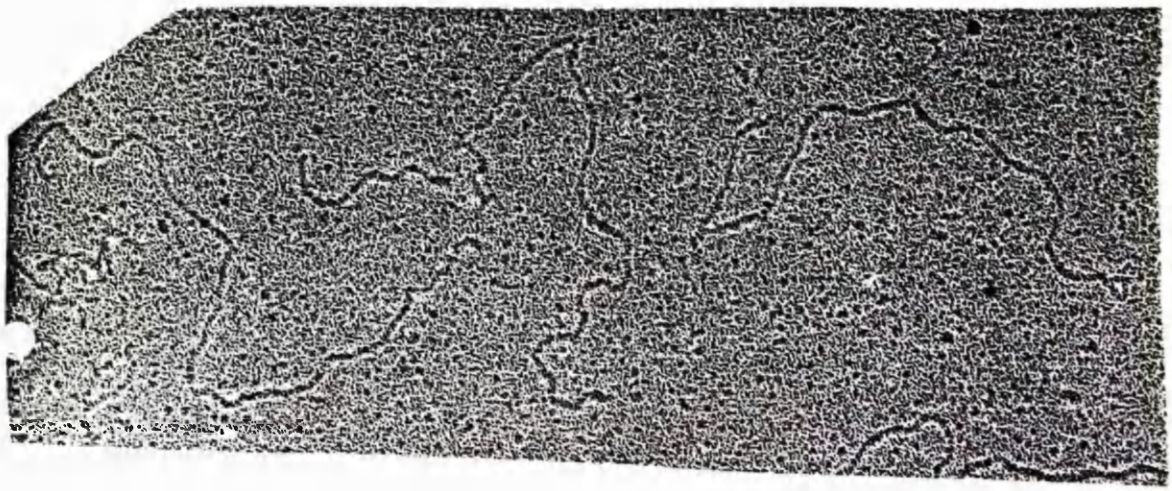

1 2 3 4 5 6 7 8

Fig. 45

Electron micrograph of DNA purified from the 0.5M NaCl nuclear extract of MVM infected BHK-21/C13 cells.

DNA purified from the 0.5M NaCl nuclear extract of MVM infected BHK-21/C13 cells prepared as indicated in the legend to Fig. 44 and analysed by agarose gel electrophoresis (Fig. 44, track 4) was spread for electron microscopy using the aqueous spreading technique. Circular molecules of pBR322 were added as internal markers.





molecules were observed without addition of the plasmid. The lengths of the molecules were measured and are expressed in base pairs assuming the plasmid pBR322 to be 4362 base pairs in length (Sutcliffe, 1978). A histogram was constructed and is shown in Fig. 46. The mean of all molecules between 4000 base pairs and 5800 base pairs was calculated and is 4636 base pairs $\pm$ 355 base pairs (mean $\pm$ 1 standard deviation). This result corresponds very well with the calculated length of MVM virion DNA which was 4863 bases in length, relative to ØX174. Therefore this DNA would appear to be MVM duplex DNA. Similarly, to the electron microscopic analysis of MVM virion DNA there is a proportion of smaller DNA molecules which may correspond to defective genomes. However, unlike the gel analysis there are not many molecules greater than unit length i.e. 4636 base pairs. This may be because of the relative infrequency of these molecules. A dimer molecule will be over-represented in a gel because of the staining procedure, because a dimer DNA molecule will take up twice as much stain as a monomer DNA molecule, therefore, for the same fluorescent intensity there will be twice as many monomer DNA molecules. Thus, although there is a detectable amount of dimer molecules on a gel they may in fact be relatively infrequent in numerical terms. In conclusion, electron microscopy has revealed that the major DNA species is a linear duplex DNA molecule of 4636 base pairs in length.

However, one major advantage of electron microscopy is the possibility to observe molecules which are present at a low frequency, i.e. possible replicating molecules. A structure which is apparent, at a level between 2-3%, is a linear duplex molecule of monomer RF DNA length with a single stranded branch (Fig. 47). The single stranded

Fig. 46

Histogram of the length distributions of MVM DNA mounted for electron microscopy by the aqueous technique.

DNA purified from the 0.5M NaCl nuclear extract of MVM infected BHK-21/C13 cells prepared as indicated in the legend to Fig. 44, was spread for electron microscopy using the aqueous spreading technique. The length of MVM DNA was calculated and is expressed in base pairs assuming a length of 4362 base pairs for pBR322 by the methods indicated in the legend to Fig. 43.

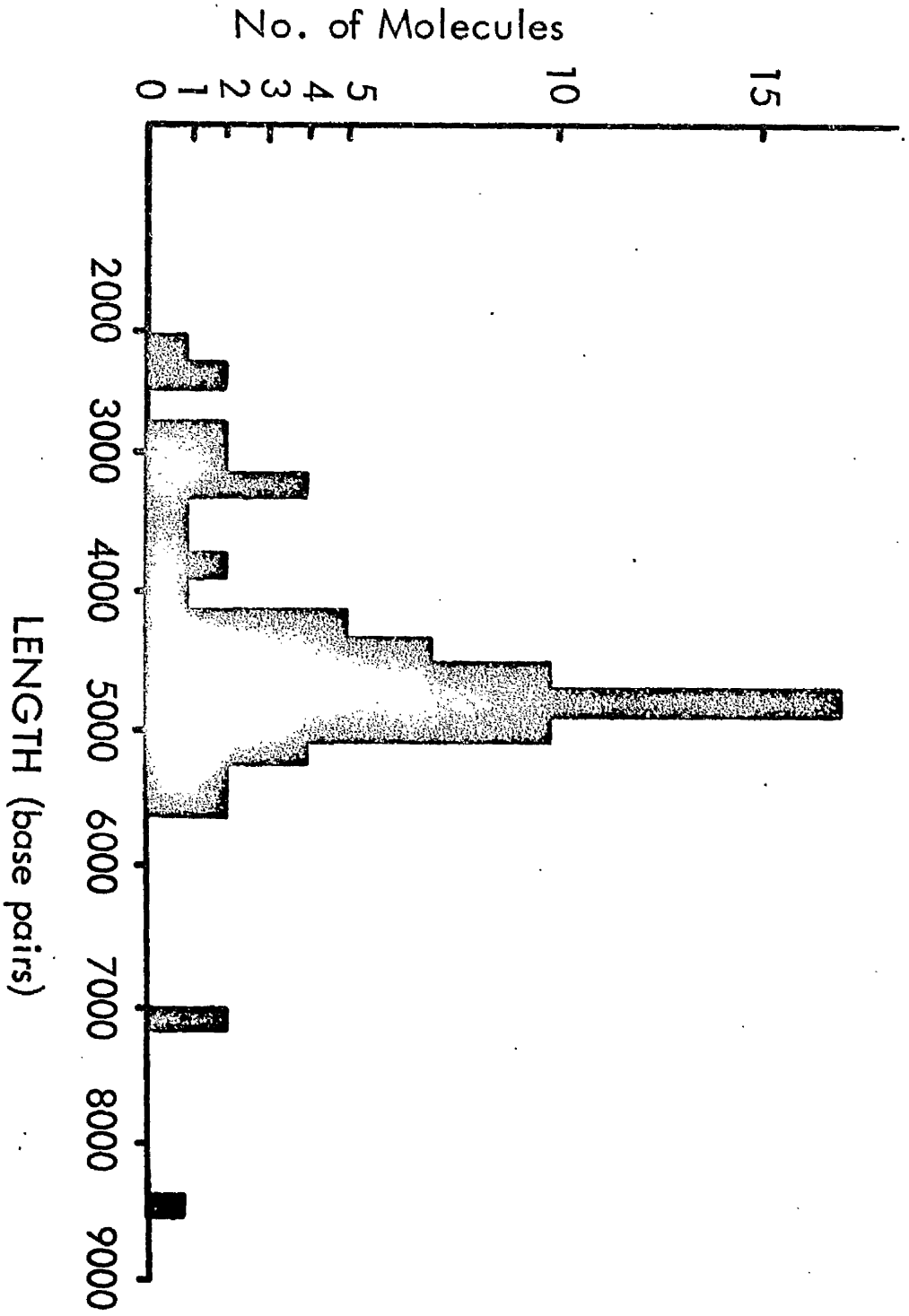

No. of Molecules
0
1
2
3
4
5
10
15
LENGTH (base pairs)
2000
3000
4000
5000
6000
7000
8000
9000

Fig. 47 Electron Micrographs of DNA purified from the 0.5M NaCl nuclear extract of MVM-infected BHK-21/Cl3 cells.

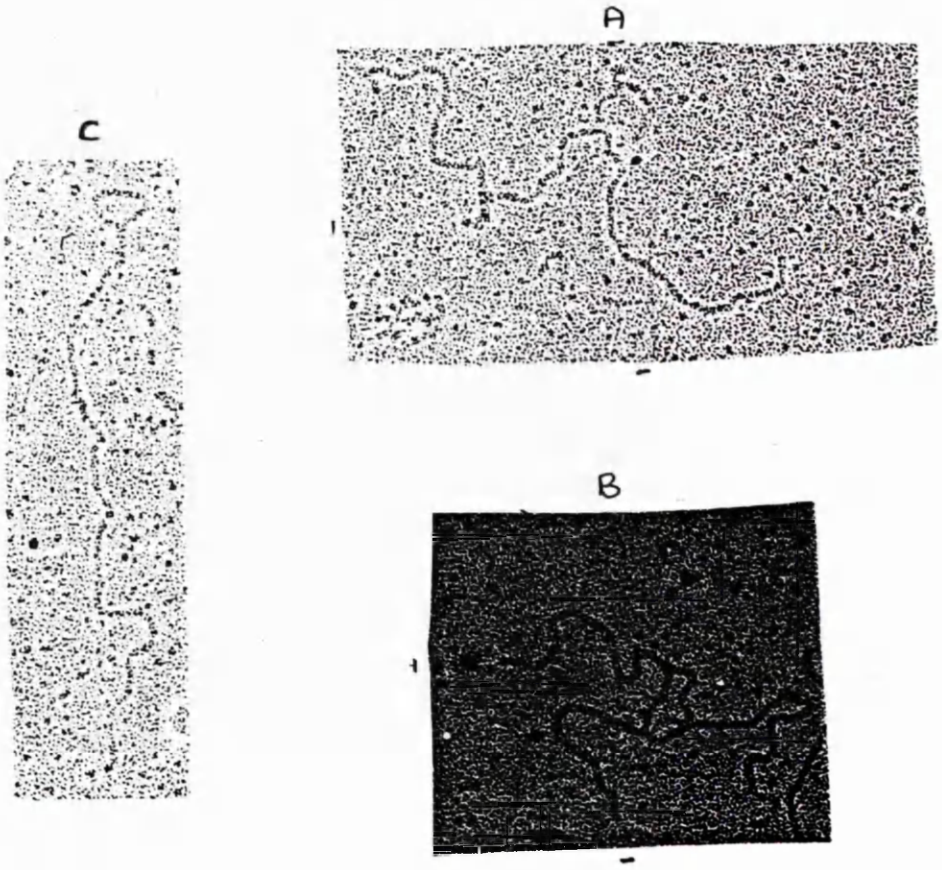
DNA purified and spread for electron microscopy as described in the legend to Fig. 45 was analysed for the presence of possible replicative intermediates.

A. double stranded DNA molecule with a bushlike (single stranded) protruberance.

B. completely double stranded Y-shaped molecule.

C. completely double stranded lariat shaped molecule.

D. double stranded DNA molecule with a bush-like (single stranded DNA) protruberance.


A
C
B
D

branch is obvious due to its bush-like appearance. Another structure which is present at lower frequencies is a completely double stranded Y-shaped molecule (Fig. 47) similar to those observed during H-1 DNA replication (Singer and Rhode, 1977). Another structure present at low frequencies is a lariat type conformation. This has also been observed previously (Rhode, 1978c; Bratosin et al, 1979) but the significance of these structures is unknown. One should stress at this point, however, that the mere observation of these molecules does not prove that they are involved in MVM DNA replication. The technique of electron microscopy is very powerful, but, confirmation of the results must be obtained by independent methods.

4. Discussion

Discussion

The time course of MVM infection in BHK-21/C13 cells is similar to that reported previously for MVM-infected A9 cells (Tattersall et al, 1973). DNA replication commences at between 8 to 10 hpi and reaches a maximum between 14 to 16 hpi, after which time there is a decrease in the rate of DNA synthesis. The infection is asynchronous because the BHK-21/C13 cells are growing logarithmically and initiation of MVM DNA replication occurs during late S or early G_2 phase of the cell growth cycle (Tattersall, 1972). Nevertheless there is a peak in the rate of DNA replication followed by a slight decrease. Indeed, Siegl and Gautschi (1973b) observed that there was a similar order of events during LuIII replication in both synchronous and asynchronous cultures although the events occurred earlier in synchronous cultures. This may be a consequence of the sensitivity of the detection techniques since in asynchronous cultures a smaller percentage of cells undergo the initiation event at any one time. Thus, for example a smaller amount of radioactive isotope is incorporated or a smaller amount of haemagglutination is produced. Therefore, at earlier times during infection of asynchronous cultures these events may not be detectable. Siegl and Gautschi (1973a) noted that between 30 to 55% of cells in asynchronous cultures were infected, as detected by fluorescent antibody staining and that a maximum rate of DNA synthesis was reached, in each case, at approximately 16 hpi. Although Parvoviruses can enter the cells at any time during infection, if entry occurs at a time too distant from the S phase of the cell cycle an abortive infection occurs. This would explain the observed maximum rate of DNA replication followed by a decrease in the rate of

DNA synthesis in asynchronous cultures (Fig.13; Tattersall et al, 1973). The major advantage in the use of logarithmically growing instead of synchronised cell cultures in the study of DNA replication is the ability to produce large quantities of material without substantially altering the metabolism of the host cell. Since the course of the infection is very similar in both instances the advantage of the synchronised culture does not seem to be required.

In order to study the complete set of virus replication and assembly events in MVM-infected cells a mild extraction procedure of the virus infected cells was sought which would result in the detection of MVM nucleoprotein complexes and the mature virion. In this way the synthesis of viral DNA and its subsequent packaging and maturation into virion structure could be followed. To achieve this aim the effect of various conditions (nonionic detergent, pH and NaCl) upon the extraction of nucleoprotein complexes from MVM-infected cells was studied.

In both BHK-21/C13 cells and A9 cells infected with MVM, the largest quantity of nucleoprotein complexes was extracted by treatment of the nuclei with a final concentration of 0.5M NaCl. The effect of the NaCl would appear to be correlated with lysis of the nuclei and may reflect the association of viral nucleoprotein complexes with the cellular chromatin. The replicative intermediate, monomer RF DNA, has been demonstrated to be closely associated with cellular chromatin during LuIII infection of HeLa cells (Siegl and Gautschi, 1976). The difference in the extractability of the viral nucleoprotein complexes between BHK-21/C13 cells and A9 cells, infected with MVM, may be related to the stronger association of MVM viral nucleoprotein complexes with A9 cellular chromatin, although there is no obvious

reason to suggest why this should be so. The nucleoprotein complexes extracted at NaCl concentrations varying from no added NaCl to 0.5M are similar when studied by sedimentation analysis using neutral sucrose gradients, containing 0.5M NaCl. However, the nucleoprotein complexes extracted at each NaCl concentration, although structurally similar, may have a different role in the nucleus, e.g. transcription or replication. Because they are analysed at 0.5M NaCl, although extracted at various concentrations between no added NaCl and 0.5M, structures which may have been present at these lower NaCl concentrations could have been dissociated on raising the NaCl concentration.

The pH of the extraction solution is important in determining the final quantity of nucleoprotein complex released from MVM infected cells. At low pH's (less than 6.5) there is an extensive degradation of cellular DNA which may reflect the presence of nucleases which are activated by low pH, e.g. lysosomal enzymes. The effect of pH appears to be mainly on the extraction of viral nucleoprotein complex, CII. CII is located in the nucleus and therefore a possible change in the nuclear structure caused by high pH may result in viral nucleoprotein complexes binding more strongly to the cellular chromatin and thus reducing the amount which is extractable. Alternatively, at the lower pH values the nucleoprotein complexes are released from larger, concatemeric forms by nuclease action. The concatemeric forms are therefore probably not extractable but as the pH is reduced, processing of these concatemers to monomeric forms, which can be extracted, occurs. This seems unlikely for two reasons. Firstly, the DNA present in CII is homogenous in size and therefore, unless the hypothetical nuclease is recognising a specific region in the proposed large concatemeric

forms of the viral DNA, it is unlikely that this DNA is a breakdown product of larger viral DNA. Secondly, the DNA present in the viral region of alkaline sucrose gradients has the same size distribution whether it is obtained from total unfractionated, MVM-infected cells or from the nucleoprotein extracted fraction.

The non-ionic detergent, NP40, is required to extract nucleoprotein complex CI and to a lesser extent CII from MVM-infected cells. This may reflect an association of these species with membranes. Virus can be released from red blood corpuscles, during haemagglutination assays, by incubation at pH 9.0 (Hallauer et al, 1972). Consistent with this observation, virus cannot be extracted from MVM infected cell nuclei unless the pH is increased to 8.7 (Tattersall et al, 1976). Therefore, MVM may be attached to a membrane bound receptor in the infected cell and since the pH of the extraction buffer is 7.0 the NP40 may be necessary in order to release CI from the membrane. This could suggest that CI is related to mature virus.

In conclusion, the greatest quantity of viral nucleoprotein complexes can be extracted from MVM-infected cells by a combination of NP40 and NaCl at pH 7.0. This quantity is 65% of the total amount of low molecular weight viral DNA which is present in the infected cell. Since MVM DNA is not integrated into the host chromosome, at any stage during the replicative cycle (Richards and Armentrout, 1979) all the viral DNA should be present in a low molecular weight forms. The extractable material could be entirely representative of the pool of viral nucleoprotein complexes. Alternatively, the 35% which cannot be extracted under these conditions could be a distinct class of viral nucleoprotein complex which is very tightly associated with the cellular chromatin.

Characterisation of the ^{3}H thymidine labelled species, designated CII, extracted by the NaCl-NP40 method, has revealed that it is a complex of DNA and protein. This was demonstrated by the change in sedimentation coefficient after treatment of CII with SDS and pronase, and also the increased susceptibility of the DNA to digestion by DNase I after treatment with SDS and pronase. It is probable that CII exists in the form of a nucleoprotein complex within the infected cell. This was demonstrated by reconstruction experiments in which the DNA, purified from CII, is added, during the extraction procedure, to MVM-infected cells. The DNA does not associate with protein under these conditions, as judged by sedimentation analysis, and therefore it is unlikely that CII arose as an artifact of the extraction protocol. Also, these experiments are performed under relatively high salt conditions (0.5M NaCl) which would prevent non-specific aggregation of protein with DNA.

The change in sedimentation coefficient of CII from 22S to 15S, upon deproteinisation, could reflect between a doubling to tripling in molecular weight due to protein binding. However, if any change in conformation resulted from protein binding events then interpretation of this data becomes more speculative. In the case of SV40, although there is a 1:1 ratio of DNA to protein in the nucleo-protein complex the sedimentation coefficient of the nucleoprotein complex, at moderately low salt concentrations (0.2M - 0.3M), is approximately 70S compared with free SV40 DNA which sediments at 21S (Keller et al, 1977). This is due to the condensation of the DNA by interaction with histones (Griffith, 1975). When the NaCl concentration is raised to 0.5M the S value of the SV40 nucleoprotein complex is reduced to between 40-50S (Keller et al, 1977). The measurement of

the sedimentation coefficient of the MVM nucleoprotein complex, CII, was performed at 0.5M NaCl. However, if CII is extracted at 0.2M NaCl (by the triton X-100 method) and sedimentation analysis performed at 0.2M NaCl then the observed size distribution is similar to that of material extracted and analysed at concentrations of 0.5M NaCl. Thus, the sensitivity of SV40 nucleoprotein complex to salt treatment is not reflected in the behaviour of MVM nucleoprotein complexes. This may suggest a difference in the structure of CII compared to the SV40 nucleoprotein complex. Indeed, the difference in sedimentation coefficient of Adenovirus DNA and Adenovirus nucleoprotein complex, 31S compared to 40S (Kedinger et al, 1978; Shaw et al, 1979), is similar to that between MVM duplex DNA and CII. Adenovirus nucleoprotein complex consists, mainly, of linear molecules, slightly condensed when compared to the length of Adenovirus DNA, with a diameter of 2 nm (Kedinger et al, 1978). MVM nucleoprotein complex may, then, more closely resemble the Adenovirus nucleoprotein complex. Consistent with this proposition is the observation that an inhibition of protein synthesis will not reduce the replication rate of either H-1 monomer RF DNA (Rhode, 1974b) or Adenovirus DNA (Horwitz et al, 1973) but will considerably reduce the rate of SV40 DNA replication (White and Eason, 1973).

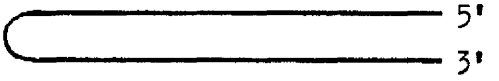
The structure of CII could be determined directly by electron microscopic analysis. This was not, however, possible due to the failure to fix CII. CII did not maintain its conformation, as judged by sedimentation analysis, after fixation with either formaldehyde or glutaraldehyde or a combination of both, if it was treated with SDS. Fixed CII did not sediment at 15S after treatment with SDS but instead sedimented at a position between 15S and 22S. These results indicated

improper fixation. The basis of the fixing procedure of chromatin with formaldehyde and glutaraldehyde has been well documented (e.g. Chalkley and Hunter, 1975). Formaldehyde gives a considerable degree of histone-DNA covalent interactions and a feasible reaction scheme would be the initial formation of the hydroxymethyl derivative of the ε amino group of lysine followed by hemiketal formation with a free carboxyl group on the C2 position of thymine. Glutaraldehyde appears to, primarily, cross link histone molecules in chromatin. This is probably because glutaraldehyde possesses two aldehyde groups and thus after reaction of one of the aldehyde groups with the ε amino group of lysine the other is then available to react with another amino group. Hemiketal formation is unlikely because of steric hindrance and the most probable reaction is thus, with another lysine of a histone molecule since there are no free amino groups in double stranded DNA. The inability of these molecules to properly fix CII suggests a radically different structure of this nucleoprotein complex compared to either SV40 nucleoprotein complex or cellular chromatin structure.

As Griffith (1978), pointed out, samples prepared for electron microscopy by direct mounting techniques are necessarily exposed to harsh chemical and physical treatment during the preparative steps required for their visualisation, and therefore proper fixation is essential if one is to be confident that the structure which is observed by electron microscopy is the one which exists in solution. Therefore little is known about either the structure of the nucleoprotein complex, CII, or the ratio of DNA to protein present in CII. This latter value is normally measured by the position of fixed nucleoprotein complexes in CsCl buoyant density gradients.

A nucleoprotein complex has also been extracted from the nuclei of AAV infected cells by treatment with 0.5M NaCl (Handa and Shimojo, 1977b; Handa and Carter, 1979). The complex extracted from Adenovirus coinfected cells, has a similar sedimentation profile to CII and the DNA would appear from preliminary observations to be monomer RF DNA (Handa and Shimojo, 1977b; Handa and Carter, 1979).

The DNA content of CII has been analysed in detail. The major component is a duplex DNA molecule of 4800 base pairs in length. This DNA is almost completely double stranded as determined by both the resistance to digestion with the single strand specific nuclease S1 and also the buoyant density in NaI gradients. Hybridisation can occur between complementary sequences during centrifugation in NaI gradients although this problem can be overcome by performing the analysis at low temperatures ($0^{\circ}C$) in the presence of ethidium bromide (Strayer, 1979). However, since the results of the NaI buoyant density analysis were confirmed by S1 nuclease resistance it would appear that CII is almost completely double stranded. Under alkaline conditions this species separates into two peaks of 4700 bases and 9300 bases in length. Also, S1 nuclease digestion of heat denatured CII DNA reveals that approximately 30% of this material is resistant to digestion. Taken together, the results of the alkaline agarose gel electrophoresis and the sensitivity to S1 nuclease after heat denaturation indicates that a portion of CII DNA exists in a hairpin like structure, i.e.



This structure would yield DNA which is monomer length

under non-denaturing conditions but is dimer length under alkaline conditions. It will also hybridise with zero order kinetics. CII DNA has also been shown to be mainly, if not exclusively, viral in origin by displacement hybridisation. CII is the major labelled DNA species in the infected cell.

Previous results using an extraction procedure which destroys protein and DNA interactions by using SDS, pronase and selective precipitation of cellular DNA with 1M NaCl (Hirt, 1967) has also revealed the presence of monomer duplex DNA (Tattersall et al, 1973; Rhode, 1974a,b; Mayor et al, 1974; Gunther and May, 1976; Handa et al, 1976; Siegl and Gautschi, 1976; Straus et al, 1976b) and hairpinned monomer duplex DNA (Gunther and May, 1976; Straus et al, 1976b; Rhode, 1977a; Ward and Dadachanji, 1978). This monomer duplex DNA is the predominant labelled DNA species (Salzman and Fabisch, 1978). Therefore, the nucleoprotein complex, CII, contains the DNA species which have been previously implicated in the DNA replication of Parvoviruses.

The sedimentation analysis profile of CII is asymmetric with material moving faster in the leading direction. This leading edge, designated CII pool B, has been demonstrated to consist of separate DNA species from the main band of CII and is not an artifact of the centrifugation conditions or harvesting techniques. A common cause of leading edges observed on peaks during sedimentation is the phenomenon of concentration dependence. This is caused by material on the leading side of the boundary sedimenting at a faster rate because it is at the lowest concentration. This occurs with large molecules at high concentrations, eg bacteriophage T7 DNA. The DNA extracted from CII pool B contains monomer (4800 base pairs in length) dimer and

tetramer DNA molecules under neutral conditions on agarose gels. These molecules are all double stranded and are not present in mock-infected cells. Similar to the main CII fraction these molecules separate into two species, under alkaline conditions. CII pool B DNA, however, contains a larger amount of the 9300 base species relative to the 4700 base species and consistent with this is the observation that a greater percentage of CII pool B DNA is resistant to digestion with S1 nuclease after heat denaturation. DNA structures with similar properties have been observed by Hirt extraction of MVM-infected cells (Ward and Dadachanji, 1978). Therefore, in conclusion, it can be stated that CII is a DNA and protein complex which contains the major replicative intermediate structures observed during Parvovirus DNA replication.

CI is a rapidly sedimenting viral nucleoprotein complex of approximately 100S. When cosedimented with virus particles which were added during the extraction procedure, CI is observed to have a mean sedimentation coefficient slightly less than that of mature virus. Either the particles in CI possess a sedimentation coefficient slightly less than that of MVM virus or there is a distribution of CI particles some of which have the same sedimentation coefficient as mature virus and some of which have a reduced sedimentation coefficient. An intermediate of sedimentation coefficient 95S (mature virus sediments at 110S) has been tentatively proposed to exist in the maturation of LuIII *in vitro* (Gautschi *et al*, 1978). A similar situation is thought to exist in the maturation pathway of SV40 virus. Mature SV40 virus sediments at 240S and an intermediate of sedimentation coefficient 200S has been detected during the assembly of SV40 nucleoprotein complexes into mature virions (Garber *et al*, 1978; Baumgartner *et al*, 1979).

Buoyant density analysis of CI in CsCl gradients demonstrates that CI bands at a position of higher density than mature virus. Also, a portion of CI bands at a density much higher than that of mature virus and perhaps consistent with these findings is that CI is 20% sensitive to digestion with micrococcal nuclease. Kongsvik et al (1979), for H-1, showed that the heavy full virus peak is 20% digestible with micrococcal nuclease if analysed immediately after centrifugation in caesium chloride buoyant density gradients. Also, further separation of both heavy full and light full virions, after isolation on CsCl gradients, on metrizamide buoyant density gradients reveals the presence of a nucleoprotein species which bands at 1.20 g/cm^3 and is sensitive to digestion with micrococcal nuclease (Kongsvik et al, 1979). Both heavy full and light full virions of MVM have a similar sedimentation coefficient (Clinton and Hayashi, 1975).

The DNA content of CI was determined by agarose gel electrophoresis (both neutral and alkaline), NaI buoyant density analysis and sensitivity to digestion with S1 nuclease. Taken together, this demonstrated that DNA extracted from CI is single stranded and of the same molecular weight as MVM viral DNA. Hybridisation analysis established that CI DNA was viral DNA and not complementary DNA.

In conclusion, it would appear that CI is probably related to mature MVM virions and may represent a maturation product. Since these experiments were all performed with newly synthesised CI (within 90 min) it did not prove possible to show the conversion of CI to mature virions.

The relationship between CI and CII was studied by using standard pulse chase techniques. This protocol involves the labelling of moieties using radioactive precursors for short periods of time and

then following the fate of the radioactive label by allowing continued, in this case DNA replication, in the presence of a large excess of unlabelled precursor. By these methods it has been demonstrated that CII is a precursor of CI, although it did not prove possible to indicate unambiguously which fraction of CII was the immediate precursor to CI. The time course of production of CI from CII is very similar to the time course of production of virions (high salt and DNase resistant molecules) from replicative intermediates both in vivo (Siegl and Gautschi, 1976) and in vitro (Gautschi and Reinhard, 1978). This adds weight to the argument that CI is a maturation product en route to mature virions.

It was not possible to demonstrate the existence of a replicative intermediate. It might have been expected that a replicative intermediate between the completely double stranded molecules of CII and the single stranded molecule CI would involve an intermediate which was partially single stranded. Indeed, a similar protein and DNA complex which exists during the production of single stranded ØX174 DNA from the double stranded replicative form template would have been a likely intermediate (Fujisawa and Hayashi, 1976). This type of structure may, however, be present in such small quantities that it would not have been detected by the analytical techniques used or alternatively it may not have been extractable under these conditions. A third alternative is that since these experiments are performed under relatively high salt conditions (0.5M NaCl) in order to extract the nucleoprotein complexes it is possible that the protein and DNA interactions which are present in these intermediates are not stable and therefore dissociate. Indeed, the assembly intermediates during SV40 maturation are very sensitive to the extraction

conditions (Fernandez-Munoz et al, 1979). A thorough investigation of the nucleoprotein species extracted at various NaCl concentrations in both BHK-21/C13 cells and A9 cells, infected with MVM, may prove to be useful in the isolation of replicative intermediates en route to assembly into virions. In conclusion, these results demonstrate that MVM replicates in the form of nucleoprotein complexes and it is possible that these DNA and protein interactions control the production of viral DNA from replicative forms.

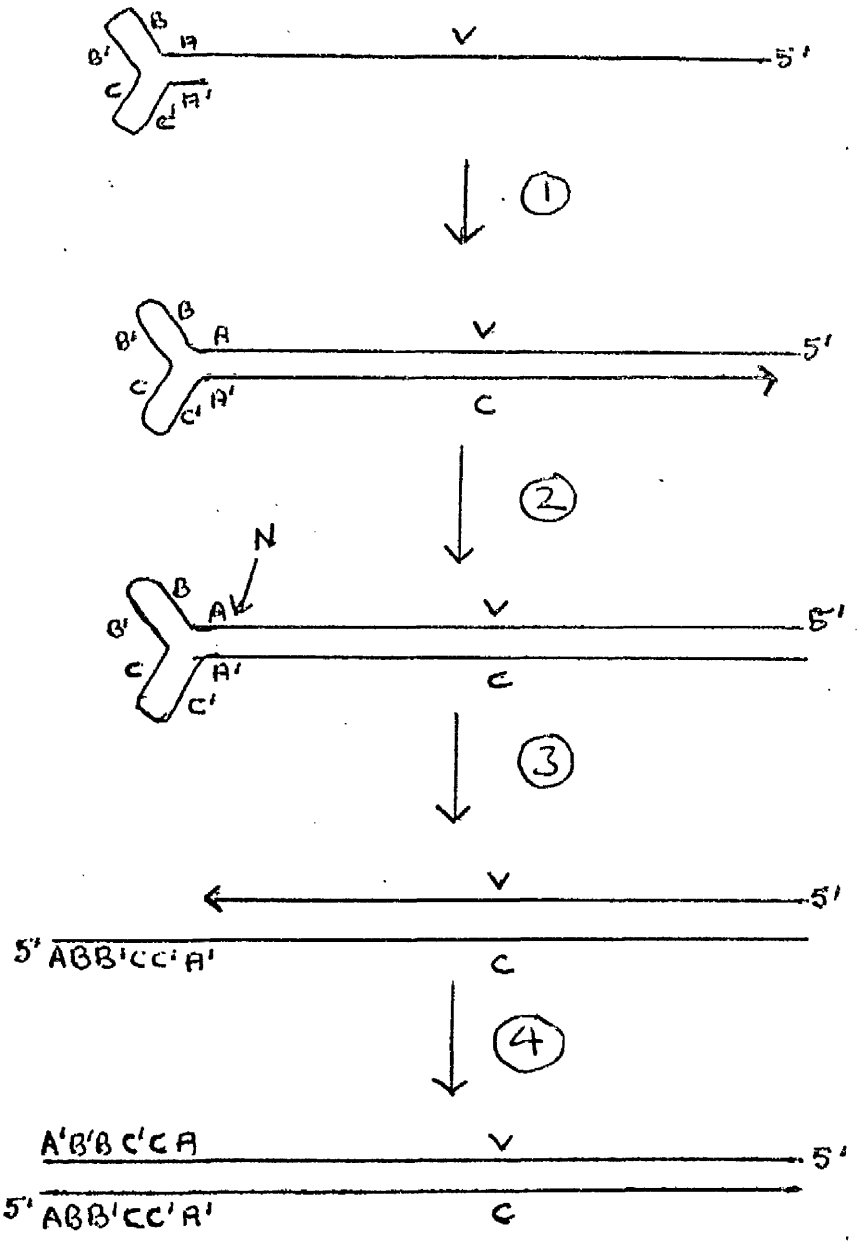
Mechanism of Parvovirus DNA Replication

The infectious unit for both autonomous Parvoviruses (Tattersall, 1972) and AAV (Blacklow et al, 1967) is one particle, therefore the initiation of the replication events of all Parvoviruses commences from a linear single stranded DNA molecule. The initial event in the DNA replication mechanism is the conversion of the input viral single stranded DNA into a duplex form (Salzman and White, 1973; Ward and Dadachanji, 1978). All known DNA polymerases require a 3' OH group to act as a primer for DNA synthesis (Kornberg, 1974; Weissbach, 1977) but if RNA was used to prime the synthesis of the complementary strand then a gap would be left at the 5' end of the complementary strand after removal of the RNA primer. An alternative mechanism for initiation, or completion of the gap left by removal of the RNA primer, arises when one considers the structure of the 3' terminal regions of Parvovirus DNA. This region has been shown to exist in the form of a stable hairpin structure (Bourguignon et al, 1976; Salzman, 1977; Chow and Ward, 1978; Salzman et al, 1978; Fife et al, 1977) and confirmed by sequencing data (Berns et al, 1978; Astell et al, 1979a,b; Salzman and Fabisch, 1979). This mechanism, illustrated in Fig. 48,

Fig. 48

Proposed mechanism for the synthesis of the complementary strand.

Input single stranded viral DNA can form a stable hairpin structure at the 3' end. This structure can act to prime DNA synthesis resulting in a completely duplex DNA molecule (stage 1). Endonuclease cleavage (N) at a position on the opposite strand adjacent to the site of initiation of DNA synthesis (stage 2) followed by strand displacement synthesis (stages 3 and 4) using the 3'-OH formed as a result of the cleavage enables complete synthesis of the complementary strand. A, B and C represent nucleotide sequences and A', B' and C' represent, respectively, their complementary sequences.


B
A
B'
C
C'
A'
V
5'
1
2
N
3
4
5' ABB'CC'A'
A'B'B C'C A
5' ABB'CC'A'

is an adaptation of the model proposed by Cavalier-Smith (1974) for replicating the 5' terminal nucleotides of linear chromosomal DNA of eukaryotes. Basically, the hairpin structure present at the 3' end of the viral DNA acts as a primer for the initiation of the complementary strand. Once synthesis of the complementary strand has been completed the hairpin structure can be regenerated by the site specific endonucleolytic cleavage of the duplex DNA, at a point on the viral strand opposite to the site of initiation of DNA synthesis, followed by strand displacement synthesis. Therefore this model solves the problem presented by RNA priming of DNA synthesis. However, a consequence of this sequence of events is the transfer of the hairpin structure at the 3' end of the viral DNA strand to the 5' end of the complementary strand, which results in the formation of virion DNA with terminal sequences of two different orientations, i.e. A'C'CB'BA and A'B'BC'CA. The observations on the terminal sequences of AAV virion DNA are entirely consistent with this model (Spear et al, 1977; Berns et al, 1978). However, the sequence data from the autonomous group of Parvoviruses indicates the presence of only one sequence orientation (Astell et al, 1979a,b; Salzman and Fabisch, 1979). This point will be discussed later. Little evidence is available to suggest if this mechanism of DNA initiation operates in the conversion of single stranded DNA to a duplex form. Ward and Dadachanji (1978) demonstrated that virions, specifically labelled in the DNA component, gave rise upon infection to a double stranded DNA molecule which re-annealed with zero order kinetics. This is consistent with the formation of a hairpinned duplex DNA molecule. However, this structure was not analysed in detail. The significance of this type of experiment must be considered doubtful because of the high particle:

infectivity ratios of Parvoviruses. Thus it is possible that the majority of the input labelled DNA, followed during this type of experiment, does not result in a productive infection. The 3' end of isolated viral DNA acts very efficiently in vitro as a primer for the initiation of DNA synthesis by many DNA polymerases (Bourguignon et al, 1976) and therefore the labelled DNA, followed during infection, could be undergoing nonspecific elongation by cellular DNA polymerases. The experiments with DNA polymerases, in vitro, do demonstrate that the 3' end can act as a primer for DNA synthesis but as yet there is no conclusive evidence that this mechanism operates in vivo.

The second stage in the DNA replication of Parvoviruses is the replication of this duplex DNA molecule, monomer RF. This would appear to be an analogous situation to ØX174 RF DNA replication and several lines of evidence support this hypothesis. Firstly, monomer RF has been isolated from Parvovirus infected cells by methods which do not allow re-annealing of complementary single stranded DNA (Tattersall et al, 1973; Rhode, 1974a,b; Gunther and May, 1976; Siegl and Gautschi, 1976; Straus et al, 1976b). Secondly, monomer RF DNA is not only a major labelled DNA species (Salzman and Fabisch, 1978) but is also a major steady state molecule, and thirdly, mutants of H-1 have been isolated which are deficient in the production of monomer RF DNA and hence produce reduced amounts of progeny DNA (Rhode, 1978a). This stage in the replication cycle can be regarded as a model system for the replication of linear chromosomes.

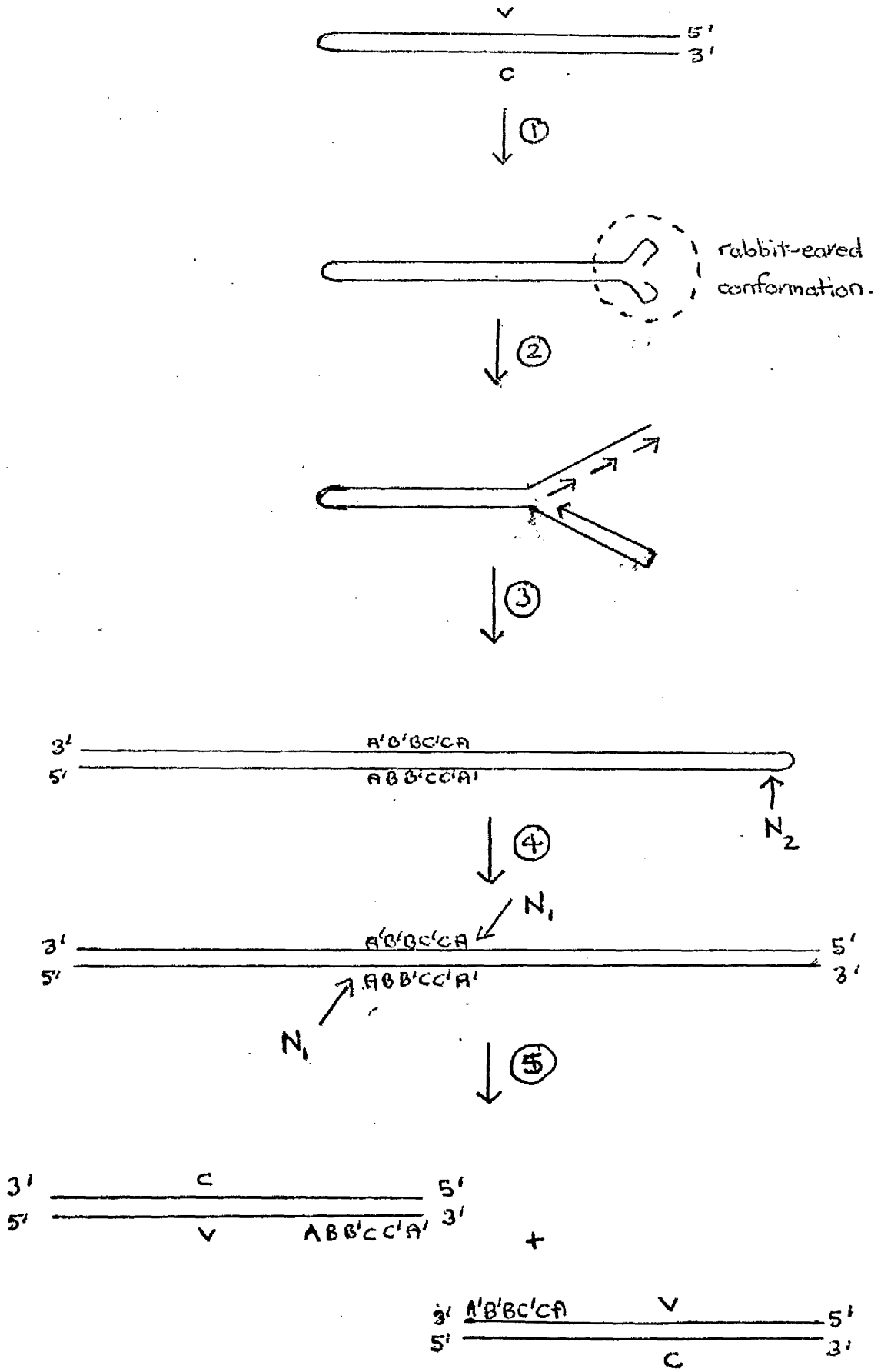
The DNA replication of monomer RF DNA has been proposed to involve hairpin priming of DNA replication and the production of concatemeric DNA molecules (Tattersall and Ward, 1976). In this model

(Fig. 49) DNA replication is commenced from the unit length hair-pinned structure, illustrated in Fig.48, which has been formed as a result of the extension of the 3' end of the viral strand. The right end of monomer RF DNA molecules contains the origin of DNA replication (Singer and Rhode, 1977b; Rhode, 1977a) and it is suggested that a "rabbit-eared" conformation could exist, because the 5' end of the viral strand has been shown to be in the form of a stable hairpin structure (Bourguignon et al, 1976). The 3' end of the complementary strand can now act as a primer for displacement DNA synthesis. In stage 2 (Fig. 49), as DNA synthesis commences, at the 3' end of the complementary strand, the displaced viral strand could be replicated by discontinuous, RNA primed DNA synthesis. This would result in the completely double stranded Y-shaped DNA molecules observed by Singer and Rhode (1977b). When replication has been completed a dimer length DNA molecule is formed (stage 3). The right end of this molecule exists in a hairpin structure but can be formed into a normal extended duplex by site specific endonuclease cleavage (N_2) and strand displacement synthesis (stage 4). The dimer molecule can then be cleaved to yield monomer RF DNA molecules by site specific endonuclease cleavage (N_1) followed by DNA synthesis to fill the gap. Clearly, endonuclease N_1 cleavage could occur before cleavage N_2. Molecules larger than dimer length could also be created by hairpin-primed replication of the dimer molecule before endonuclease cleavage. Processing of these larger molecules would also yield monomer RF DNA molecules. This model describes a mechanism for the completion of the 5' ends of newly synthesised DNA molecules. However, if one considers the sequence arrangement at the 3' end of

Fig. 49

Proposed mechanism for the replication of intracellular Parvoviral duplex DNA.

Hairpinned duplex DNA, formed as described in the legend to Fig. 48, can adopt a "rabbit-eared" conformation at the right end of the DNA molecule (stage 1). Displacement DNA synthesis can now begin using the 3'OH group of the complementary strand as the primer. Discontinuous, RNA primed, DNA synthesis can commence on the displaced viral strand (stage 2). Once replication is completed a dimeric duplex DNA molecule is formed which is hairpinned at the right end (stage 3). Site specific endonuclease cleavage (N_2) and strand displacement synthesis, identical to that described in the legend to Fig. 48, results in the formation of a normal extended duplex (stage 4). Another site specific endonuclease cleavage (N_I) followed by DNA synthesis at the exposed, internal, 3'OH groups results in the formation of two progeny duplex DNA molecules (stage 5). Letters representing nucleotide sequences are as described in the legend to Fig. 48.


V
5'
3'
C
①
rabbit-eared
conformation.
②
③
3'
A'B'BC'CA
5'
ABB'CC'A'
N2
④
N1
3'
A'B'BC'CA
5'
5'
ABB'CC'A'
3'
N1
⑤
3'
C
5'
5'
V
ABB'CC'A'
3'
+
3'
A'B'BC'CA
V
5'
5'
C
3'

the viral strand then one can see that the same situation which occurs in Fig.48, that is the formation of two sequence orientations, operates here. A further prediction of this model is the formation of concatemeric DNA molecules which act as a precursor to monomer RF DNA. Concatemeric DNA molecules, of similar structures as predicted by this model do exist at a low frequency, relative to monomer RF DNA molecules, in Parvovirus infected cells (Gunther and May, 1976; Siegl and Gautschi, 1976; Straus et al, 1976b; Rhode, 1977a; Ward and Dadachanji, 1978; Hauswirth and Berns, 1979) but as yet no convincing relationship between the concatemeric DNA and monomeric DNA has been reported for the autonomous Parvovirus group, although concatemeric DNA would appear to be a precursor to monomeric DNA in the AAV group (Straus et al, 1976b). Indeed Rhode (1977a) has suggested that the replication rates of dimeric and monomeric RF DNA may be different. The mere existence, however, of concatemeric DNA molecules does not necessarily indicate that they are involved in the replicative process of the virus. Concatemeric molecules of SV40 DNA have been shown to exist, up to a maximum of 20% of the amount of SV40 DNA monomers, at late times during the infectious cycle (Goff and Berg, 1977). These closed circular and supercoiled concatemeric molecules, up to four times the genome size, are arranged as head to tail oligomers of monomer molecules (Martin et al, 1976; Goff and Berg, 1977). They are equally effective in producing plaques/DNA molecule as monomer circles (Israel et al, 1978) and can give rise to monomers upon infection (Goff and Berg, 1977). Although most of the dimers arose by events occurring during replication, e.g. homologous crossover between sister strands of replicative intermediates or a failure to segregate the two daughter molecules

properly at the termination of replication, a small proportion (approximately 7%) of dimers arose by a reciprocal recombinational event (Goff and Berg, 1977). The function of these concatemers is unknown although the association of concatemers with SV40 transcriptional complexes suggests that they can function as templates for mRNA synthesis (Shani et al, 1977). Therefore since replication of SV40 DNA does not necessarily require concatemers as replicative intermediates, the presence of concatemers during Parvovirus DNA replication, by analogy, may also be an unnecessary by-product of replication or recombination.

The failure to demonstrate a role for concatemers during replication of the autonomous Parvoviruses, though the converse is also true, means that another mechanism has to be found to completely replicate the 5' end of the complementary strand. As illustrated in Fig. 50 DNA synthesis is proposed to initiate at the right end of monomer RF DNA molecules via a hairpin priming mechanism. The existence of a "rabbit-eared" conformation at this end of the molecule (Fig. 49) facilitates this mechanism. As indicated previously this synthesis will result in the displacement of the viral strand which can be replicated, simultaneously, by RNA primed discontinuous DNA synthesis giving rise to Y-shaped DNA molecules as intermediates. Completion of this process will result in the formation of a hairpinned duplex molecule and a normal duplex molecule with a gap at the 5' end of the newly synthesised complementary strand. The hairpinned duplex can be formed into a normal extended duplex by site specific endonuclease cleavage (N_2), opposite the site of initiation, followed by strand displacement synthesis. The gap on the normal extended duplex can be completed by taking advantage of the formation of a stable

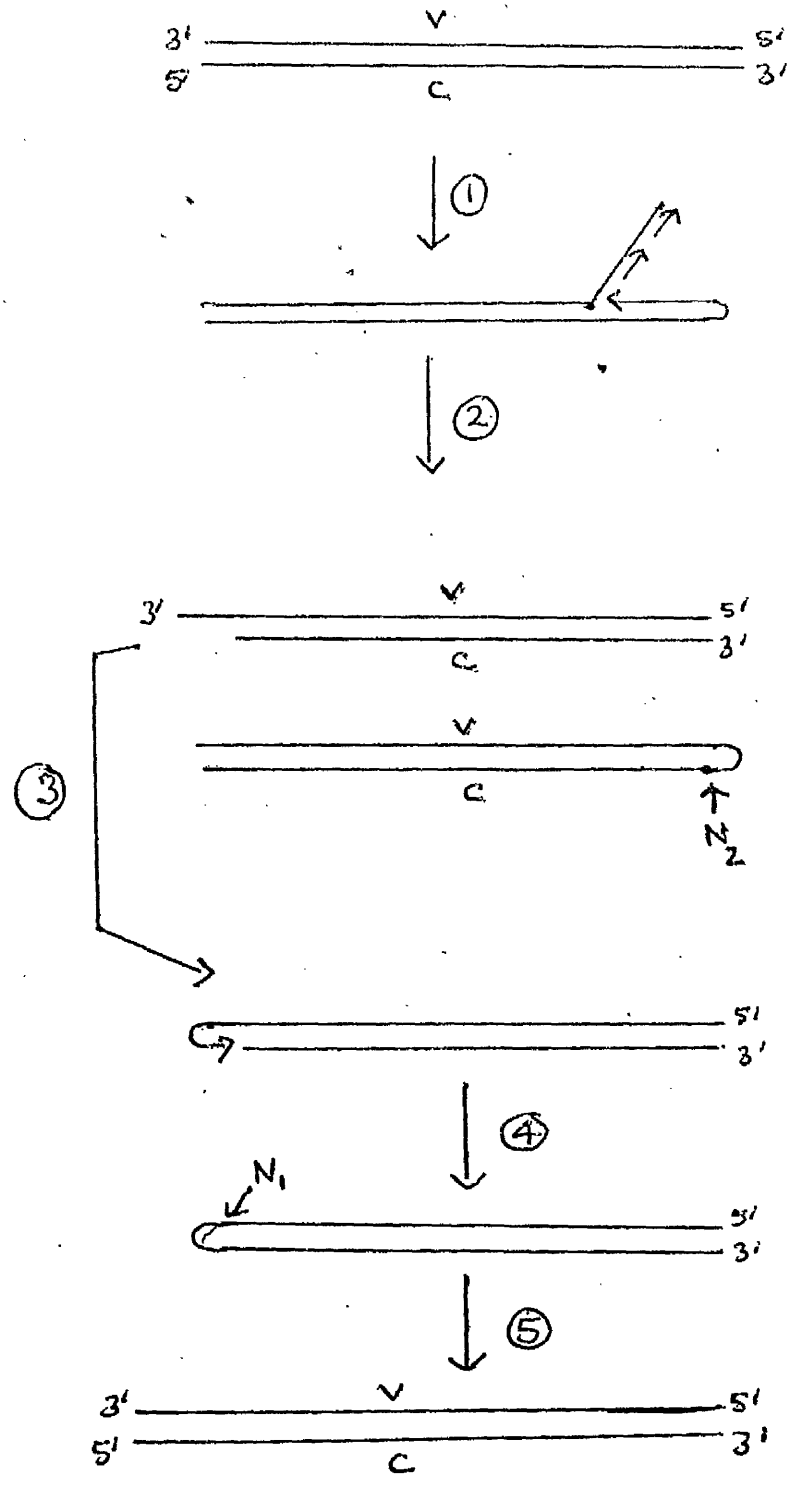
Fig. 50

Proposed mechanism for the replication of intracellular Parvoviral duplex DNA.

Normal extended duplex DNA is primed for DNA synthesis by the hairpin structure at the 3'end of the complementary strand followed by displacement synthesis and RNA primed discontinuous DNA synthesis on the displaced viral strand, as outlined in the legend to Fig. 49 (stage 1). Completion of replication results in the formation of a hairpinned duplex and a normal extended duplex with a gap at the 5' end of the newly synthesised strand (stage 2). Site specific endonuclease cleavage (N_2) and strand displacement synthesis will result in the conversion of the hairpinned duplex into a normal extended duplex. The gap at the 5' end of the newly synthesised strand can be filled by the formation of a haipin structure at the 3' end of the viral strand followed by gap fill DNA synthesis and ligation (stages 3 and 4). Site specific endonuclease cleavage (N_L) followed by strand displacement synthesis will result in the formation of a normal extended duplex (stage 5).

LEFT END

RIGHT END



hairpin structure at the 3' end of the viral strand. This structure can be extended to complete the gap. Ligation results in the formation of a hairpinned duplex which can be converted to a normal extended duplex by site specific endonuclease cleavage (N_1) and strand displacement synthesis. Since the 5' and 3' ends of autonomous Parvovirus DNA have different sequences the two site specific endonucleases would recognise different sequences. However, as yet no site specific endonucleases have been discovered in mammalian cells. The specificity of the proposed nucleases may not reside in direct sequence recognition, as occurs for bacterial endonucleases involved in replication, e.g. ØX174 gene A product, but may instead reside in the recognition of a specific structure or alternatively a region which has been preferentially exposed due to protein-DNA interactions. The finding of hairpin structures located at both the right and especially the left end of monomer RF DNA molecules is entirely consistent with this model (Rhode, 1977a; Ward and Dadachanji, 1978). However, a prediction of this model of hairpin priming is that two sequence orientations will occur in the 3' and 5' ends of the viral strand (and clearly in the complementary strand as well). This change in sequence orientation would also occur if monomer RF DNA was formed from concatemers. The 3' ends of the viral strands of four autonomous Parvoviruses have been sequenced and no heterogeneity of sequence orientation has been observed (Astell et al, 1979a,b; Salzman and Fabisch, 1979). This could be explained by assuming that the production of viral strands is restricted to one sequence orientation of the 3' end and that the two sequence orientations do exist in monomer RF DNA. This proposition is unlikely because viral DNA is likely to be packaged from the 5' end of the viral strand and thus it is this end which must

be recognised, otherwise complementary strand synthesis may begin on this displaced strand. Also, Astell et al (1979b) cite evidence from restriction endonuclease mapping studies that the 3' end of the viral strand in monomer RF DNA contains only one sequence orientation. They also cite evidence that the 5' end of the viral strand in monomer RF DNA does contain sequence orientation heterogeneities. Therefore, it is proposed that hairpin priming only occurs at the right end of monomer RF DNA. Clearly, in the case of AAV DNA replication since both ends of monomer RF DNA are equivalent initiation will occur with equal probability at either end of the molecule and thus two origins of DNA replication and two sequence orientations in the 3' end of viral DNA will be found. Both of these situations have been shown to occur (Hauswirth and Berns, 1977; Berns et al, 1978). A consequence of the proposition that hairpin priming only occurs at the right end of monomer RF DNA in autonomous Parvoviruses is that an asymmetry in the replication process can be introduced. This asymmetry of replication of the ends of the DNA is necessary in order to explain the highly asymmetric product of Parvovirus replication, i.e. single stranded viral DNA. However, another consequence of not allowing hairpin priming at the left end of the molecule is that a gap will be left at the 5' end of the discontinuously synthesised complementary strand. Recently, Revie et al (1979) described protein linked strongly, and probably covalently, to the 5' ends of both the viral and complementary strands in monomer RF DNA. Protein was not found linked to DNA extracted from virions. The protein could function to initiate DNA synthesis at the 5' end of the complementary strand to fill the gap left by removal of the RNA primer, in a similar fashion to that described for Adenovirus DNA replication (see section

1.4.2; Rekosh et al, 1977). There is no evidence to suggest that this model operates but clearly the formation of concatemers as precursors to monomer RF DNA molecules and hairpin priming at the left end of monomer RF DNA are not consistent with all of the experimental evidence.

The production of single-stranded DNA has been proposed to occur from both concatemeric and monomeric RF DNA molecules. Tattersall and Ward (1976) suggested that the concatemeric DNA molecules functioned as the precursor to progeny single stranded DNA (Fig. 51). The first step is a site specific endonuclease cleavage (N) to yield an internal initiation point for progeny strand displacement synthesis using the 3' OH of the "nick" as a primer for synthesis of the new viral strand. As the DNA is displaced it is immediately packaged into virion-like structures by recognition of the 5' end of the genome by the assembled capsid structure. Once the entire length of the genome has been packaged the 3' OH end is cleaved to yield mature single stranded DNA and simultaneously the dimeric duplex structure is regenerated. The substrate for this reaction could be higher oligomeric forms of the DNA. A second proposal is that monomer RF DNA is the immediate precursor to progeny single stranded DNA (Fig. 52). In this model the 3' end of the complementary strand functions as a hairpin primer for displacement synthesis of the viral strand. As displacement synthesis continues the displaced viral strand is recognised by a pre-virion structure thus preventing RNA primed discontinuous synthesis of the complementary strand. Once synthesis is completed a hairpin duplex is formed and also a virion structure containing the progeny viral DNA. The hairpin duplex is converted to a normal extended duplex by site specific endonuclease cleavage and strand displacement

Fig. 51

Proposed mechanism for the production of progeny single stranded DNA from concatemeric DNA molecules.

Dimeric duplex DNA is cleaved with a site specific endonuclease (N) at a site immediately before the 5' end of the viral strand to produce a 3'OH group capable of priming DNA synthesis (stage 1). DNA synthesis results in the displacement of the 5' end of the viral strand which is recognised by a pre-formed capsid-like structure (stage 2). Displacement synthesis continues until the 5' end of the viral strand is recognised by an endonuclease and cleavage produces mature virions or a product en route to mature virions and regenerates dimeric duplex DNA (stage 3). Virion DNA is represented by the heavy lines. Progeny single stranded DNA can also be produced from larger concatemeric molecules than the dimer represented here. Adapted from Tattersall and Ward (1976).

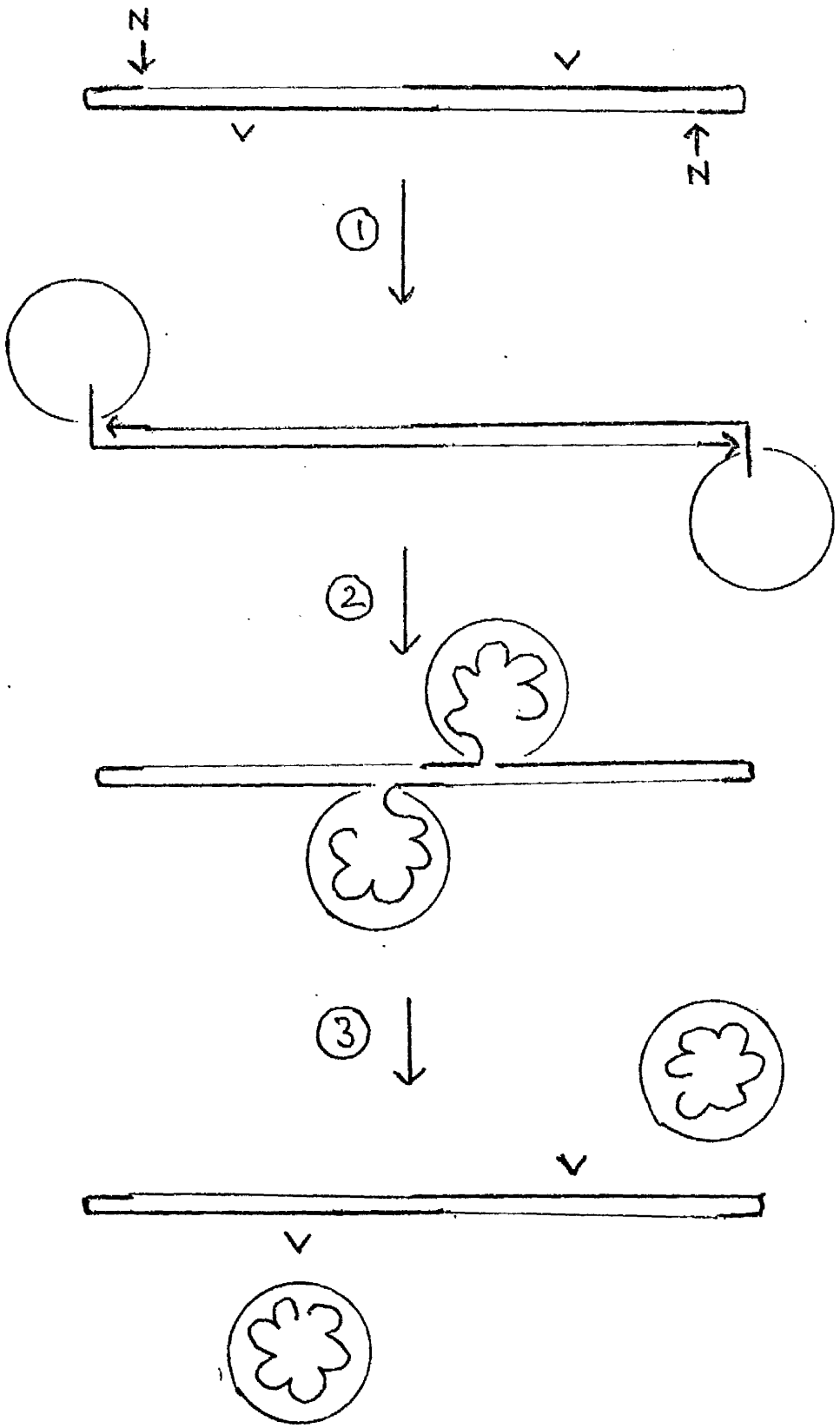
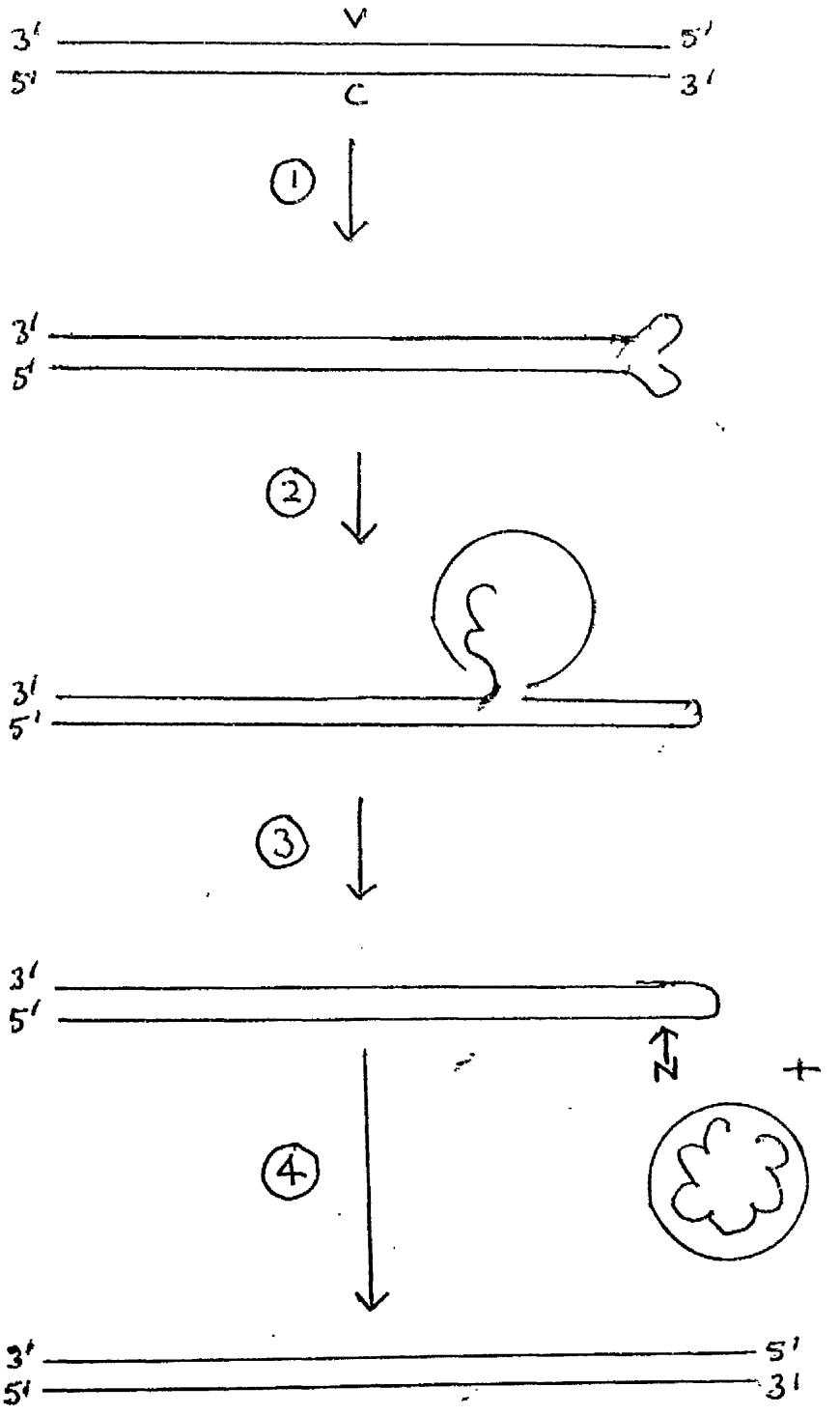

N
V
V
N
①
②
③
V
V

Fig. 52

Proposed mechanism for the production of progeny single stranded DNA from monomer duplex DNA.

Normal extended duplex DNA can form a "rabbit-eared" conformation at the right end (stage 1). This enables hairpin-priming to occur at the 3' end of the complementary strand resulting in DNA synthesis causing displacement of the viral strand which is then recognised by a pre-formed capsid-like structure (stage 2). Continued DNA synthesis results in the production of mature virions or a product en route to mature virions and hairpinned duplex DNA (stage 3). The hairpinned duplex is converted to a normal extended duplex by site specific endonuclease cleavage (N) and strand displacement synthesis (stage 4).


V
3'
5'
5'
3'
C
1
3'
5'
2
3'
5'
3
3'
5'
N
+
4
3'
5'
5'
3'

synthesis. Both these models predict that the synthesis of progeny DNA requires DNA synthesis and also site specific endonucleases. Progeny virus production does require DNA synthesis (Richards et al, 1977) but as previously mentioned no site specific endonucleases capable of performing this function have been isolated. The evidence for displacement synthesis on monomer RF DNA molecules includes asymmetric labelling, with BUdR, of the viral strand of monomer RF DNA during a period of progeny DNA synthesis (Rhode, 1974a,b) and preliminary electron microscopic data which demonstrated the existence of single stranded DNA branches on duplex monomer RF DNA (Rhode, 1978c; Fig. 47). Pulse chase experiments have not conclusively revealed whether the template for progeny DNA synthesis is monomer RF DNA or concatemeric DNA (Ward and Dadachanji, 1978; Fig. 39). The basic reason for this discrepancy may be the inability to distinguish between monomer RF DNA replication and progeny single strand DNA synthesis. The development of mutants, temperature sensitive if possible, would enable one to clearly separate these events and, hopefully, yield less ambiguous results. Since viral proteins are required for both RF DNA replication and progeny DNA synthesis it should be theoretically possible to achieve this aim. Thus, one must await further experimentation involving the use of mutants and also possibly the use of extraction conditions which will permit one to follow the synthesis and packaging of viral DNA.

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