

ELECTRON MICROSCOPY OF BOVINE PARVOVIRUS

DNA REPLICATION INTERMEDIATES

by

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## 1. Introduction

The family Parvoviridae consists of small nonenveloped, icosahedral viruses 20-25 nm. in diameter. The capsid contains a linear, primarily single-stranded, DNA genome with a molecular weight of 1.2 to  $2.2 \times 10^6$  daltons. These viruses have been shown to replicate within the nuclei of infected cells and are reported to cause fetal and neonatal diseases in various animal species (Tattersall and Ward, 1978).

Three genera constitute the Parvoviridae family: 1) Parvovirus, 2) Adeno-associated viruses, and 3) Densoviruses. These divisions are based primarily upon the requirement of a helper virus for their replication. The genus Parvovirus, as opposed to the Adeno-associated viruses, are characterized by their ability to replicate without the presence of a helper virus. These viruses are also referred to as the nondefective or autonomous parvoviruses. Members of the Parvovirus genus include bovine parvovirus (BPV), Kilham rat virus (KRV), H-1, Lu III, and minute virus of mice (MVM) (Siegl, 1976). Another characteristic of the autonomous parvoviruses is the packaging of DNA strands of only one polarity in the virions (the minus strand). This is in contrast to the Adeno-associated and denso viruses which separately encapsidate both plus and minus strands (Tattersall and Ward, 1978).

Although the autonomous parvoviruses are able to complete their replication cycle without helper virus functions, they are dependent on

a cellular function(s) or event(s) associated with late S or early G2 phase of the cell cycle (Siegl, 1976). Various investigators, working with parvovirus-infected cells have isolated several low molecular weight DNA species that could not be demonstrated in mock infected cells. These DNA species have been termed replicative forms and intermediates. Parvoviruses for which replicative structures have been isolated include Lu III (Siegl and Gautschi, 1976), Kilham rat virus (Lavelle and Li, 1977), H-1 (Rhode, 1974), and minute virus of mice (Tattersall et al., 1973; Dobson and Helleiner, 1974), however replicative structure studies have not been reported for bovine parvovirus (BPV).

Characterization of the replicative form and intermediate DNA from parvovirus-infected cells was accomplished by several techniques. The visualization and determination of contour length of the DNA was performed by electron microscopy. Molecular weight was also determined by this method. Centrifugation studies were used to determine the sedimentation characteristics of the DNA including sedimentation coefficient and bouyant density. The presence of secondary structure in the replicative structures was demonstrated by BND-cellulose or hydroxyapatite chromatography.

Because of the lack of published information regarding the replicative forms of BPV, as mentioned earlier, the overall objective of the research reported in this thesis was the isolation and characterization of the replicative forms of BPV.

Specific goals included the following:

1. To determine the suitability of the guanidine-hydrochloride method and the Hirt procedure for extracting low molecular weight DNA replicative structures from BPV-infected cells.
2. To determine the contour length and molecular weight of the DNA replicative structures by electron microscopy.
3. To determine the secondary structure of the DNA by hydroxyapatite chromatography.
4. To determine the sedimentation coefficient of the DNA replicative structures in neutral and alkaline sucrose.
5. To determine the bouyant density of the DNA replicative structures by centrifugation in neutral CsCl.

## 2. Literature Review

### 2.1 Prologue

Members of the Parvoviridae family are the smallest known DNA-containing animal viruses. The virions are 18-25 nm. in diameter and contain a single-stranded DNA genome. Over 25 different viruses have been identified since the first isolation in 1952, and many have been characterized extensively. In the interest of brevity and in consideration of the mortality of man, this thesis will be concerned primarily with the replication of the autonomous parvoviruses. For further information, the reader is referred to The Replication of the Mammalian Parvoviruses edited by Tattersall & Ward (1978).

### 2.2 The Autonomous Parvoviruses

Over 20 different autonomous parvoviruses have been isolated. This group includes Kilham rat virus (KRV), minute virus of mice (MVM), H-1, Lu III, and bovine parvovirus (BPV). Although these viruses have been isolated from different sources (see Table 1) they have two characteristics that justify their classification as nondefective parvoviruses. The first of these characteristics is their ability to replicate without requiring the presence of a helper virus, as is the case with adenovirus coinfection with the adeno-associated virus genus. Also, the autonomous parvoviruses encapsidate predominantly the minus strand whereas adeno-associated viruses package both plus and minus strands separately (Tattersall and Ward, 1978).

Table 1. The Autonomous Parvoviruses

| Virus                      | Isolation Source <sup>a</sup>    | Date <sup>a</sup> | Virion <sup>b</sup> Size (nm) | Molecular <sup>b</sup> Weight (d) |
|----------------------------|----------------------------------|-------------------|-------------------------------|-----------------------------------|
| Bovine parvovirus (BPV)    | Calf feces                       | 1961              | 23-28                         | --                                |
| H-1                        | Human Hep-1 transplantable tumor | 1960              | 20-30                         | $6.6 \times 10^6$                 |
| Kilham rat virus (KRV)     | Rat liver sarcomas               | 1959              | 18-28                         | $6.6 \times 10^6$                 |
| Lu III                     | Human Lu 106 cell line           | 1971              | 19-22                         | $5.7 \times 10^6$                 |
| Minute virus of mice (MVM) | Mouse adenovirus stock           | 1966              | 19-28                         | --                                |

<sup>a</sup>Tattersall and Ward, 1978

<sup>b</sup>Siegl, 1976

### 2.3 In Vitro Characteristics

Parvoviruses are able to replicate within the nuclei of various cell types including cell lines and primary cell cultures. These viruses, however, replicate most efficiently in actively dividing cultures. Further replication studies showed that the virus requires some cellular function(s) that is expressed in late S phase of the cell cycle. Infection of non-mitotic cells does not result in production of progeny virus particles. The parvoviruses are also unable to stimulate cells to enter S phase (Tattersall and Ward, 1978). These observations are not surprising since in vivo studies had demonstrated that these viruses were responsible for a number of diseases in fetuses and neonates which are known to have large populations of mitotic cells. Adult animals, are, for the most part, not susceptible to these diseases (Tattersall and Ward, 1978).

### 2.4 Physico-chemical Characteristics of Single-Stranded DNA of the Autonomous Parvoviruses

The DNA isolated from the autonomous parvoviruses has been shown by various investigators to be predominantly linear and single-stranded. Siegl (1973) reported that approximately 70% of Lu III DNA was sensitive to the exonuclease from the mushroom Verongia aerophoba, a single strand specific enzyme. BND-chromatography demonstrated similar results with 86% of the total radiolabeled virion DNA being recovered in the fractions eluted with 1 M NaCl plus 2% caffeine. This suggested that the majority of the DNA was single-stranded. No DNA was eluted with 1 M NaCl which elutes double-stranded DNA. Using the Lang microdiffusion

technique for electron microscopy (EM) in which formaldehyde is added to prevent single strand collapse, molecules were seen that had characteristics of single-stranded DNA. Length measurements estimated average contour length to be  $1.61 \pm 0.125 \mu\text{m}$ . Bouyant density of virion DNA in CsCl was 1.725 g/cc and the molecular weight was calculated to be  $1.59 \times 10^6$  daltons (Siegl, 1973).

Bourguignon et. al. (1976) obtained similar results with MVM. The molecular weight of the linear virion DNA determined by EM was  $1.48 \times 10^6$  daltons. Hydroxyapatite column chromatography showed an elution pattern characteristic of single stranded DNA with some secondary structure. Upon digestion with S1 endonuclease and exonuclease I, 6% of the MVM genome appeared resistant to these single strand specific enzymes. Since exonuclease I is specific for the 3' end of single-stranded DNA molecules and because it digests MVM DNA to the same extent as S1, it was suggested that the 5' end of the virion DNA contained duplex region with characteristics of palindromes. Polyacrylamide gel analysis of the S1 resistant fraction suggested a size of approximately  $130 \pm 20$  base pairs. Further, S1 digestion studies with  $^{32}\text{P}$  labeled 5' phosphate confirmed the presence of the hairpin structure at the 5' end. Because MVM DNA both serves as a template-primer for a number of DNA polymerases and is digested with exonuclease I enzyme, it was suggested that the 3' terminus forms an unstable hairpin structure.

Characterization studies of KRV DNA have also demonstrated predominantly linear, single stranded DNA molecules. (Salzman, et. al., 1970; Salzman, et. al., 1971). The molecular weight of KRV is  $1.6 \times$

$10^6$  daltons. Contour length measured by EM was  $1.505 \pm 0.206 \mu\text{m}$ . Digestion of KRV DNA with exonuclease I demonstrated that 70-80% of the DNA was sensitive to this single strand specific enzyme and thus demonstrates its linearity. Salzman (1977) reported that S1 endonuclease resistant fragments containing 5' terminal  $^{32}\text{P}$  labeled phosphate could be generated in KRV DNA by incubation of the labeled DNA with the enzyme. These fragments could be eluted from BND cellulose columns under the conditions for elution of double-stranded DNA, thus establishing a hairpin or duplex structure at the 5' terminus.

Furthermore, treatment of double-stranded  $^{32}\text{P}$  labeled KRV DNA from infected cells with exonuclease III reduced the fraction of DNA resistant to S1 digestion, after denaturation, by half indicating the possibility of a hairpin or duplex structure at the 3' terminus. Lavelle & Mitra (1978) demonstrated that KRV DNA was resistant to exonuclease I, which is specific for 3' end of single-stranded DNA thus confirming the presence of the 3' hairpin. These investigators further demonstrated that prior treatment of the DNA with exonuclease III rendered the DNA susceptible to exonuclease I. Polyacrylamide gel electrophoresis resolved two fragments; one of 135 nucleotides and one of 110 nucleotides. Previous studies (Salzman, 1977) reported an exonuclease I sensitivity that was used as evidence for the linearity of the molecules. The contradictory observations by Lavelle & Mitra of exonuclease I resistance is possibly due to the contamination of exonuclease I with double-stranded specific enzyme in the previous studies by Salzman (Bern & Hauswirth, 1978).

BPV DNA has been shown to be linear single-stranded molecules with a molecular weight of  $1.7 \times 10^6$  daltons and bouyant density of  $1.721 \text{ g/cm}^3$  (Saemundsen, 1978). It has also been shown to contain duplex regions that comprise about 7% of the genome which suggests the presence of hairpin structures. BPV appears to encapsidate plus strands into 15-20% of the virions. This phenomenon appears to be dependent upon cell type in which the virus is propagated. This is contrary to findings for other autonomous parvovirus which encapsidate predominantly the minus strand.

In summary, the physico-chemical properties of autonomous parvoviruses are as follows:

1. The DNA genome consists of single-stranded molecules containing hairpins or palindromes at both the 5' and 3' termini.
2. Molecular weight of the genome ranges from  $1.4 \times 10^6$  to  $1.7 \times 10^6$  daltons.
3. Contour length of the single-stranded DNA is approximately 1.6  $\mu\text{m}$ .
4. Most autonomous parvovirus encapsidate predominantly the minus strand except for BPV which also encapsidates the plus strand in 15-20% of the virions depending upon cell type.

#### 2.5 Physico-chemical Characteristics of Viral DNA Isolated From Infected Cells

Since replication of the autonomous parvoviruses occurs without the aid of helper virus but is dependent upon some cellular function of late S phase of the cell cycle, various investigators have looked to the infected cell for answers to the question of parvovirus replication.

### 2.5.1 Parvovirus Lu III

Siegl and Gautschi (1976) isolated radiolabeled low molecular weight DNA by the Hirt procedure (1967) from HeLa cells infected early in S phase with Lu III. Isopycnic sedimentation of the Hirt supernatant DNA showed that RF DNA could be detected as early as 9 hours post infection. Characterization of this RF DNA from the CsCl gradient was accomplished by S1 nuclease digestion, which showed a peak of primarily S1 resistant molecules that corresponded to the peak of radioactively labeled DNA from the gradient. These results suggested that this DNA was double-stranded. Electron microscopic studies demonstrated that the molecules were linear. Two different type of molecules were seen; completely double-stranded molecules which were designated replicative forms (RF) and double-stranded molecules that had single-stranded side chains which were called replicative intermediates (RI). Rate zonal sedimentation analysis in sucrose gradients revealed that the RF had a sedimentation coefficient of 14.7S. Sedimenting just in front of the RF DNA were the RI's with a S value of 18 to 22S. Another type of molecule was also seen by EM that was approximately 3 times as long as the RF DNA and has an S value of 24. Bouyant density in CsCl of the 14.7S DNA was 1.714 g/ml. The molecular weight for this DNA as measured by sedimentation and EM was reported to be  $2.8 \times 10^6$  to  $3.1 \times 10^6$ d, which is approximately twice the molecular weight for single-stranded Lu III virion DNA. No length measurements were reported.

Further studies by the same investigators (Siegl and Gautschi, 1978), using HeLa or NB cells, demonstrated as before that virus specific

DNA could be isolated as early as 9-10 hours post infection in both cell types. Sedimentation analysis of the low molecular weight DNA in neutral sucrose gradients showed that at 9-10 hour post infection, there appeared to be only one predominant peak of DNA for both the NB and HeLa cells. This DNA sedimented at 14.7S, as before. As the infection progressed the sedimentation profile became more complex. Several other types of molecules appeared in both alkaline and neutral CsCl gradients that had different sedimentation characteristics. This phenomenon seemed to be more pronounced in HeLa cells. In alkaline gradient analysis of low molecular DNA from HeLa cells, molecules with a S value of 16S, 20.4S (dimers) and 26.8S (tetramers) were observed. The relative number of each increased with time. NB cells yielded low molecular weight DNA molecules only of monomer and dimer size during the same time period. RF molecules eluted from a BND cellulose column with 1 M NaCl were further characterized by EM and shown to be predominantly linear molecules. RF's from NB cells had a length 1.2 to 1.7  $\mu$ m whereas RF's from HeLa cells were more heterogenous in length. Two thirds of these double-stranded RF molecules had covalently linked strands which formed a hairpin structure. About 20-30% of the RI's eluted from BND-cellulose with caffiene, were shown to be y-shaped by electron microscopy. These molecules were primarily monomer length and the complimentary strands were not covalently linked. Because these branched RI molecules had single strands of varied lengths, it was suggested that these molecules were the most active in the replication sequence.

### 2.5.2 Parvovirus Minute Virus of Mice (MVM)

Tattersall et. al. (1973) was able to isolate replicative form DNA from MVM-infected mouse L cells. Hirt extraction of low molecular weight DNA from infected cells showed an increase in the amount of radiolabeled DNA in the supernatant at 8-14 hour that could not be detected in mock-infected cells. The appearance of this labeled DNA in the supernatant corresponded to the production of cell-associated virus hemagglutinin. This DNA eluted from HAP column in two peaks; one in the position of single-stranded DNA and the other in the position of double-stranded DNA. Alkaline sucrose gradients revealed that the single-stranded peak from HAP behaved identically to single-stranded virion DNA suggesting that it is progeny single-stranded DNA. Neutral sucrose gradient sedimentation of the double-stranded peak from HAP resolves one major peak corresponding to an S value of about 16S. A small amount of apparently heterogenous length DNA was also detected sedimenting in front of the main peak which was postulated to be complexes of genome length viral DNA. Approximately all of the double-stranded HAP peak sedimented as a single band in alkaline sucrose and had an S value slightly lower than the polyoma DNA included as a marker. The Hirt supernatants were also subjected to benzoylated DEAE-cellulose chromatography. This procedure resolved two peaks, one eluting under conditions that elute double stranded DNA and the other peak at a position corresponding to partially single-stranded molecules. These double-stranded and partially single-stranded molecules were shown by hybridization to contain virus specific base sequences.

Ward and Dadachanji (1978), working with  $^{32}\text{P}$ -labeled MVM DNA, showed its conversion to a duplex form 6-8 hours post infection in parasynchronous culture of mouse L cells. This DNA will spontaneously renature suggesting that the two strands of the duplex are covalently linked. The amount of this spontaneously renaturing duplex DNA decreased as the infection continued. The authors speculated that the covalent linkage is an important event early in the replication process. When double-stranded monomers and dimers were digested with the restriction enzyme EcoRI, fragments are resolved on polyacrylamide gels which contained the 3' and 5' termini of the genome. These fragments were also capable of spontaneous renaturation suggesting a covalent linkage between the virus and complimentary strand. Ward and Dadachanji mention, however, that it is not certain that the linkages occur at the termini of the genome. They also state the renaturation is consistent with the proposed models for parvovirus replication through a self-priming mechanism (see Section 2.6). EcoRI digestion of double-stranded dimer RFs yielded a fragment not present upon digestion of monomers are digested. The fragment contained a 3' terminus of the genome, was the same size as monomer RF DNA, and underwent spontaneous renaturation, which suggested that the viral and complimentary strands in dimers were covalently attached through the 3' end of the virus strand. Pulse-chase experiments involving monomer and dimer RF demonstrated that these radioactive molecules lost their radioactivity when chased with non-labeled thymidine. The loss corresponded to an increase in radioactivity in single-stranded DNA, which suggested that the progeny DNA

could be produced from both monomer and dimer forms. The authors point out that this observation is consistent with the proposed replication model (see Section 2.6).

### 2.5.3 Parvovirus Kilham Rat Virus (KRV)

Gunther and May (1976) reported that Hirt extraction of KRV infected rat cells yielded a single peak of radioactively labeled DNA when subjected to isopycnic centrifugation. These molecules were subsequently analyzed on neutral sucrose gradients and two peaks were obtained. Characterization of these two peaks by HAP showed that both contained double-stranded molecules. Hybridization demonstrated that both peaks were virus specific. Further rate zonal centrifugation analysis showed that one of the double-stranded peaks corresponded to monomer length molecules with a molecular weight approximately twice that of single strand KRV virion DNA. The other peak consisted of dimer length molecules. Results from sedimentation of the monomeric and dimeric forms in alkaline sucrose gradients led the authors to postulate that the viral and complementary strand were covalently linked suggesting a self-priming process for replication.

The Hirt extraction does not recover progeny DNA because it is encapsidated as it is made and is precipitated in the Hirt pellet. This was overcome by Lavelle and Li (1977) who developed a method for obtaining low molecular weight DNA from infected cells that would also yield progeny DNA. Briefly, the infected or mock-infected cells were lysed in the presence of 4M guanidine-hydrochloride (G-HCl) and sedimented in a sucrose gradient that also contained G-HCl. The authors reported that

KRV-infected cells pulse-labeled with  $^3\text{H}$ -thymidine ( $^3\text{H}$ -TdR) and subsequently chased with non-labeled TdR demonstrated two peaks of radio-labeled low molecular weight DNA upon extraction by this method. One peak corresponded to the 16S replicative form and the other cosedimented with  $^{32}\text{P}$  labeled 25S virion DNA. The radioactivity in the 16S peak was also shown to decrease as the infection progressed. This decrease was concomitant with an increase in the 25S peak. Thus, part of the 16S peak could be chased into the single-stranded position which suggested that the 16S DNA included molecules that are intermediate in the synthesis of progeny DNA. Another DNA species was also found in the leading edge on the 16S peak. It also decreased as the infection progressed and was double-stranded with some single-stranded nature as determined by S1 endonuclease digestion. This DNA was postulated to be replicative intermediates. Mock-infected cells treated in the same manner failed to yield any of the three DNA species described above. The authors point out that contamination due to sheared cellular DNA is greatly reduced as evidenced by the large amount of radioactivity in the bottom of the centrifuge tubes in comparison to that of the supernatant.

Hayward et. al. (1978) were able to isolate a double-stranded DNA from KRV infected cells which had an approximate molecular weight and length corresponding to that of monomer molecules. The molecules were found to consist of monomer length single strands upon denaturation. Another type of DNA species was also recovered that was again, a double-stranded monomer length and had a molecular weight of twice the virion DNA. However, these molecules were shown to form dimer length single

strands upon denaturation indicating that these molecules consisted of viral and complimentary strand covalantly attached. Linear dimer double-stranded DNA was also seen which had a molecular weight of twice the duplex monomer RF. These molecules consisted of equal amounts of covalently linked dimers and monomer single strands. The dimers were shown to renature to form duplex monomers. The data presented did not demonstrate any precursor-product relationship between the different DNA species.

Further characterizations were reported in 1978 by Li, Lavelle and Tennant for KRV. Again, using the G-HCl method for extracting low molecular weight DNA from KRV infected cells, three pools were obtained from the preparative G-HCl sucrose gradient. These were designated Pools I, II, and III with Pool I being the fastest sedimenting peak. Neutral sucrose gradient analysis showed Pool I to be 25S which is the sedimentation coefficient for single stranded virion DNA. Pool III has an S value of 17S which corresponds to a similar value obtained for H-1 double-stranded RF. Pool II seemed to be more heterogenous than the other two pools, and had a sedimentation coefficient between 17S and 23S.

Velocity sedimentation in alkaline sucrose gradients was also performed with  $^{32}\text{P}$ -label AAV DNA and  $^{14}\text{C}$ -label KRV virion DNA used as markers.  $^3\text{H}$ -labeled Pool I DNA cosedimented with the  $^{14}\text{C}$ -KRV virion. DNA that was longer than genome length was not detected. The majority of the DNA from Pool III also cosedimented with the KRV virion DNA, however, some DNA was observed that was twice unit length. DNA that was

shorter than unit length was not observed. Pool II again appeared to be more heterogenous than the other two pools. Approximately 35% was observed to be longer than unit length and 25% was shorter than unit length. Genome length DNA (Pool I) made up 40% of the total DNA in the gradient.

S1 endonuclease digestion and BND-cellulose chromatography yielded similar results as to the secondary structure of the molecules within the three pools. Pool I appeared to be single-stranded DNA with characteristics consistent with those of virion DNA. Pool III was similar to the linear double-stranded replicative forms which were described for several other parvoviruses. Pulse-chase experiments, in addition to the nuclease digestion and column chromatography, showed that Pool II seemed to be the precursor for the other two classes of molecules and consisted of double-stranded DNA with some single-stranded regions, characteristic of replicative intermediates. The authors contend that these results are consistent with the proposed model of parvovirus replication (see Section 2.6).

#### 2.5.4 Parvovirus H1

Rhode (1974a) reported the isolation of a DNA species from H-1 infected hamster embryo cells that was not of cellular origin. Prior to infection with H-1 the cells had been preincubated with  $^{14}\text{C}$ -TdR to label cellular DNA. At 20 hours post infection, the cells were again labeled, but with  $^3\text{H}$ -TdR. After isolation of the nuclei from these cells, the nuclei were placed on a controlled pore glass bead column, lysed and viral DNA was subsequently eluted with 1% SDS, 0.1M NaCl, 50 M Tris-HCl

pH 7.4 and 1 mM EDTA. Both the NaCl and SDS elution fractions were subjected to sedimentation in neutral sucrose gradients. H-1 virion DNA was also run separately as a marker. Both eluants yielded a single peak of  $^3\text{H}$ -DNA, but no peak of prelabeled  $^{14}\text{C}$ -cellular DNA. The S value of the SDS eluant was 16.5 S, which suggested a weight of  $3.7 \times 10^6$  daltons or twice that of virion DNA. Hirt extraction of H-1-infected hamster embryo cells also demonstrated similar results upon velocity sedimentation analysis. DNA-DNA hybridization of the 17S peaks confirmed that it was of viral origin. Bouyant density in neutral CsCl was 1.705 g/cc.

Studies using RNA synthesis inhibitors, actinomycin D and  $\alpha$ -amanitin, and the protein synthesis inhibitor, cycloheximide (Rhode, 1974b), showed that the initiation of RF synthesis is dependent upon protein synthesis in late S phase. However, its continuation is not dependent upon protein synthesis. Furthermore, progeny synthesis appears somewhat dependent upon viral protein(s).

Studies by electron microscopy of H-1 RF synthesis were reported by Singer and Rhode (1977). Low molecular weight DNA was extracted from parasynchronous cultures of hamster embryo fibroblasts or human NB cells infected with a temperature-sensitive mutant, ts-1, of H-1 deficient in the synthesis of progeny single-stranded DNA at the restrictive temperature. The purpose of using the temperature sensitive mutant was to reduce confusion in identification of RF DNA and RI DNA involved in progeny synthesis when viewed under the electron microscope. The DNA isolated from the wild type or ts-1-infected cells was subjected to rate-zonal sedimentation in neutral sucrose gradients and then banding

in  $\text{Cs}_2\text{SO}_4$  density gradients. The DNA was spread for electron microscopy by the aqueous or formamide techniques. Single-stranded virion DNA was cospread by the formamide technique with single-stranded  $\phi\text{X174}$  DNA to determine the molecular weight of H-1 virion DNA.

Monomer and dimer length linear double-stranded RF molecules were observed which had mean lengths of  $1.53 \pm 0.04 \mu\text{m}$  and  $3.10 \pm .28 \mu\text{m}$  respectively. Only 16 dimer length molecules were measured and 20-100 are needed for statistical accuracy (Kay, 1976) therefore, this value must be viewed with some caution. Also observed under the EM were linear Y-shaped double-stranded molecules which were postulated to be involved in synthesis of daughter RF DNA molecules. The authors term these branched molecules replicative intermediates, but they must not be confused with the replicative intermediates involved in the synthesis of single-stranded progeny DNA. It is unfortunate that the term "replicative intermediate" was used in this manner, as it increases confusion.

Both monomer and dimer branched molecules were measured and were determined to have mean lengths of  $1.55 \pm .10 \mu\text{m}$  and  $2.94 \pm .22 \mu\text{m}$  respectively. These lengths were obtained by adding the length of the longest branch to that of the unreplicated portion of the molecule. To insure that these Y-shaped molecules were double-stranded and did not contain single-stranded regions, they were spread for EM by the aqueous method. No pooled bushes characteristic of single-stranded DNA were observed, suggesting that these molecules were indeed double-stranded.

The branched molecules were ordered based on the proportion that had been replicated. The replicated portions ranged from 0.15-0.88 genome lengths with replication forks distributed randomly. The authors

conclude that the origin of replication is therefore with 0.15 genome lengths from the end(s) of the molecules. Since "eye" structures were not observed, they suggest that the replication proceeds unidirectionally in 73% of the RF molecule. However, they could not disregard the possibility that the ends are replicated by a fork moving in the opposite direction. Based on measurements of single-stranded virus  $\phi$ X174 DNA, the molecular weight of the single-stranded H-1 virion DNA was  $1.40 \times 10^6 - 1.56 \times 10^6$  daltons. The double-stranded monomer RF DNA has a molecular weight of  $2.95 \times 10^6$ , based upon  $\phi$ X174 RF II DNA. Molecular weight values were not reported for dimer length double-stranded RF molecules.

#### 2.5.5 Bovine parvovirus (BPV)

Parris and Bates (1976) reported that optimum conditions for bovine parvovirus (BPV) replication in bovine fetal spleen (BFS) cells occurred when the cells were beginning S phase. Low molecular weight DNA extracted by the Hirt method was detected in BPV-infected BFS cells 8 hrs. post release from the synchronization block. This is 2 hours after the division of cellular DNA. The authors postulated that this DNA was due to virus replication. However, they pointed out that hybridization should be performed to confirm the presence of viral sequences. BPV-infection appeared to have little effect on cellular DNA synthesis or the progression of the cells through S-phase. RNA and protein synthesis, however, were reduced. Characteristics of the low molecular weight DNA from infected cells was not reported.

It has also been reported (Bates, et. al., 1978; Pritchard, et.

al., 1978) that BPV lacks a virion-associated DNA polymerase. Further, there was also a temporal correlation between the levels of DNA polymerase and viral DNA synthesis (Pritchard, et. al., 1978). The authors postulated that DNA polymerase  $\alpha$  was involved in the replication of BPV. Again, characterization of the replicative forms from BPV-infected cells was not done.

## 2.6 Model for Parvoviral Replication

The fact that the parvoviruses have a single-stranded DNA genome has lead to a number of questions concerning their replication since all known DNA polymerases required a 3'-OH for chain elongation and are unable to initiate synthesis of DNA without this primer (Klein, 1973). Cavalier-Smith (1974) suggested that eukaryotic DNA replicated by using palindromic sequences which form duplex hairpin structures. This hairpin is then used as a primer for DNA synthesis.

The current model for parvovirus replication proposed by Berns and Hauswirth (1978) is similar to that suggested by Cavalier-Smith. In the Berns and Hauswirth model, (see Figure 1) the single-stranded virion DNA is converted to a duplex form using the 3' hairpin as a primer. The hairpin must then be opened at a specific site and the replication completed so that terminal sequences may be conserved. This RF can then undergo semi-conservative replication to form daughter RF's, is subsequently nicked again forming a 3' hairpin primer, and is used as a template for progeny strand synthesis by a single strand displacement mechanism. Dimers and trimers are also possible through extended synthesis of the parental or daughter RF's.



This model is consistent with the available data on autonomous parvoviruses in that the single stranded virion DNA is able to form hairpin structures at both the 3' and 5' ends. Also, various investigators (see section 2.4) have been able to isolate and characterize these replicative forms and intermediates. The enzymes required for site specific nicking have not, as yet, been identified.

## 2.7 Electron microscopy

### 2.7.1 Early techniques.

Original techniques for visualization of nucleic acids involved drying droplets of DNA or RNA solutions upon specimen grids, however, these methods proved unsatisfactory in that aggregation of the nucleic acid molecules occurred upon drying. In addition, structural artifacts and preferential orientation were also common. Because of these problems electron microscopy initially was not very useful for characterization of nucleic acids (Lang, 1971).

### 2.7.2 Protein-film technique.

In 1959, Kleinschmidt and Zahn devised a method for visualizing nucleic acids that has proven to be the cornerstone for the use of electron microscopy in nucleic acid characterization. This technique utilized a protein plus DNA solution (the spreading solution) that was allowed to flow down a glass ramp onto the surface of an aqueous solution of ammonium acetate (the hypophase). A portion of the film was subsequently transferred to a coated grid and stained or shadowed to enhance contrast. An advantage to this technique is that only 2-5  $\mu\text{g/ml}$

of DNA or RNA is required (Evenson, 1977). It is known as the aqueous spreading technique (Davis, 1971). Since 1959, various other methods have been developed for spreading both single- and double-stranded nucleic acids for electron microscopy. Although reagents and/or concentrations may vary, the protein film-DNA or RNA complex is shared by most spreading techniques (Lang, et. al., 1971).

The protein normally used for spreading is cytochrome C, a basic protein which binds to the negatively charged nucleic acid through the positively charged side groups and constituent amino acid residues (Kleinschmidt, 1968). The protein film formed on the surface of the aqueous hypophase is denatured at the surface/air interface and results in a stable insoluble monolayer which fixes the attached nucleic acid in a two-dimensional position (Lang, et. al., 1967). Thus, aggregation and distortion caused by drying encountered in previous techniques is avoided. The stable protein monolayer that fixes the nucleic acid molecules may also be compressed or decompressed. It is the different degrees of compression that allow the film to be transferred to specimen grids for visualization. Experiments involving repeated compression and decompression of the film have demonstrated the elastic properties of the protein monolayer. It is this elasticity that provides flexibility for absorbed nucleic acids and prevents strand breakage (Kleinschmidt, 1968).

Although cytochrome C is the most common protein employed in spreading techniques, other basic globular proteins have also been shown satisfactory. These alternate proteins include trypsin, chymotrypsin, ribonuclease, methylated albumin and lysozyme (Kleinschmidt, 1968).

### 2.7.3 Secondary Structural Differentiation

An aqueous spreading technique developed by Kleinschmidt permits the visualization of double stranded DNA and RNA (Davis, 1971). These molecules appear as gently curved filaments. If single-stranded DNA or RNA is spread by this method, it appears as bushes in the electron microscope. This is due to the random intramolecular base pairing which results in the collapse of single strands.

### 2.7.4 Technique for Spreading both Single and Double-Stranded Nucleic Acids

Many techniques have been reported for spreading both single- and double-stranded nucleic acids (Westmoreland, et. al., 1969; Davis et. al., 1971). The methodology of spreading relies on the basic protein-film technique developed by Kleinschmidt and Zahn in 1959. However, several different reagents are used to prevent intramolecular base pairing occurring in single stranded nucleic acids while leaving double stranded molecules in their duplex form.

One of these techniques was developed by Inman and Schnöss in 1970 for use in denaturation mapping experiments with phage  $\lambda$  DNA. In this procedure, the double-stranded DNA of bacteriophage  $\lambda$  was mixed with sodium carbonate, EDTA and formaldehyde and adjusted to the desired pH. The mixture was allowed to incubate for 10 minutes at room temperature and then placed on ice for 5 minutes. This solution was adjusted to a lower pH with addition of a phosphate buffer, an equal volume of formaldehyde was added and the mixture was again cooled. A 5 $\mu$ l sample of the nucleic acid suspension was allowed to flow down a tapered glass rod

onto the surface of a drop of distilled water that was formed in an indentation in a teflon block. The protein film was compressed by removing a small amount of water from the well and the samples were picked up by carbon coated mica. The mica disks were rotary shadowed and the carbon film floated onto the surface of clean distilled water and picked up by specimen grids. Electron micrographs of  $\lambda$  DNA spread by this method revealed long strands of double-stranded DNA that contained "bubbles" or single-stranded regions rich in adenine and thymine pairs which had been denatured by the high pH during spreading. The single-stranded areas appeared thinner than the double-stranded regions, thus both single and double-stranded DNA could be visualized. Visualization of separate single and double-stranded molecules is also possible with the Inman & Schnöss technique, (D.R. Allison, personal communication) by keeping the DNA-formaldehyde solution at a lower pH than necessary to denature double-stranded DNA. The formaldehyde and formamide present in the spreading solution prevent formation of the intramolecular hydrogen bonding in single-stranded DNA and thus prevent single strand collapse. Formamide also enhances contrast (Westmoreland, 1969) and seems to reduce tangling of the single strands at low ionic strengths (Davis et. al., 1971).

#### 2.7.5 Grids and Support Films

The grids used for electron microscopy of nucleic acids are generally made of copper or nickel. The mesh size will vary depending upon the strength of the support film, size of the molecules being viewed and the type of electron microscope (Evenson, 1977). Generally, when

molecules of 1-2  $\mu\text{m}$  are being examined, a mesh size of 300 will be satisfactory. Larger molecules (30-80  $\mu\text{m}$ ), however, will require a mesh size between 50-100. Support film characteristics on larger mesh grids (50-100) may present problems especially when performing contour length measurements, in that the film may stretch or break when it is exposed to the electron beam. Thin and fragile support films should be used with the smallest mesh in order to minimize errors introduced through the aforementioned stretching or breakage (Evenson, 1977).

The type of support film will influence the success of any spreading procedure employed (Evenson, 1977). Consideration of many factors is necessary when choosing the type of support film to be used with a particular nucleic acid sample. These include strength of the film itself, composition, thickness, and surface charges.

Parlodion (Collodion) is one of the most widely used plastic support films for nucleic acid observation. Strips of parlodion are dissolved in dessicated amyl acetate and a drop placed on the surface of distilled water (Bradley, 1965). The parlodion layer is lowered onto grids which are resting on a wire screen or filter paper. The grids are dried and are ready for use. One of the major disadvantages of this film is that it will stretch when exposed to the beam (Lang, 1970), which will introduce error into length and molecular weight calculations. Also, if care is not taken to prevent water condensation in the stock parlodion solution or when drying the grids, "holey" films may result that are unsuitable for visualization and characterization of any nucleic acids (Evenson, 1977).

Formvar, (poly [vinyl] formol) is another support film used in electron microscopy. It is usually a thinner film than parlodion and thus provides better resolution of nucleic acids. It has the disadvantage, however, of being hydrophobic which inhibits the wetting action of the spreading solution (Kay, 1976). Like parlodion, it also has the disadvantage of stretching and/or shrinking when exposed to the electron beam (Lang et. al., 1967).

Carbon support films seem to provide the best results for nucleic acid visualization (Kleinschmidt, 1968). Briefly carbon is evaporated at high vacuum onto sheets of freshly cleaved mica. Mica provides the smoothest natural surface available. The carbon is floated onto the surface of clean distilled water and picked up with specimen grids (Carol Baker, personal communication). These films provide a strong, yet thin, support for nucleic acid samples (Evenson, 1977) and also will not stretch or shrink when exposed to the electron beam (Lang et. al., 1967).

Surface characteristics of the carbon support film are not well understood. Carbon films are usually hydrophobic and are sometimes difficult to use for nucleic acid spreading. Various methods are available for enhancing absorptive characteristics of carbon films. One technique involves placing carbon filmed grids under ultraviolet-light source for 30 minutes. These grids are wettable with the spreading solution for approximately 1 hour after treatment (Kay, 1976). Another method involves treating carbon films with a glow discharge, which is carried out under a partial vacuum in the presence of air or amylamine

atmosphere. This places positive charges on the grids which will attract the negatively charged nucleic acids. This positive charge is stable for approximately 20 minutes after glow discharge (Evenson, 1977).

### 2.7.6 Contrast.

#### 2.7.6.1 Staining

There are several staining procedures for contrast enhancement of nucleic acids. The most widely used is positive staining with uranyl acetate. Uranyl acetate may be dissolved in several organic solvents such as ethanol and acetone (Evenson, 1977). If an alcohol solution is used, staining and dehydration may be accomplished in one step (Kleinschmidt, 1968). Various times have been reported for proper staining of nucleic acids, for example, 15-30 seconds is suggested by Gordon & Kleinschmidt (1968). The staining solution may also be prepared using a diluted hydrochloric acid solution (Davis *et. al.*, 1971). Longer staining times are required, however, for aqueous uranyl acetate solutions (Kleinschmidt, 1968). All uranyl acetate solutions must be stored in dark containers to prevent a precipitation which will result in large electron dense crystals in electron microscope samples (Kay, 1976).

#### 2.7.6.2 Metal Shadowing

Metal shadowing is the most common procedure used to enhance contrast in nucleic acid samples. Briefly, a metal or metal alloy is heated and allowed to evaporate at an angle of  $5-10^{\circ}$  onto specimen grids located 10-15 cm away from the metal source. The grids are mounted on a

rotary table that is turning at a speed of 30-60 rpm. Shadowing may be performed without rotation of the specimen if done from several different angles simultaneously (Kleinschmidt, 1968). Either technique will allow metal to be preferentially deposited around the nucleic acid molecules. Subsequent resolution of the nucleic acids is dependent upon the vacuum achieved during evaporation. Several metals and metal alloys may be used for metal shadowing. These include platinum, platinum-palladium and uranium. Platinum-palladium (80:20) is the most widely used and results in a small grain size (Kleinschmidt, 1968).

#### 2.7.7 Uses of EM in Nucleic Acid Characterization

The major use of EM in the characterization of nucleic acid is in the measurement of contour length and molecular weight. Contour length is determined by measuring the length of a large number of molecules photographed at a known magnification. Magnification may be calibrated using a grating replica and/or internal nucleic acid marker of known length (Kay, 1976). The number of molecules are usually measured for statistical accuracy is 20 to 100. These contour length measurements can then be used to estimate molecular weight (Lang, 1970). This is done by using a nucleic acid standard of known molecular weight from which molar linear density in daltons/cm or daltons/ $\mu\text{m}$  may be calculated. Substitution of the contour length (L) of the unknown DNA sample into the following equation results in a value for molecular weight:

$$M = M'L$$

In this equation M is molecular weight and M' is molar linear density (Lang, 1970).

Lang (1970), using the diffusion technique for preparing nucleic acids for EM calculated the molar linear densities for bacteriophages T2, T4, and T7 DNA molecules. Several assumptions (Kay, 1976) were made during these calculations.  $M'$  was assumed to be independent of base composition. Methylation of any of the bases present was assumed to have no effect on the configuration. Although the "B" configuration may be true for DNA in solution, this was not necessarily believed to be true for the DNA molecules on specimen grids. With these assumptions, Lang calculated the  $M'$  for T7 to be  $2.07 \pm 0.04 \times 10^{10}$  dalton/cm. The value for standard molecular weight and contour length was  $25.1 \times 10^6$  d and 12.15  $\mu$ m respectively. This value was believed to be applicable to DNA's with similar base compositions. An  $M'$  value of  $2.28 \times 10^{10}$  d/cm was obtained for T4 and  $2.23 \times 10^{10}$  d/cm for T2.

In addition to contour length and molecular weight measurements, several other parameters may also be determined. These include degree of secondary structure, circularity, and degree of branching (Lang et. al., 1967).

#### 2.7.8 Factors Influencing Contour of Double-Stranded DNA

In 1967, Lang and coworkers defined several factors that can influence the contour length of DNA. Various measurements were made of double-stranded T1, T3 and BPV (bovine papilloma virus) DNA spread by the diffusion technique which employs a solution of DNA onto which a cytochrome C film is allowed to form. DNA molecules diffuse through the solution to complex with the film at the surface of the solution (usually a droplet). It was noted that while the method of nucleic acid extrac-

tion showed little influence on contour length, the ionic strength demonstrated a marked effect. For example T3 DNA prepared for EM by the diffusion method at an ionic strength of 0.00003 had a contour length of 13.77  $\mu\text{m}$ , whereas the T3 prepared at an ionic strength of 0.20 measured 11.83  $\mu\text{m}$  in length. A similar decrease in contour length with increasing ionic strength was also observed when samples were spread by the aqueous technique of Kleinschmidt. Lang suggested that this linear extension is a result of electrostatic repulsion between the like charges within a DNA molecule. These repulsive forces increase at ionic strengths less than 0.10 M.

Ionic strength also was shown to have an effect on the stability of the protein film. DNA molecules spread by the diffusion method had a flower-like appearance at ionic strength less than 0.10, which Lang suggested was due to instability of the protein film. This effect was not as pronounced when spread by the aqueous technique at ionic strength greater than 0.05. It was most common when the DNA solution was spread onto water.

Inman (1967) also reports similar findings with regard to ionic strength in contour length measurements of double-stranded phage  $\lambda$  DNA in which the DNA is spread by the aqueous technique onto hypophases of various ionic strengths. He also noted that the influence of ionic strength on contour length was considerably reduced if the hypophase contain 0.5% formaldehyde. Looping and aggregation were also reduced. In addition, Inman suggested that it was the different spreading conditions that resulted in length discrepancies for DNA of other sources that had been reported by various investigators.

### 2.7.9 Factors Influencing the Length of Single Stranded DNA

Bujard has reported several major factors that will influence the length of single-stranded DNA. Single-stranded DNA was unfolded in the presence of formaldehyde (final concentration of 5%) and dimethylsulfoxide. The DNA sample was prepared for EM by the diffusion method of Lang, et. al. (1967) using varying ionic strengths in the hypophase DNA mixture. One of the factors that was observed to influence molecular length of single-stranded DNA was that of unstable protein films which may result from contamination or convection. Preferential orientation of molecules characteristic of such an influence is indicative that the molecules were subjected to some type of mechanical stress. Linear expansion results from this stress. Bujard noted that single-stranded DNA is highly susceptible to this type of stress and observed that in some instances of film instability, single-stranded fd DNA measured 3.1  $\mu\text{m}$  as compared to 1.25  $\mu\text{m}$  observed in experiments in which the protein film was undisturbed.

Ionic strength was also shown to have a marked affect on contour length of single-stranded DNA. Bujard reported that the length of single-stranded DNA increased by 74% if the molarity of the ammonium acetate solution used in preparation of the DNA for EM was decreased from 0.4 to 0.1. He also suggested that it is the effect of the ionic strength on contour length that accounts for the length variations reported in the literature for various single-stranded viral nucleic acids.

### 2.7.10 Sources of Error During Contour Length Measurements

There are various sources of error in contour measurements which may compromise the accuracy of the results (Lang et. al., 1967). One of these sources is in the determination of magnification using a grating replica. The grating constant given by the manufacturer was shown to be 2.7% lower than Lang measured by light microscopy. Pincushion distortion in both the electron microscope and projector or enlarger is also a source error. Lang demonstrated pincushion distortion in the electron microscope to be 1.7% and 0.5% for the negative projector. Other sources of error include determination of enlargement factor in the negative projector or enlarger and tracing. The determination of these errors will be described in the Materials and Methods section.

### 3. Materials and Methods

#### 3.1 Cells

Bovine fetal spleen (BFS) cells were obtained from bovine fetuses 3 to 6 months of age. After aseptic removal, the spleens were cut into small pieces and placed in a sterile Erlenmeyer flask (500 ml) to which was added approximately 250 ml of 0.25% trypsin. This mixture was agitated using a sterile magnetic stir bar until the tissue was almost fully digested. The cells were then pelleted at 1500 rpm in an IEC HN-S table top centrifuge for 10 minutes. The cell pellet was resuspended in Eagle's minimum essential medium (MEM; Flow Laboratories, Inc.) and seeded into glass tissue culture bottles (TC; Brockway Glass Company) in MEM plus 10% lamb serum (LS; Flow Laboratories, Inc.). Upon formation of a monolayer, the cells were dispersed with a solution of trypsin-versene (TV) and either dispensed into TC bottles or frozen and stored in liquid nitrogen.

BFS cells were maintained in MEM plus 10% fetal bovine serum (FBS; Flow Laboratories, Inc.). The cells were dispersed and dispensed in a ratio of 1:3-5 after formation of a monolayer (5-7 days after seeding). Only cells between passages 10-20 were used for experimentation.

#### 3.2 Bovine Parvovirus

BPV was isolated and maintained according to Bates, et. al. (1972) and Bates and Storz (1973).

Virus stocks were propagated in BFS cells that had been seeded in 75 cm<sup>3</sup> plastic flasks (1-1.5 x 10<sup>6</sup> cells/flask; Falcon) in MEM plus 10% LS. The cells were observed for 100% cytopathic effects at which time they were frozen and thawed 3 times. The cell suspensions were combined and centrifuged at 6500 rpm for 10 minutes in a Sorvall superspeed RC2-B automatic refrigerated centrifuge. The titer of the resulting supernatant was determined by plaque assay. Average titers were 5 x 10<sup>7</sup> plaque forming units (pfu)/ml. Virus stock was stored at -20°C.

### 3.3 Synchronization, Infection and Radiolabeling

BFS cells were synchronized as described by Parris *et. al.* (1975). Briefly, BFS cells were seeded into 15 mm x 60 mm petri dishes (1.5 x 10<sup>6</sup> cells/plate) or plastic roller bottles (1.7-2.0 x 10<sup>7</sup> cells/roller bottle; 850 cm<sup>3</sup>; Corning) containing MEM and 10% dialyzed LS or FBS, which had been screened for the presence of BPV-antibodies by hemagglutination-inhibition testing. The medium also contained 2 mM hydroxyurea (HU; Sigma). Approximately 32 hours after addition of the HU block, the cells were released by washing 3X with warm (37°C) Dulbecco's salt solution.

Upon release the cells were infected with stock BPV at a multiplicity of infection (m.o.i.) of 10. After 1 hour incubation at 37°C, MEM plus 10% dialyzed LS or FBS was added back to the cells (3 ml/petri dish; 20 ml/roller bottle) and then reincubated at 37°C.

At 13.5 hours past infection (h.p.i.) the cells in petri plates or roller bottles were labeled with <sup>3</sup>H-TdR (200 µCi/plate or roller bottle; ICN) and then incubated for various times and either immediately harvested

or subsequently chased with unlabeled TdR ( $1 \times 10^{-4}$  M); for 2 or 4 hours and harvested.

### 3.4 Extraction of Low Molecular Weight DNA

#### 3.4.1 Guanidine - Hydrochloride Method

BFS cells that had been synchronized, released, infected and labeled in 15 x 60 mm petri plates as previously described were harvested by removing the medium from the plates and adding 2 ml of saline A solution (see appendix). After agitating the plates and removing the saline A, 2 ml of Trypsin-versene (TV) was added and the cells were observed for characteristic "rounding" morphology with an inverted microscope (Olympus). The TV solution was removed and 0.1 ml of TV was again added and the cells reincubated at 37°C for 1-2 minutes. To each plate was added 0.4 ml of phosphate buffered saline (PBS; pH 7.0) and 50 µl of proteinase K (2 mg/µl; Merck). The cells were gently scraped from the plates and the 0.5 ml suspension was layered onto a 5-20% sucrose gradient containing 4 M guanidine hydrochloride (G-HCl; Lavelle and Li, 1977), and 10 mM Tris-HCl and 1 mM EDTA (TE, pH 8.0) to lyse the cells. These gradients were incubated for a minimum of 17 hours at room temperature before centrifugation.

The sucrose-G-HCl gradients were centrifuged in an SW27.1 rotor at 20°C for 16 hours at 27,000 rpm in a Beckman L5-50 ultracentrifuge. The gradients were fractionated from the bottom and 0.5 ml fractions were collected. Aliquots of 100 µl from each fraction were precipitated onto Whatman #3 filter papers by 3, 10 minute washes in cold trichloroacetic acid (TCA; Sigma). After dehydration by 3, 10 minute washes in cold 95%

ethanol and 1, 10 minute wash in diethylether, the filters were air dried and placed in 5 ml toluene-PPO-POPOP scintillation fluid and counted for 1 minute in a Packard Tri-Carb liquid scintillation counter. DNA in remaining portion of the fractions was precipitated with 95% ethanol containing 3 M sodium acetate.

In some cases the nuclei from infected cells were isolated by the method of Lynch (1975) and used for isolation of viral replicative forms. BFS cells were seeded in 15 x 60 mm petri plates ( $1.5 \times 10^6$  cells/plates), synchronized and infected as previously described in Section 3.3. At 13.5 h.p.i., 200  $\mu$ Ci/plate of  $^3\text{H-TdR}$  was added and the cells incubated for 2 hours at  $37^\circ\text{C}$ . The cells were harvested by scraping and pelleted at 1500 rpm in an IEC HN-S tabletop centrifuge. The pellet was resuspended in 0.3M sucrose containing 4 mM  $\text{CaCl}_2$  and 5 mM dithiothreitol and homogenized with 60-65 strokes in a Potter-Elvehjem homogenizer at  $4^\circ\text{C}$ . Removal of the cytoplasm was monitored by phase-contrast microscopy. The nuclei were then pelleted at 1000 x g for 10 minutes in a Sorvall superspeed RC2-B automatic refrigerated centrifuge and resuspended in PBS plus 100  $\mu\text{g/ml}$  of proteinase K. A total of  $3 \times 10^6$  nuclei were then layered on top of a 5-20% sucrose gradient containing 4M G-HCl, incubated, centrifuged and fractionated as before (see Section 3.4.1.2).

#### 3.4.2 Hirt Procedure

BFS cells were seeded into plastic roller bottles, synchronized and infected as previously described in Section 3.3. The cells were labeled at 13.5 h.p.i. for 2 hours with 200  $\mu\text{Ci/roller}$  bottle of  $^3\text{H-TdR}$ .

The cells were harvested by scraping and pelleted by centrifugation at 1500 rpm in a IEC-HN-5 tabletop centrifuge. The low molecular weight DNA was extracted by Hirt method (Hirt, 1967) as modified by Siegl and Gautschi, (1978). The cell pellet was resuspended in 5 ml of 0.6% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, 10 mM EDTA (pH 7.5). To this suspension was added 300 µg/ml of proteinase K. It was then incubated for 1 hour at room temperature and subsequently made 1 M NaCl. The cell preparation was placed on ice in a refrigerator, incubated overnight, and then centrifuged for 30 minutes at 17,300 x g at 4°C in a Sorvall superspeed RC2-B automatic refrigerated centrifuge in SS-34 rotor. The pellet was discarded, the supernatant placed back on ice, and the incubation and centrifugation was repeated twice more. The final supernatant was stored at -20°C until further purification.

### 3.5 Purification of Hirt Supernatant DNA

#### 3.5.1 Phenol Extraction

The Hirt supernatant was mixed with 2.5 volumes of phenol saturated with 0.01 M Tris-HCl (pH 8.0) in a plastic centrifuge tube and agitated for 3 minutes at room temperature. The mixture was then placed on ice for 5-10 minutes to aid separation of the layers and centrifuged for 10 minutes at 2000 rpm in an IEC HN-S tabletop centrifuge. The phenol layer was removed and the procedure repeated. The aqueous phase was dialyzed against TE (pH 8.0). This procedure for deproteinization was a modification of that described by Penman (1969).

### 3.5.2 Hydroxyapatite Chromatography

Hydroxyapatite (HAP; DNA-grade; BioRad) that had been prepared in 0.01 M sodium phosphate (NaP) buffer, pH 7.2 was used. Approximately 8-10 ml of the HAP suspension was placed in plastic 10 cc syringes that contained a glass wool support. The resulting packed volume was 1-2 ml. After washing the column with several volumes of 0.01 M NaP, the DNA sample was allowed to adsorb to the column and washed with 1.0 ml of 0.05 M NaP (pH 7.1). A gradient of between 0.05 M -0.4 M NaP was allowed to flow over the column at a rate of about 1 ml/min. The rate was adjusted with a Buchler peristaltic pump. Fractions of 2 ml were collected and 0.05 ml aliquots were placed in 3 ml of ACS scintillation fluid with 0.2 ml of distilled water and counted in a Beckman LS-230 liquid scintillation counter. The elution molarity of the peak fractions was established by determining the refractive index of each sample using a Bausch and Lomb refractometer and a standard curve. Fractions to be used in further purification steps or other procedures were dialyzed overnight against TE (pH 8.0; 10 mM Tris-HCl, 1 mM EDTA).

### 3.5.3 Preparative Neutral Sucrose Gradients

Dialyzed samples from HAP were concentrated by polyethylene glycol. This DNA was then placed on 5-30% (w/v) neutral sucrose gradient (pH 8.0) containing 1 M NaCl, 10 mM Tris, 1 mM EDTA, and 0.15% sarkosyl. The gradients were centrifuged in a SW41 rotor at 20°C for 14 hours at 30,000 rpm in L5-50 ultracentrifuge. Tubes were fractionated from the bottom and 0.5 ml fractions collected. Aliquots of 50  $\mu$ l were placed in 3 ml of ACS scintillation fluid plus 0.4 ml of distilled water. The

samples were counted as described in section 3.5.2 Individual fractions were dialyzed overnight against TE pH 8.0 and then stored at  $-20^{\circ}$  until further use.

### 3.6 Electron Microscopy

#### 3.6.1 Preparation of Carbon Films

Carbon was evaporated onto sheets of freshly cleaved mica (Fullam) in a Ladd Vacuum evaporator (Model 40000) and subsequently placed at  $4^{\circ}\text{C}$  for at least 1 hour. The carbon film was floated onto a surface of distilled water. Rhodium backed copper grids (300 mesh; Ladd) that had been dipped into a 50 ml beaker of acetone containing 12-18 inches of Scotch Brand Magic Tape (Carol Baker; personal communication) were used to pick up the film from below and the coated grids were allowed to air dry at least 1 hour before use. The grids were stored in glass petri dishes until used.

#### 3.6.2 DNA Spreading Procedures

##### 3.6.2.1 Kleinschmidt Aqueous Technique

DNA at a concentration of 2-5  $\mu\text{g}/\text{ml}$  was mixed with 20  $\mu\text{l}$  of 0.1 mM EDTA pH 7.5, 5  $\mu\text{l}$  of 5 M ammonium acetate ( $\text{NH}_4\text{Ac}$ ) and 20  $\mu\text{l}$  of cytochrome C (1 mg/ml; Sigma). This DNA spreading solution was allowed to flow down a clean glass microslide onto the surface of 0.25 M  $\text{NH}_4\text{Ac}$  hypophase that was contained in a plastic trough (9 cm x 9 cm x 1.5 cm). Two teflon bars supported the slide. A carbon-filmed grid was used to pick up the DNA from the surface of the hypophase and was then stained for 30 seconds with a 1:50 dilution in 95% ethanol of uranyl acetate (UrAc) stock solution (0.05 M UrAc in 0.05 M HCl). The grid was subse-

quently placed in 95% ethanol to remove excess stain, air dried and viewed under the electron microscope. The aqueous technique allows visualization of double-stranded nucleic acid (Kleinschmidt, 1959).

### 3.6.2.2 Inman Technique

The Inman technique (Inman, et. al., 1970) allows for visualization of both double- and single-stranded DNA. This procedure is a slight modification (D.P. Allison; personal communication) of the original technique. All DNA spread by this technique was suspended in TE pH 8.0. A titration curve was worked out for this TE buffer in order to determine the amount of 5 N NaOH or 1 N HCl that must be added to the Inman buffer (described below) in order to achieve the proper pH for spreading the DNA.

The Inman buffer consisted of 4 ml of formaldehyde (Mallinkrodt), 0.4 ml of 0.126 M EDTA and 0.32 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> adjusted to the proper pH by the addition of 0.1 ml of 1N HCl. To 0.07 ml of DNA sample (2-5 µg/ml) was added 0.030 ml of the Inman buffer (final pH spreading solution was pH 8.7). This mixture was allowed to incubate for 10 minutes at room temperature and was then placed on ice for 5 minutes. At the end of this 5 minute incubation, 0.1 ml of formamide (Mallinkrodt) and 0.02 ml of cytochrome C (1 mg/ml; Sigma) was added, followed by another 10 minutes incubation at room temperature.

To spread the DNA, 0.005 ml of the spreading solution was allowed to flow down a tapered glass rod onto the surface of the hypophase. The hypophase consisted of water which had been placed in a well (8.5 cm x 1.0 cm) formed in a Teflon block. The DNA-cytochrome C film was picked

up by carbon-filmed grids and the grids dehydrated by washing serially in distilled water, 50% ethanol and 95% ethanol. The grids were then stained for 30 seconds with UrAc (see section 3.6.1.1) and then further dehydrated by washing for 10 seconds in 2-methyl butane (Eastman). The prepared grids were subsequently rotary shadowed at 60 rpm in a Ladd vacuum evaporator at angle of  $10^{\circ}$ . Between 3.5 and 4.0 cm of platinum: palladium alloy (80:20; E. Fullam) was used. The samples were then ready for viewing.

### 3.6.3 Visualization and Photography

All grids were viewed in a JEOL 100C electron microscope at 20,000X. Magnification was calibrated with a grating replica (54,864 lines/in; Fullam). The grating replica was checked for accuracy by light microscopy. Pincushion distortion was measured by determining the distance between bars on the grating replica as viewed on the negatives. Micrographs were taken using Kodak electron microscope plate film (#4483) and enlarged using an Omega Pro-Lab enlarger equipped with a Schneider-Kreuznach lens (S 5.6/135). Prints were made on Kodabromide RC II F ultra-hard paper developed with an Ektamatic rapid print processor and fixed with Kodak Rapid Fix. Pincushion distortion within the enlarger was determined by measuring the distance between bars of the grating replica as the negatives were viewed through the enlarger.

### 3.6.4 Measurements

The negatives were enlarged in an Omega Pro-Lab enlarger and traced onto white paper. The lengths of the tracings were measured using a

Kelsh 600-A digitizer. Tracing error was determined by measuring a molecule 5-6 times.

### 3.7 Velocity Sedimentation

#### 3.7.1 Neutral Sucrose Gradients

Gradients of 5-20% (w/v) or 5-30% (w/v) sucrose (Beckman) contained 1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 0.15% sarkosyl (Lavelle and Li, 1977). The pH was adjusted to 8.0. Gradients were centrifuged for 3 hours at 39,000 rpm in SW 50.1 rotor at 20°C in a Beckman L5-50 ultracentrifuge or in a SW 41 rotor as described in Section 3.5.3.

Tubes were fractionated from the bottom and approximately 0.2 ml fractions collected directly into counting vials. ACS scintillation fluid (3 ml) and distilled water (0.2 ml) were added and the vials counted in a Beckman LS-230 liquid scintillation counter.

#### 3.7.2 Alkaline Sucrose Gradients

Alkaline 5-20% (w/v) or 5-30% (w/v) sucrose gradients contained 0.3 N NaOH, 0.7 M NaCl 1 mM EDTA (pH 8.0) and 0.15% sarkosyl (Lavelle & Li, 1977). The pH was adjusted to 12.0. Gradients were centrifuged in SW 50.1 rotor at 42,000 rpm for 4 hours at 20°C in a Beckman L5-50 ultracentrifuge or in SW 41 rotor as described in Section 3.5.3. Tubes were fractionated and counted as described in section 3.7.1.

### 3.8 Isopycnic Sedimentation

Neutral CsCl gradients to determine bouyant density were prepared by mixing the DNA sample with CsCl that had been dissolved in TE pH 8.0

(1.70 g/cc) in 5 ml cellulose nitrate tubes. These were then centrifuged for 48 hours in SW 50.1 rotor at 35,000 rpm at 18°C. Fractions of 0.2 ml were collected from the bottom of these tubes directly into mini-vials. The refractive indices of each fraction was measured to determine the density of the CsCl. To each vial was added 4 ml of Gradient-Solv (Beckman) and 0.2 ml of distilled water and counted as before (see Section 3.7.1).

### 3.9 Hybridization

DNA used for hybridization was dialyzed against 0.1 X SSC (0.15 M NaCl, 0.015 M sodium citrate pH 7.3). The <sup>14</sup>C-DNA samples were then denatured by adding 0.1 volume of 1 N NaOH and incubating for 10 min. at room temperature. They were brought to a neutral pH by the addition of 0.1 volume of 1.5 M NaH<sub>2</sub>PO<sub>4</sub>.

This DNA was allowed to adsorb to nitrocellulose filters (5 & 5:25 mm) according to the method of Gillespie and Spiegelman (1965). After presoaking the filters in 6 X SSC for 20 minutes, they were placed on a Millipore 12 place manifold and washed with 10 ml of 6 x SSC. DNA samples were made 6 x SSC and brought to a volume of 10 ml. The DNA (2 µg/filter) was adsorbed to the filters and subsequently washed 5 times with 10 ml of 2 x SSC. These filters were then placed in clean glass scintillation vials and incubated overnight at 60°C.

The hybridization procedure that was used is that described by Green et. al. (1969). <sup>3</sup>H-labeled Peak II BPV virion DNA and cellular DNA were used as probes and were denatured as previously described. The samples were made 6 x SSC and the volume brought to 1 ml. This DNA (0.1

ug) was made 0.1% SDS and incubated with the filters for 24 hours at 66°C. The filters were then washed twice with 300 ml of 0.003 M Tris-HCl pH 9.2 and placed on the manifold where they were again washed 10 times with 10 ml of the Tris-HCl pH 9 buffer. The filters were dried under a heat lamp and then counted in 3 ml of toluene-PP0-POPOP.

## 4. Results

### 4.1 Guanidine - Hydrochloride Preparation of Viral DNA

The sucrose-G-HCL gradient method for extracting low molecular weight DNA from parvovirus-infected cells was shown to provide excellent separation without shear of cellular and viral nucleic acids (Lavelle and Li, 1977). In addition, this method also allowed for the isolation of encapsidated single-stranded progeny DNA and its separation from double-stranded RF's. This is unlike the Hirt procedure in which only the RF's and RI's are isolated.

BPV-infected BFS cells were pulse labeled with  $^3\text{H}$ -TdR for 1 hour at 13.5 h.p.i. and subsequently harvested or chased with unlabeled TdR before harvest as previously described in Section 3.3. Lysis of the cells and sedimentation through sucrose gradients containing 4 M G-HCl resulted in the gradient profile seen in Figure 2. When cells were pulse-labeled and harvested, a single peak of radiolabeled DNA appeared toward the top of the tube (Figure 2a) or in a position corresponding to double-stranded RF DNA based on previous reports by Lavelle & Li (1977). Sedimentation of BPV-infected cells that were pulse-labeled and then chased for 2 hours with unlabeled TdR demonstrated two peaks of radio-labeled DNA (Figure 2b). There was a peak in the region of double-stranded RF DNA toward the top of the tube and, in addition, a smaller second peak toward the bottom of the tube in the position of single-stranded progeny DNA. The gradient profile of labeled BPV-infected cells

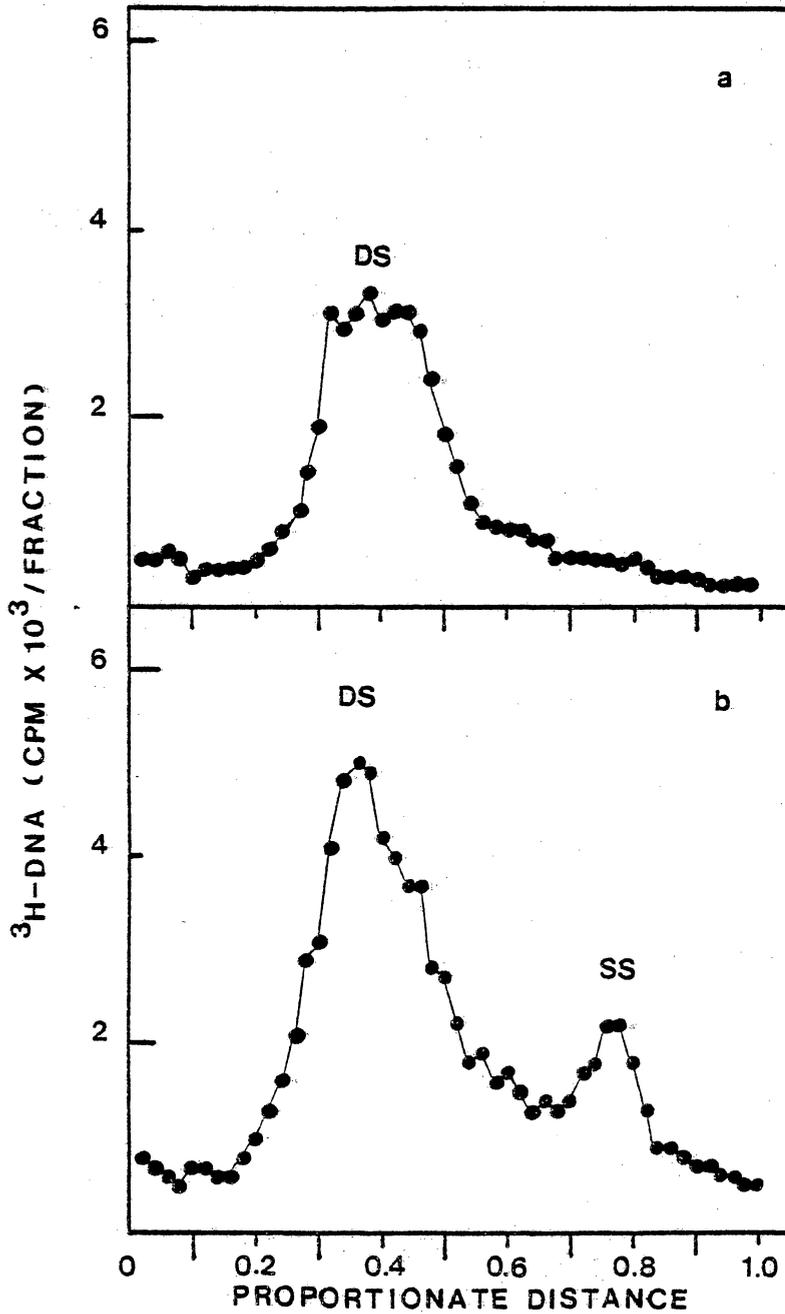


Figure 2. G-HCl-sucrose gradient profile of BPV RF DNA from infected and mock-infected cells. a) 2 hr pulse with  $^3\text{H-TdR}$  and no chase. b) 2 hr pulse with  $^3\text{H-TdR}$  and a 2 hr chase with nonlabeled Tdr.

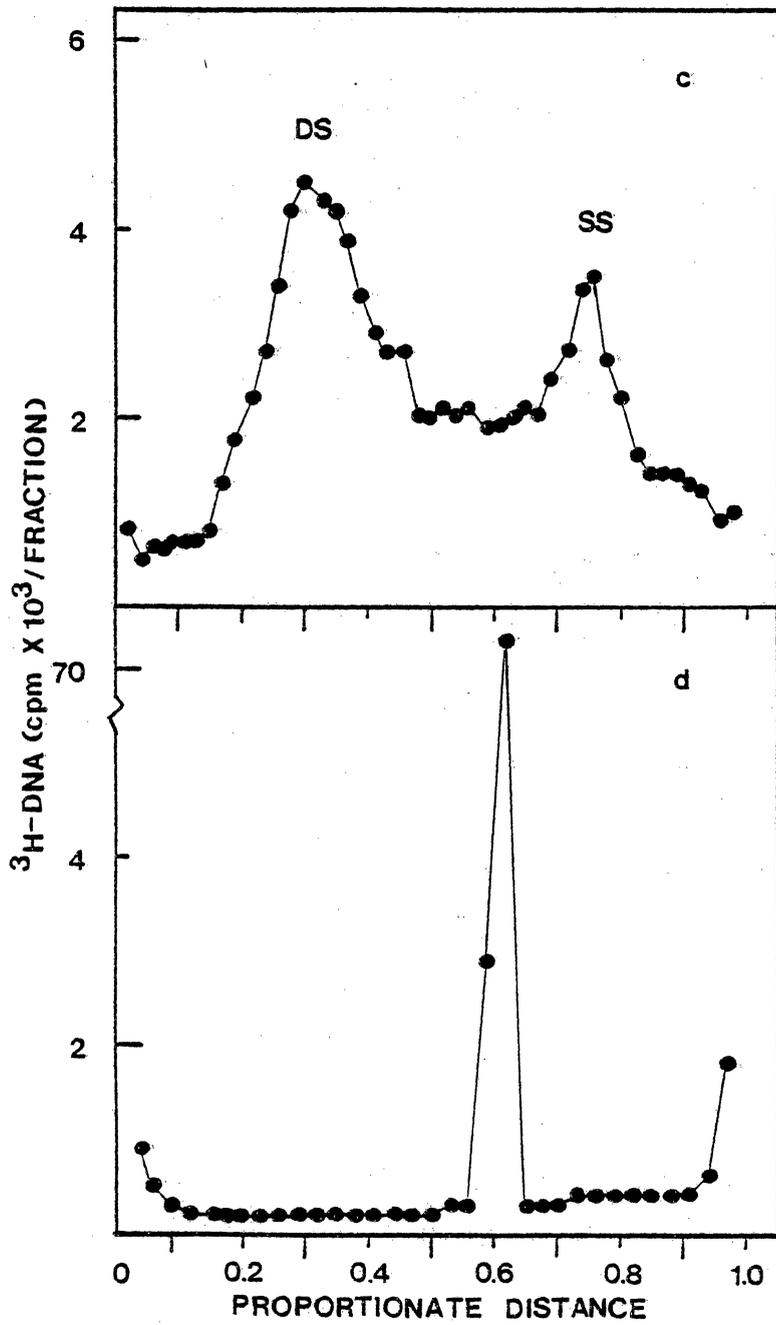


Figure 2. Continued. c) 2 hr pulse with  $^3\text{H}$ -TdR and a 4 hr chase with nonlabeled TdR. d)  $^3\text{H}$ -TdR labeled DNA from mock-infected cells.

that were chased with unlabeled TdR for 4 hours demonstrated similar results as those chased for 2 hours (Figure 2c). Again, there were two peaks of labeled DNA; a double-stranded peak and a single-stranded peak. There appears, however, to be a decrease in the amount of radioactivity in the double-stranded region with a concomitant increase in the single-stranded region. This suggests a precursor-product relationship between DNA species found within each peak when compared to the 2 hour chase period. The double-stranded regions from the three time periods are much broader than the single-stranded regions suggesting some heterogeneity within the double-stranded region. The shoulder on the double-stranded peak from the 2 hour chase could be due to the presence of RI which would be expected to sediment to an intermediate position between double and single-stranded DNA. This shoulder was not, however, clearly observed in every preparation.

Sedimentation of mock-infected cells under identical conditions failed to demonstrate any peaks of radiolabeled low molecular weight DNA. Occasionally, as seen in Figure 2d, a spike of radioactivity considerably higher in comparison to the peaks of the low molecular weight DNA seen for infected cells was observed. In conjunction with this observation was the presence of a viscous material adhering to the sides of the centrifuge tubes after fractionation. Both observations suggest that cellular DNA contamination could also be occurring during sedimentation of the BPV-infected cell samples through this type of gradient.

#### 4.1.1 Guanidine-HCl Method of Viral DNA Extraction from Isolated Nuclei

Even though several of the peak fractions of the double-stranded regions from the G-HCl-sucrose were pooled to be used for further experimentation, the total amount of material and total counts obtained was relatively low when one considers that at least 10,000-20,000 cpm are used, for example, on a single neutral sucrose gradient. In order to increase the yield of material, a procedure was employed to extract nuclei from infected cells as described in section 3.4.1.3. A total of  $3 \times 10^6$  nuclei could be layered onto the sucrose-G-HCl gradient without overloading the gradient as compared to only  $1.5 \times 10^6$  cells which could be used for the isolation of low molecular weight DNA in the original procedure.

The results of the sedimentation, by G-HCl method of nuclei from cells that had been pulse labeled with  $^3\text{H}$ -TdR and chased for 2 hours with unlabeled TdR were similar to that of the whole cell profile treated identically (Figure 3). Two peaks were obtained; one in the double-stranded region and one in the region corresponding to single-stranded DNA. As would be expected, approximately twice as much material were obtained from these nuclei preparations as from whole cell preparations. However, the problem of possible cellular DNA contamination was not eliminated as viscous material could still be detected in association with the walls of the centrifuge tubes. Nuclei from mock-infected cells yielded a sedimentation profile corresponding to only a basal level of incorporation of  $^3\text{H}$ -TdR into DNA and void of any peaks of low molecular weight DNA (data not shown).

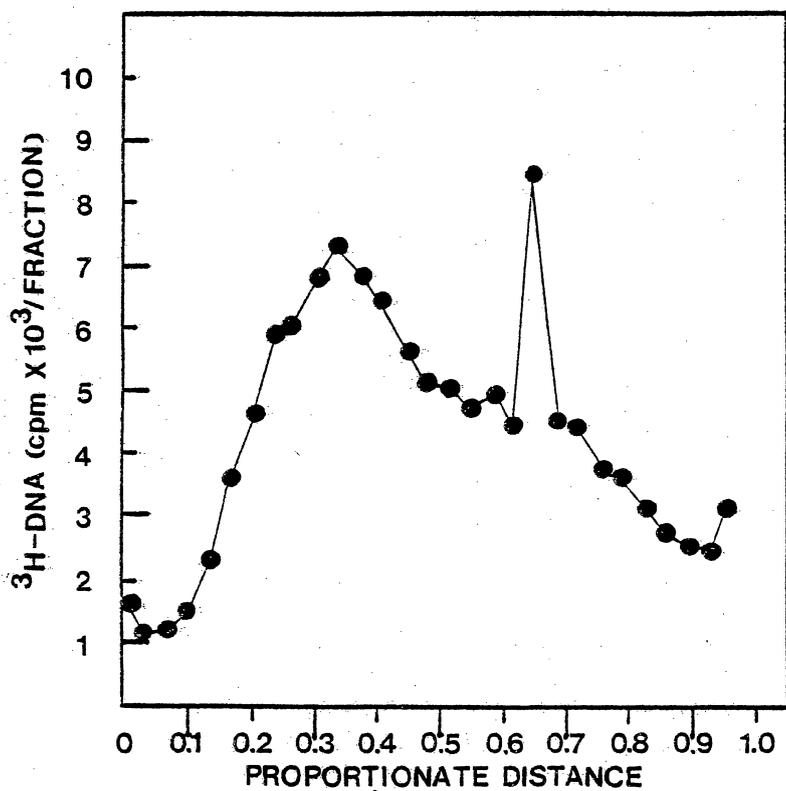


Figure 3. G-HCl-sucrose gradient profile of BPV RF DNA isolated from infected nuclei.

#### 4.1.2 Physico-Chemical Characteristics of DNA Isolated by the Guanidine-Hydrochloride Method

##### 4.1.2.1 Velocity Sedimentation in Sucrose

It was of interest to determine the sedimentation characteristics of the double-stranded RF DNA isolated from BPV-infected cells in alkaline and neutral sucrose gradients. Information from the data obtained would be indicative of the conformation and molecular weight of this DNA (this will be discussed in more detail in Section 4.5).

Sedimentation of the double-stranded RF region in both alkaline and neutral sucrose gradients in SW50.1 rotor as described in Section 3.7.1 and 3.7.2 failed to demonstrate any distinct peak of radiolabeled DNA. The DNA appeared to be distributed throughout the gradient. This observation was consistent for double-stranded RF DNA isolated either from whole cells or nuclei by the G-HCl method.

In an effort to further purify the RF DNA, it was subjected to hydroxyapatite (HAP) column chromatography which separates DNA on the basis of secondary structure (Benardi, 1971). The RF DNA was found to elute from the column at a NaP concentration of 0.232 M which is the molarity at which double-stranded DNA elutes from HAP (see Figure 4). This confirmed the presence of secondary structure in the molecules within the RF region of the G-HCl gradients.

When this DNA was dialyzed and sedimented on alkaline sucrose gradient with  $^{32}\text{P}$ -BPV single-stranded virion DNA as a marker, the gradient profile seen in Figure 5 was obtained. A portion of the RF DNA appeared to cosediment with the single-stranded BPV marker DNA, however, the peak had a leading shoulder indicating some heterogeneity present

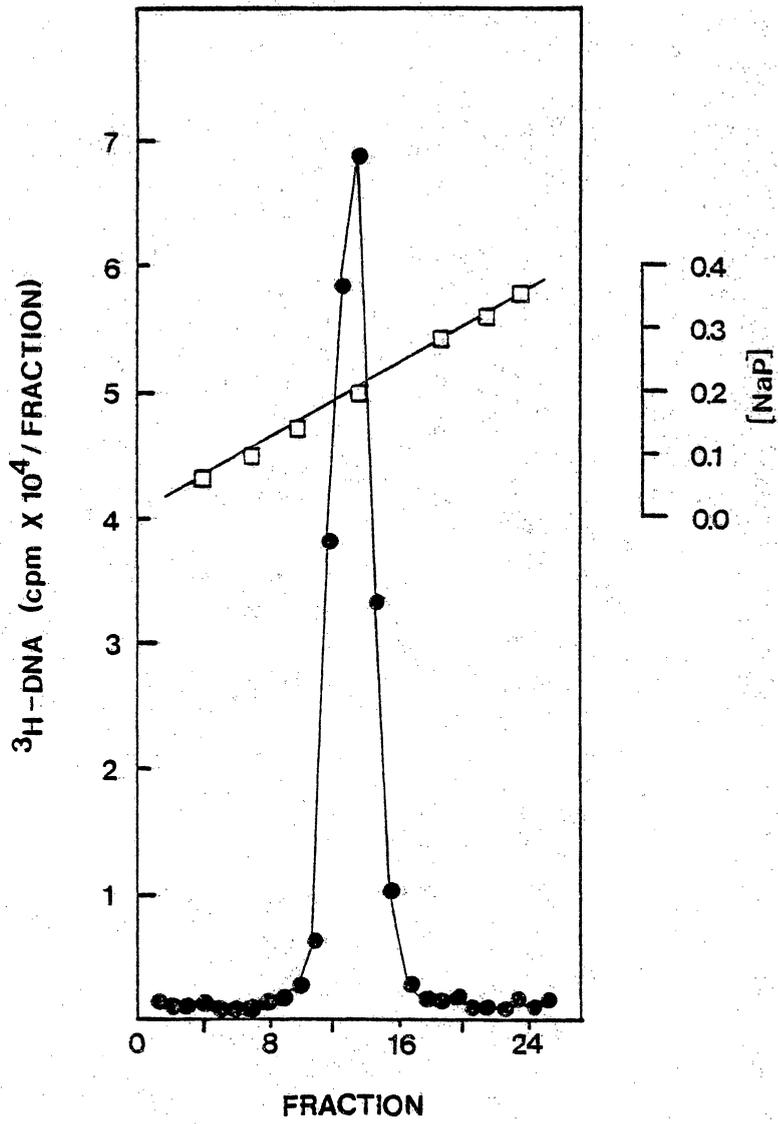


Figure 4. HAP chromatography elution pattern of BPV RF DNA.

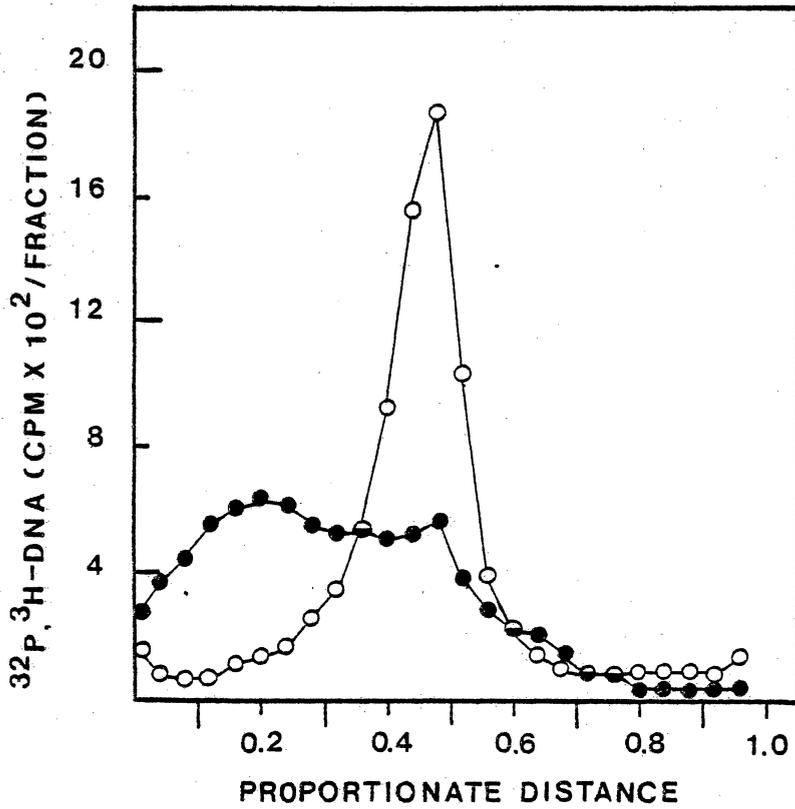


Figure 5. Alkaline sucrose gradient profile of BPV RF DNA (●—●) BPV RF <sup>3</sup>H-DNA (○—○). Peak I BPV virion <sup>32</sup>P-DNA used as a marker.

within the DNA even when further purified by HAP.

#### 4.1.2.2 Electron Microscopy

In order to determine the length of the molecules isolated by the G-HCl method and to help in the clarification of the results obtained from the velocity sedimentation studies, the double-stranded RF DNA was purified by HAP chromatography and spread for electron microscopy by the Kleinschmidt aqueous technique (Kleinschmidt, 1968). This procedure allows for the visualization of double-stranded nucleic acid molecules. Figure 6 is a representative micrograph of the double-stranded RF DNA and illustrates the variety of lengths present in the DNA sample. This is consistent with the data from the sucrose gradients previously described. Length measurements of this DNA were not performed due to heterogeneity in the sample.

#### 4.2 Hirt Extraction of Low Molecular Weight DNA

The procedure developed by Hirt in 1967 has been used extensively to isolate low molecular weight DNA from parvovirus-infected cells (Siegl & Gautschi, 1976; Tattersall, *et. al.*, 1973; Gunter & May, 1976). In this method, the cells are gently lysed and the cellular DNA is precipitated in the cold by the addition of SDS and NaCl. The cellular DNA is pelleted by low speed centrifugation while low molecular weight DNA remains in the supernatant. This procedure however, does not release encapsidated virion DNA as does the G-HCl method (Lavelle and Li, 1977). This is due possibly to the fact that the progeny DNA is rapidly encapsidated upon synthesis and is precipitated with the Hirt pellet.

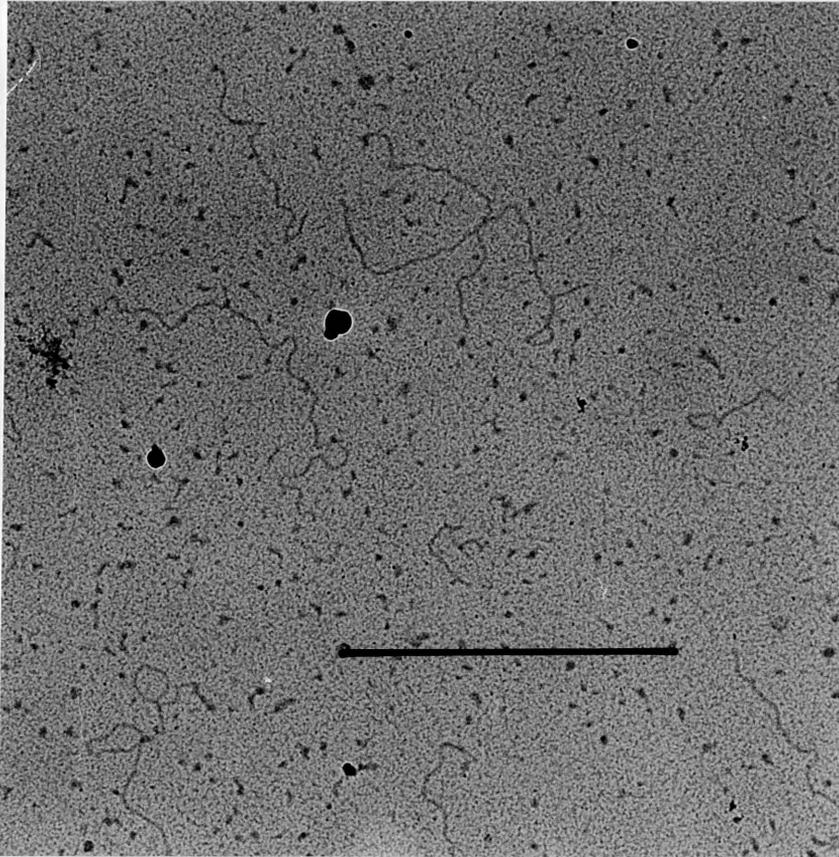


Figure 6. Electron micrograph of BPV RF DNA from G-HCl-sucrose gradients. Bar represents 1  $\mu\text{m}$ .

Because of the inconclusive results from physico-chemical studies with the RF DNA obtained by the G-HCl method and the low yield of material even when nuclei are employed, it was believed that the Hirt procedure would provide a reasonable alternative for the isolation of the replicative structures from infected cells, even though the single stranded progeny DNA would not be released. The possibility of cellular contamination, however, would not be eliminated since some breakage in cellular DNA can occur during the various manipulations involved.

#### 4.2.1 HAP Chromatography

The Hirt supernatant DNA that had been isolated from BPV-infected BFS cells and deproteinized by two phenol extractions was subjected to HAP chromatography which, as stated previously, will separate DNA on the basis of secondary structure (Bernardi, 1971). This procedure should also separate RF molecules from the RI molecules since partially single stranded molecules elute in an intermediate position between double and single-stranded DNA (Wilson and Thomas, 1973). Figure 7 is a representative of HAP elution pattern for Hirt DNA. There is a single sharp peak of radiolabeled DNA which elutes at a sodium phosphate (NaP) concentration of 0.198 M. This concentration falls within the range of elution molarities corresponding to double-stranded DNA as determined by chromatography of known DNA samples. It is interesting to note that a separate peak for RI's was not observed. This, however, does not negate the possibility that RI's may be present in this preparation.

When the peak fraction DNA was spread for electron microscopy by the Inman technique, a variety of lengths, both long and short, were

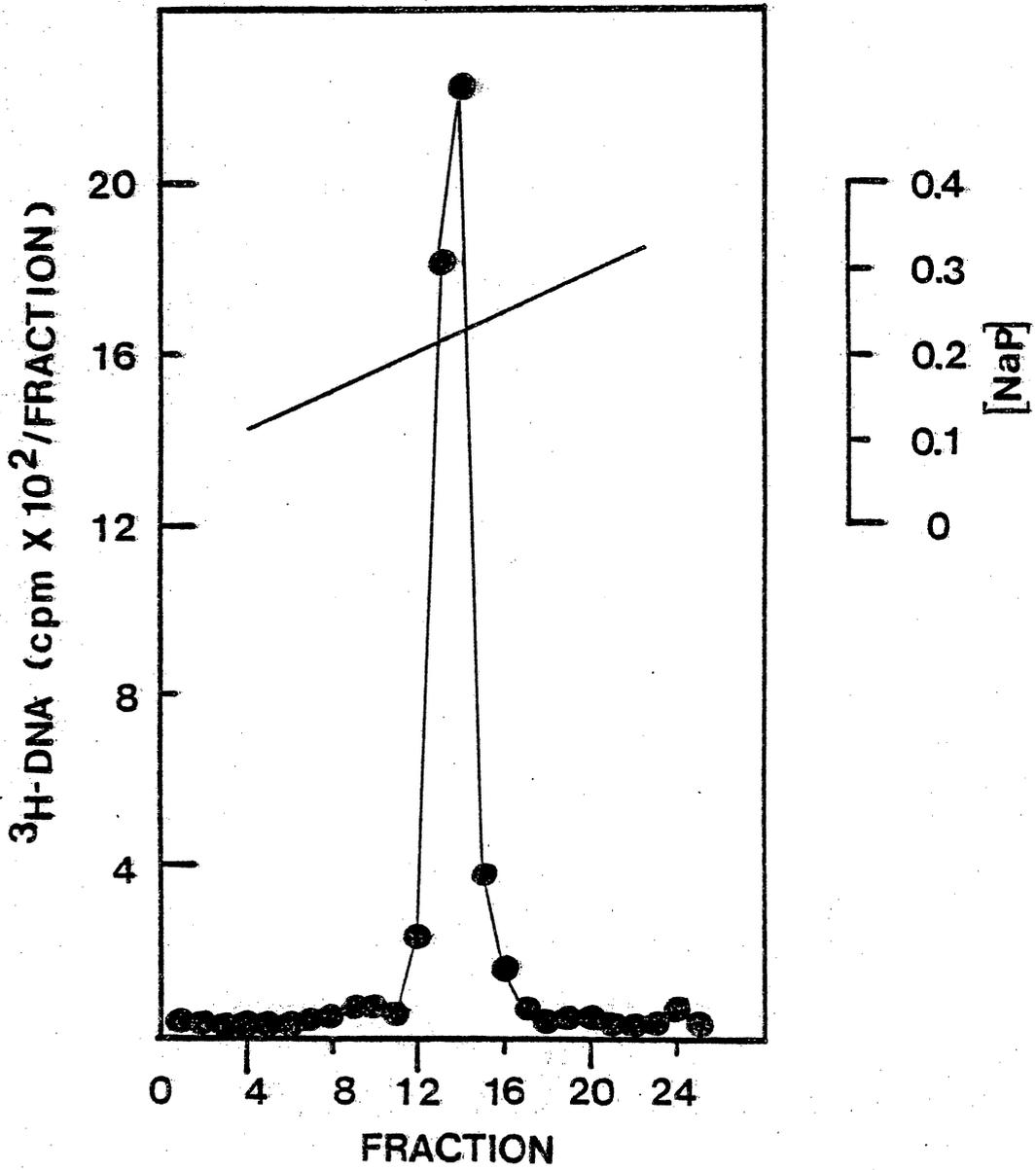


Figure 7. HAP chromatography elution pattern of BPV RF DNA present in Hirt supernatants.

observed. An interesting micrograph is shown in Figure 8. The most striking aspect of this micrograph is the presence of the extremely long piece of DNA in a pattern which resembles a fingerprint. This is most probably cellular DNA within the sample and therefore may well be a "telltale fingerprint" of contamination. A majority of the micrographs, however, did not show contamination to such dramatic extent, since some of the molecules were also very short (data not shown).

#### 4.2.2 Preparative Neutral Sucrose Gradients

Because of the variety of lengths present in samples purified only by HAP chromatography, and the strong evidence for some cellular contamination, it was felt that further purification of the Hirt DNA was needed before characterization studies could be performed. Sedimentation on neutral sucrose gradients was chosen to accomplish this goal due to its ability to separate DNA on the basis of size, shape, and density.

Figures 9 and 10 are gradient profiles obtained from two separate Hirt preparations (Prep. I and Prep. II respectively). A major peak of radiolabeled double-stranded RF DNA can be observed in the middle of both gradients with smaller peaks of DNA sedimenting slightly faster than the major peaks also observed. Broad minor bands can also be observed sedimenting somewhat slower than the major peak. The band is more distinct in Figure 9 than Figure 10 and may represent a variability in the cellular DNA contamination present in Hirt DNA samples. This, however, would not account for the extremely long pieces observed previously since the molecules of this minor band are in the top portion of

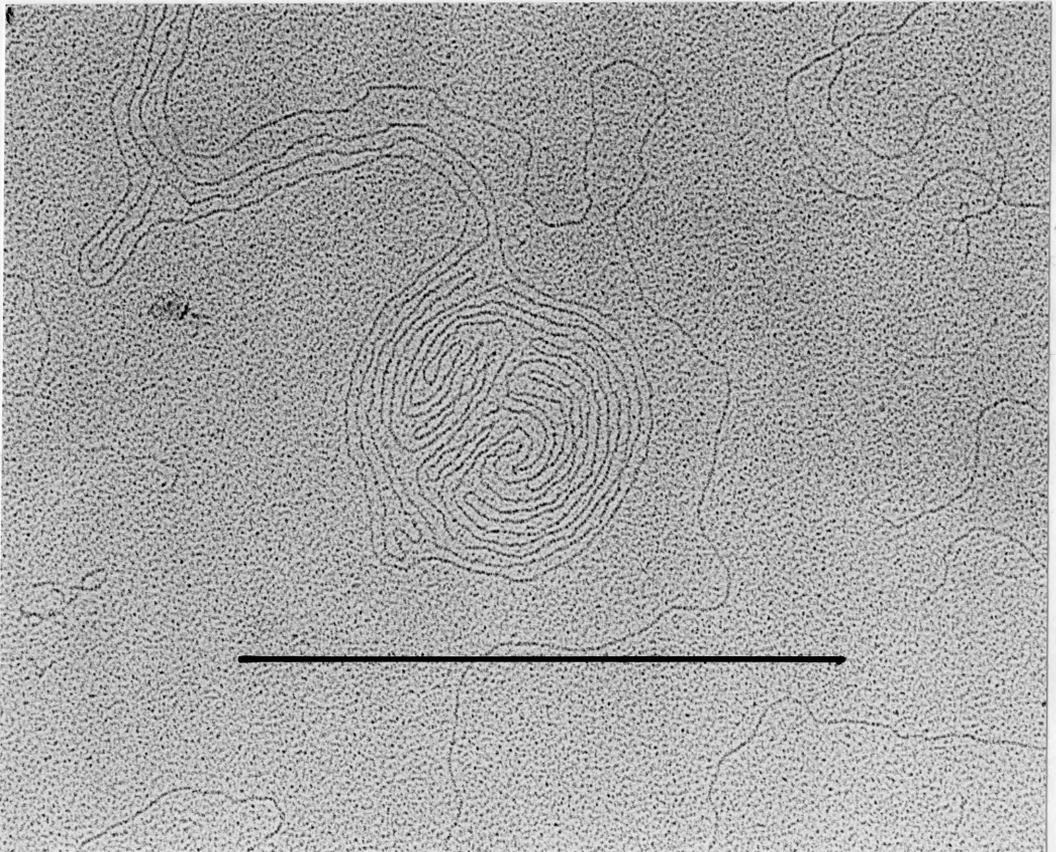


Figure 8. Electron micrograph of BPV RF DNA from HAP column. Bar represents 1  $\mu\text{m}$ .

05

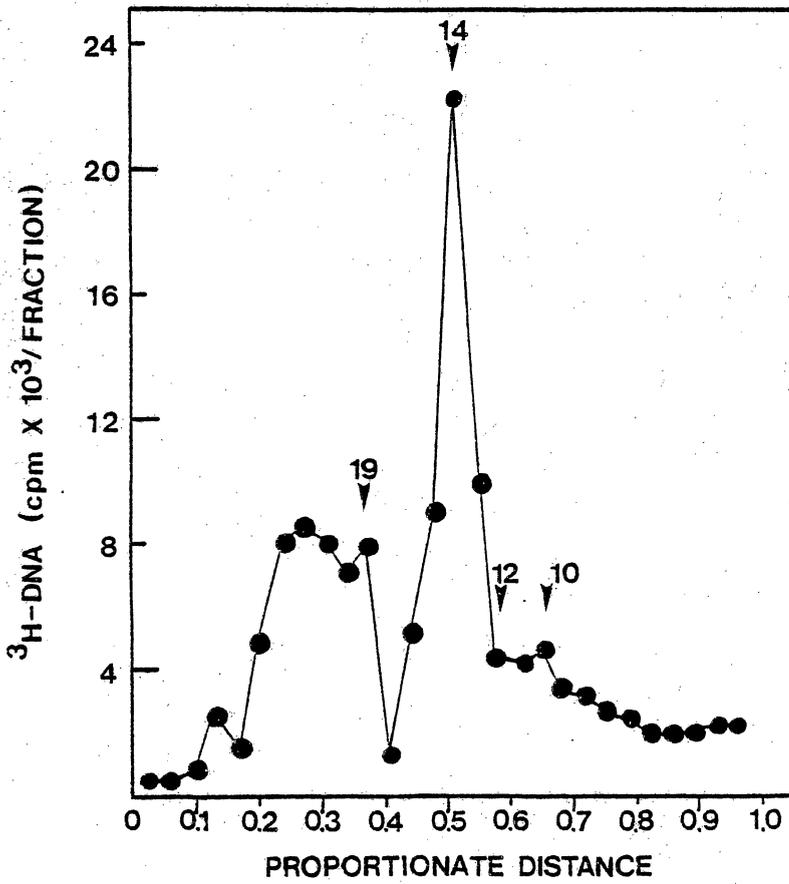


Figure 9. Preparative neutral sucrose gradient of Hirt supernatant DNA: Prep I.

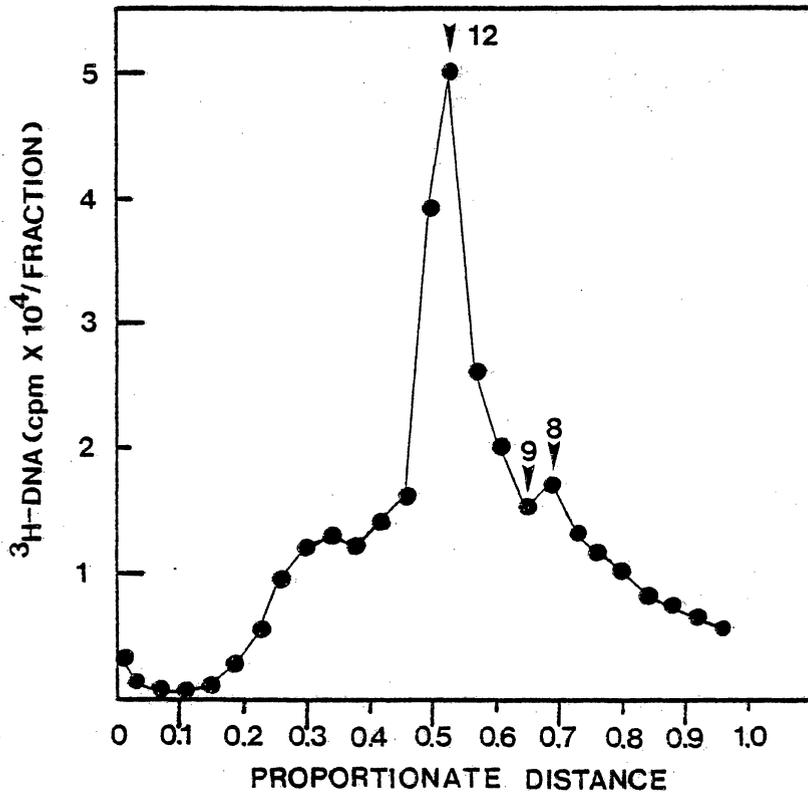


Figure 10. Preparative neutral sucrose gradient of Hirt supernatant DNA: Prep II.

the gradient and should be short. Long molecules such as those in Figure 8 would be expected to sediment much faster and band more toward the bottom of the gradient. This broad band is, however, consistent with the very short molecules that were observed in micrographs of Hirt preparations subjected only to HAP chromatography. The possibility that this broad band is viral cannot be disregarded.

### 4.3 Electron Microscopy

To determine the contour length of the Hirt RF DNA purified by neutral sucrose gradient sedimentation, fractions 10, 12, and 14 from Prep I illustrated in Figure 9 and fractions 8, 9, 12 from Prep II as seen in Figure 10 were spread for electron microscopy by the Inman technique. This spreading procedure allows for the visualization of double- and single-stranded DNA (Inman & Schnöss, 1970). Therefore, any RI's present should be distinguishable as branched molecules with the single strands appearing slightly thinner than the duplex regions.

#### 4.3.1 Contour Lengths

Figure 11 is a representative micrograph of DNA from Fraction 14 of Prep I. All the molecules visualized from this Prep I fraction were linear. Branched molecules were not observed. Figure 12 is representative of fraction 12 from Prep II. Again, the molecules from the main peak of RF DNA were linear. Several branched molecules were observed from this fraction and a representative can be seen in Figure 13.

Representative micrographs of molecules from fraction 12 from Prep I and fraction 8 from Prep II are illustrated in Figures 14 and 15,

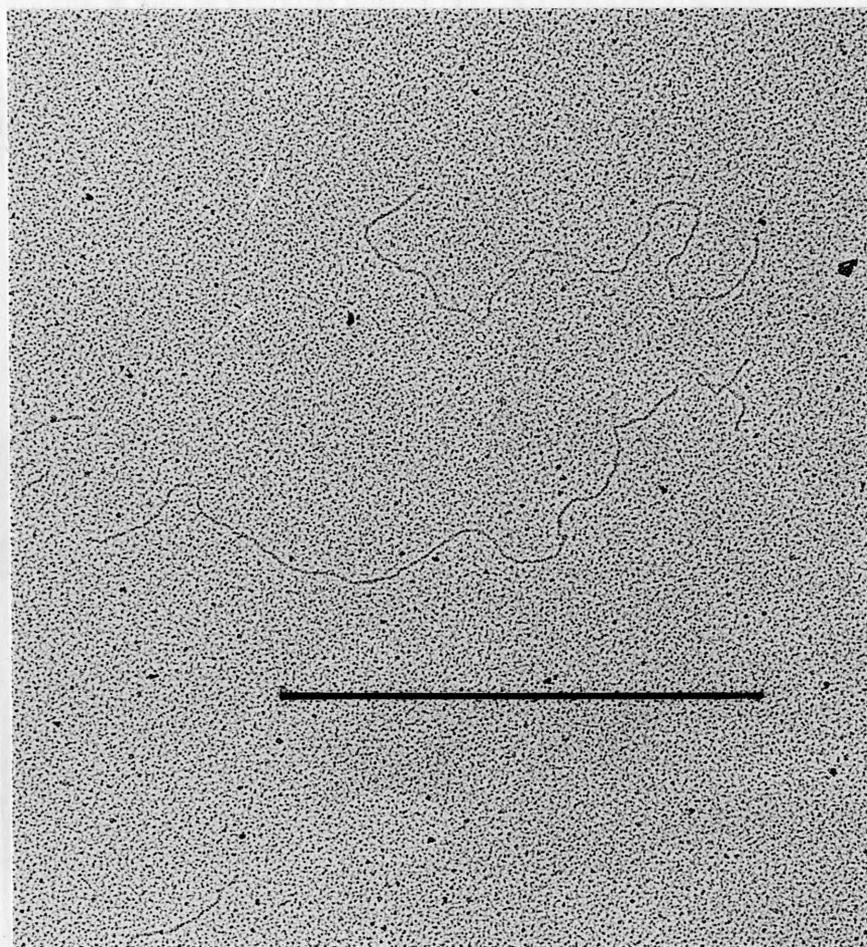


Figure 11. Electron micrograph of BPV RF DNA from Prep I, fraction 14.  
Bar represents 1  $\mu\text{m}$ .

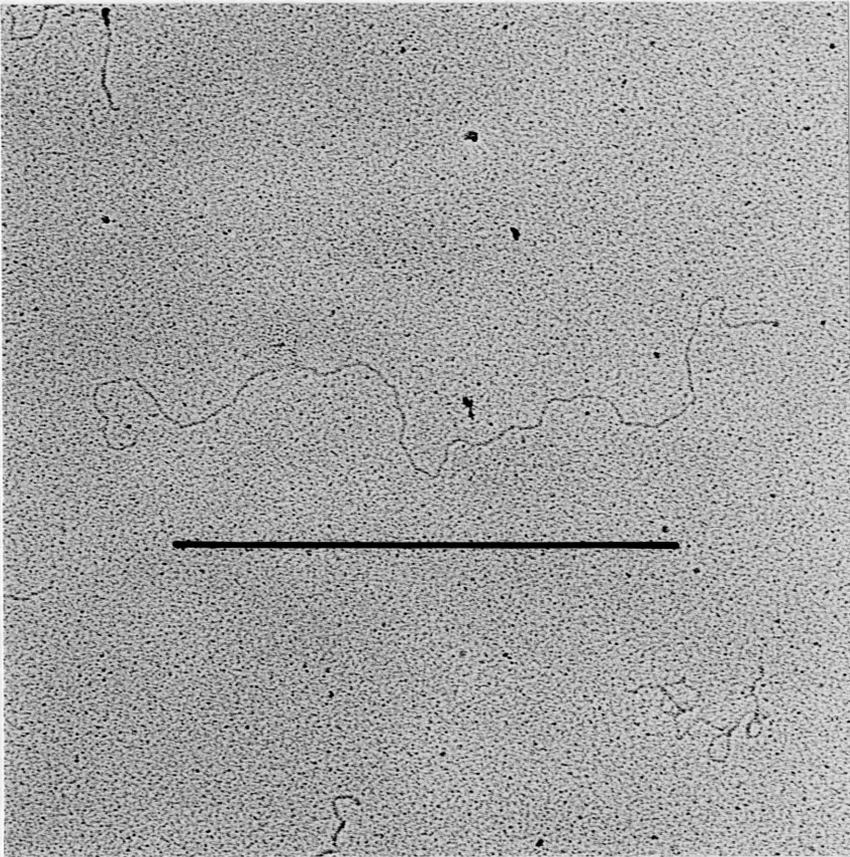


Figure 12. Electron micrograph of BPV RF DNA from Prep II, fraction 12.  
Bar represents 1  $\mu\text{m}$ .

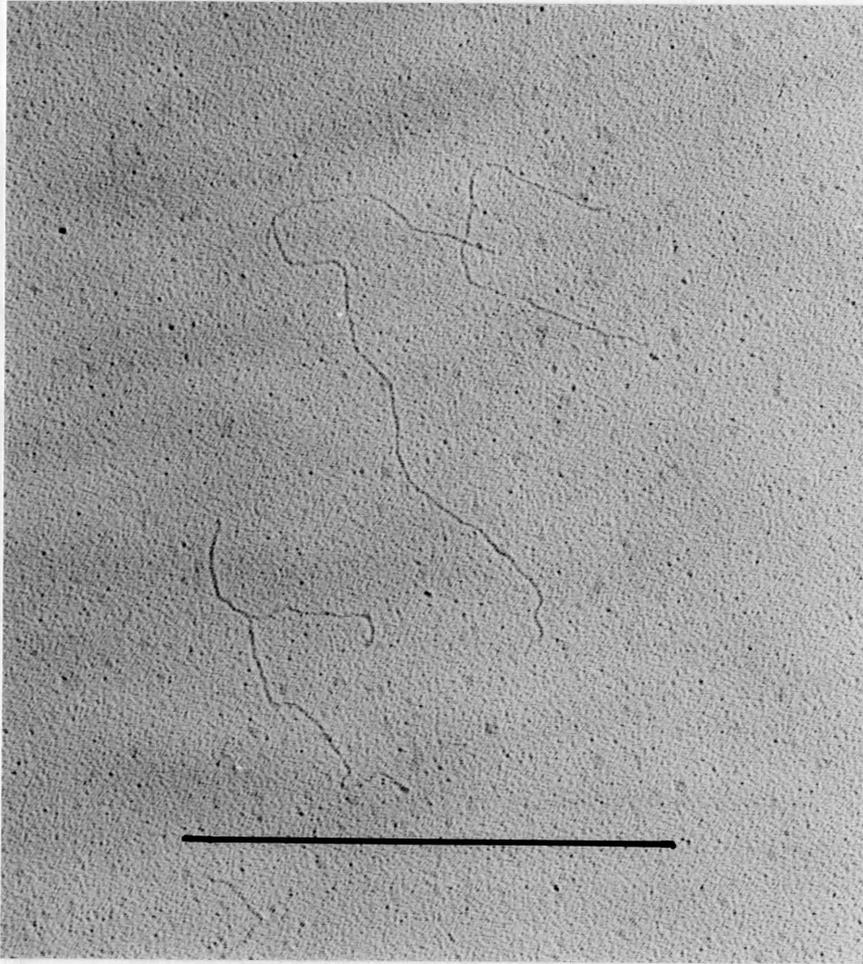


Figure 13. Electron micrograph showing branched DNA molecules present in Prep II, fraction 12. Bar represents 1  $\mu\text{m}$ .

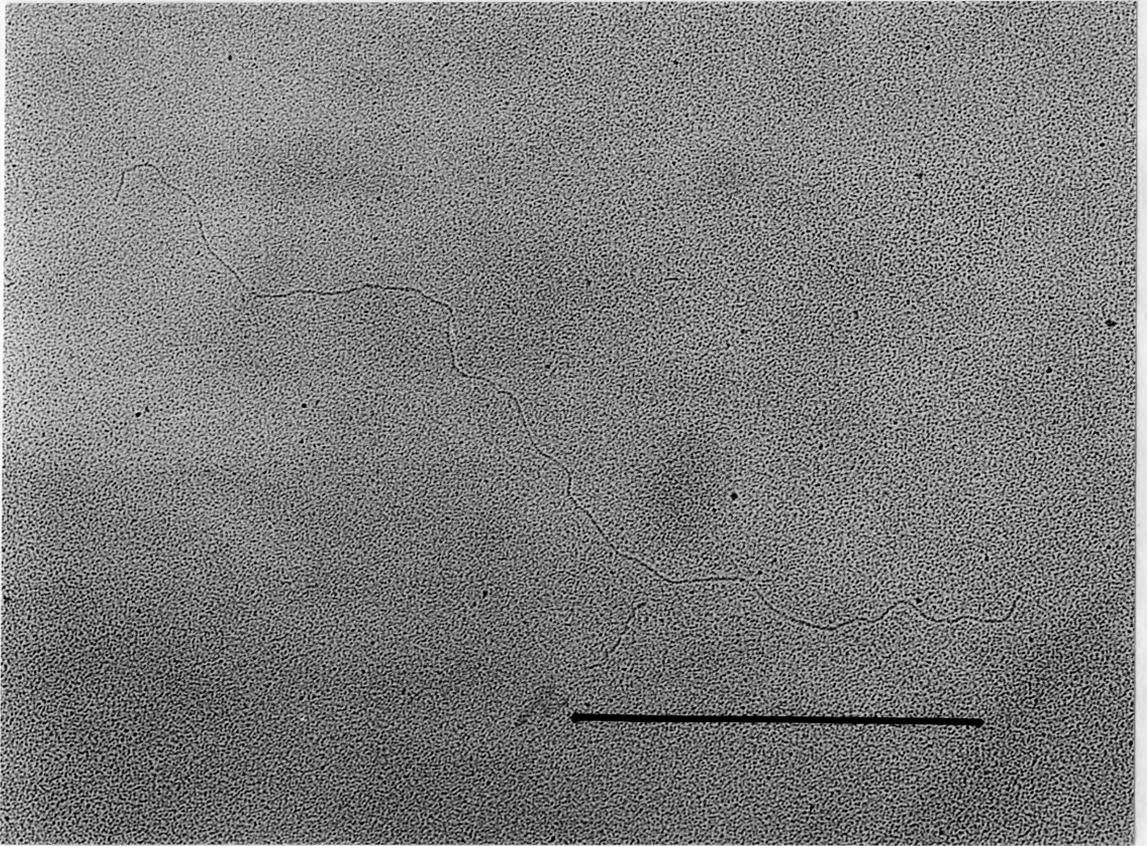


Figure 14. Electron micrograph of BPV RF DNA from Prep I, fraction 12.  
Bar represents 1  $\mu\text{m}$ .

MS

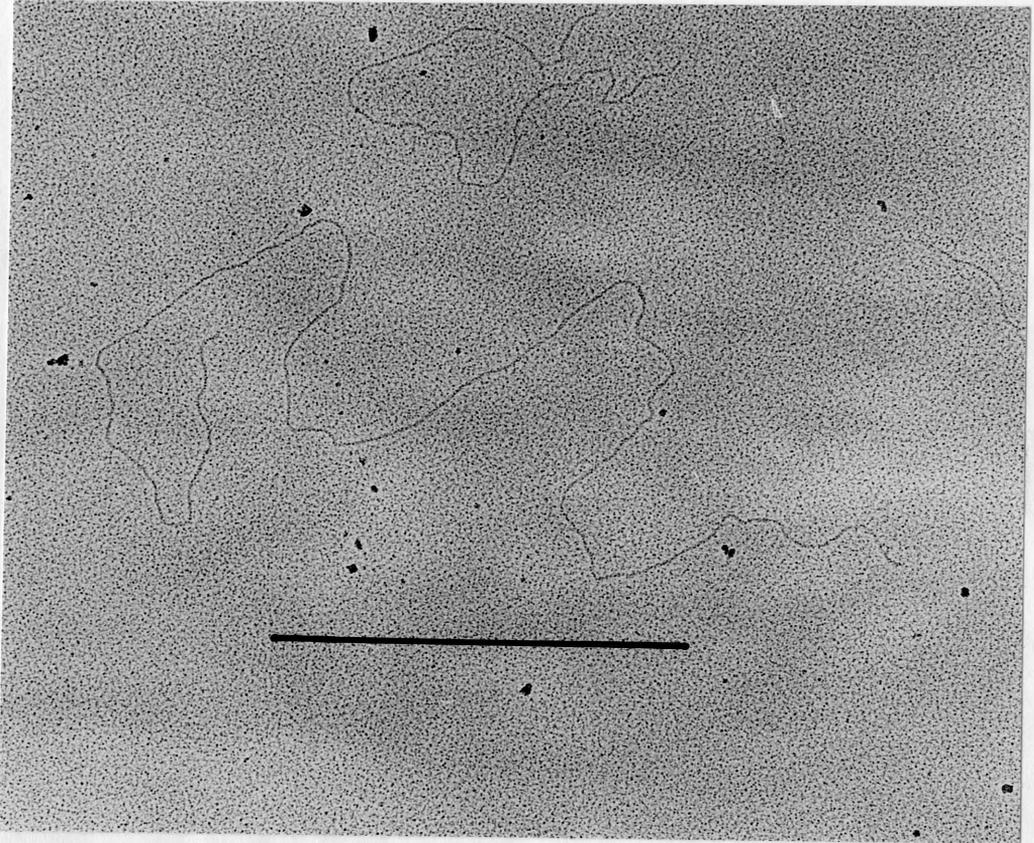


Figure 15. Electron micrograph of BPV RF DNA from Prep II, fraction 8.  
Bar represents 1  $\mu\text{m}$ .

respectively. DNA from these fractions were also linear and branched molecules were not observed.

The lengths were determined as described in Section 3.6.4. A histogram of the lengths obtained from fraction 14, Prep I is seen in Figure 16. The lengths show a normal distribution with a single population of molecules present. A histogram of the lengths from fraction 12, Prep II is shown in Figure 17. Again, the lengths show a normal distribution with the curve shifted slightly toward the longer lengths.

Histograms of lengths of molecules from Fraction 12, Prep I and Fraction 8, Prep II are seen in Figure 18 and Figure 19 respectively. There appears to be two possible populations of molecules present in Fraction 12, Prep I. The histogram of Fraction 8, Prep II could also suggest the existence of two populations, however, the case for this is not clear-cut. The histogram of Fraction 10, Prep I is similar to that of Fraction 12, Prep I in that there appears to be two size populations (data now shown).

A summary of the mean lengths obtained for all fractions is found in Table 2. The lengths of the RF DNA from the major peak fractions (Fraction 14, Prep I, and Fraction 12, Prep II) are 1.69  $\mu\text{m}$  and 1.82  $\mu\text{m}$  respectively. A rather large length discrepancy appears to exist if these molecules represent unit length RF DNA. These molecules should be the same size since they sediment to similar positions in neutral sucrose gradients. If, however, one looks at the lengths obtained for  $\Phi\text{X174}$  RF II DNA, which served as the control for the spreading procedure, a length discrepancy is also evident. The length of 1.63  $\mu\text{m}$

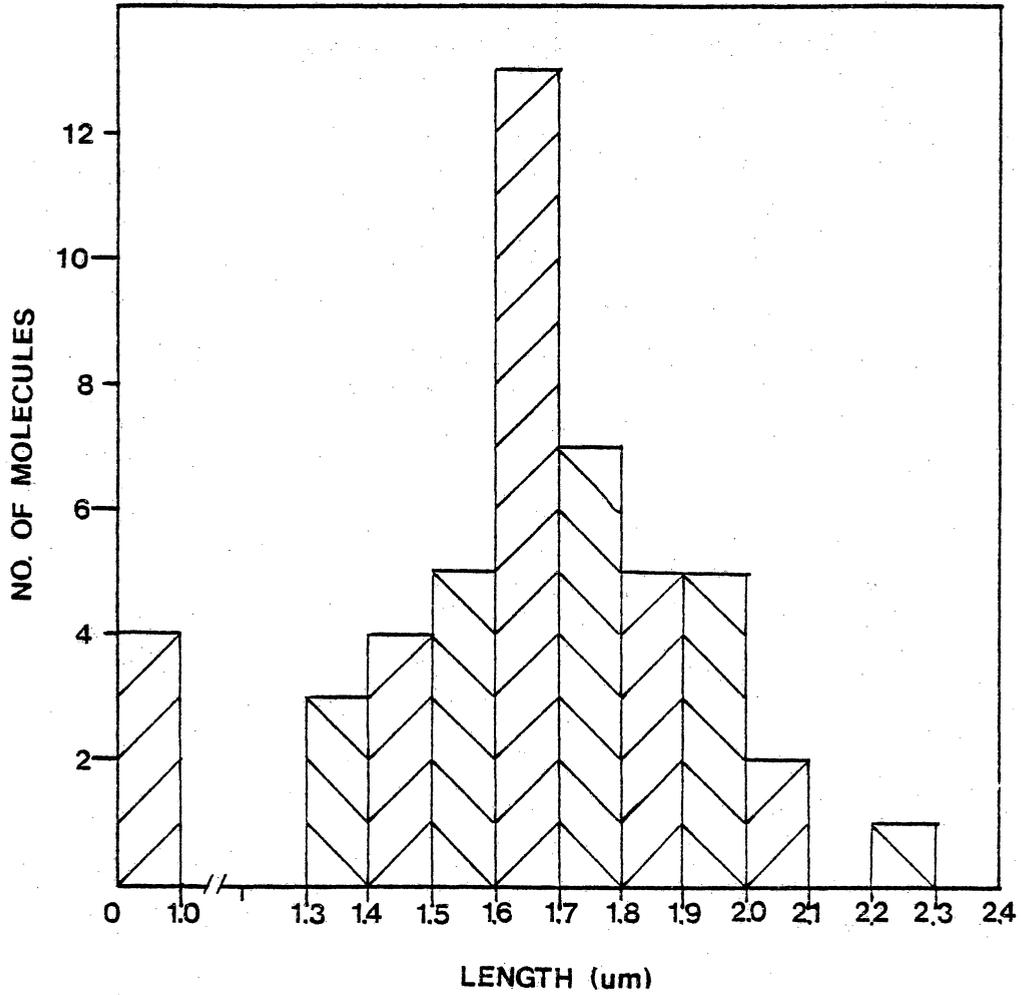


Figure 16. Histogram of length distribution for Prep I, fraction 14.

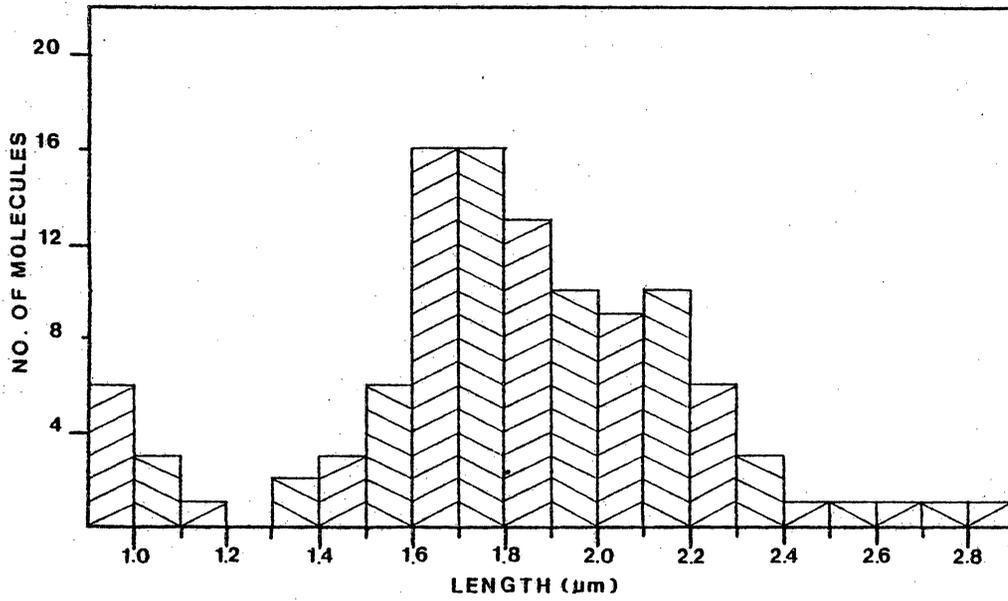


Figure 17. Histogram of length distribution for Prep II, fraction 12.

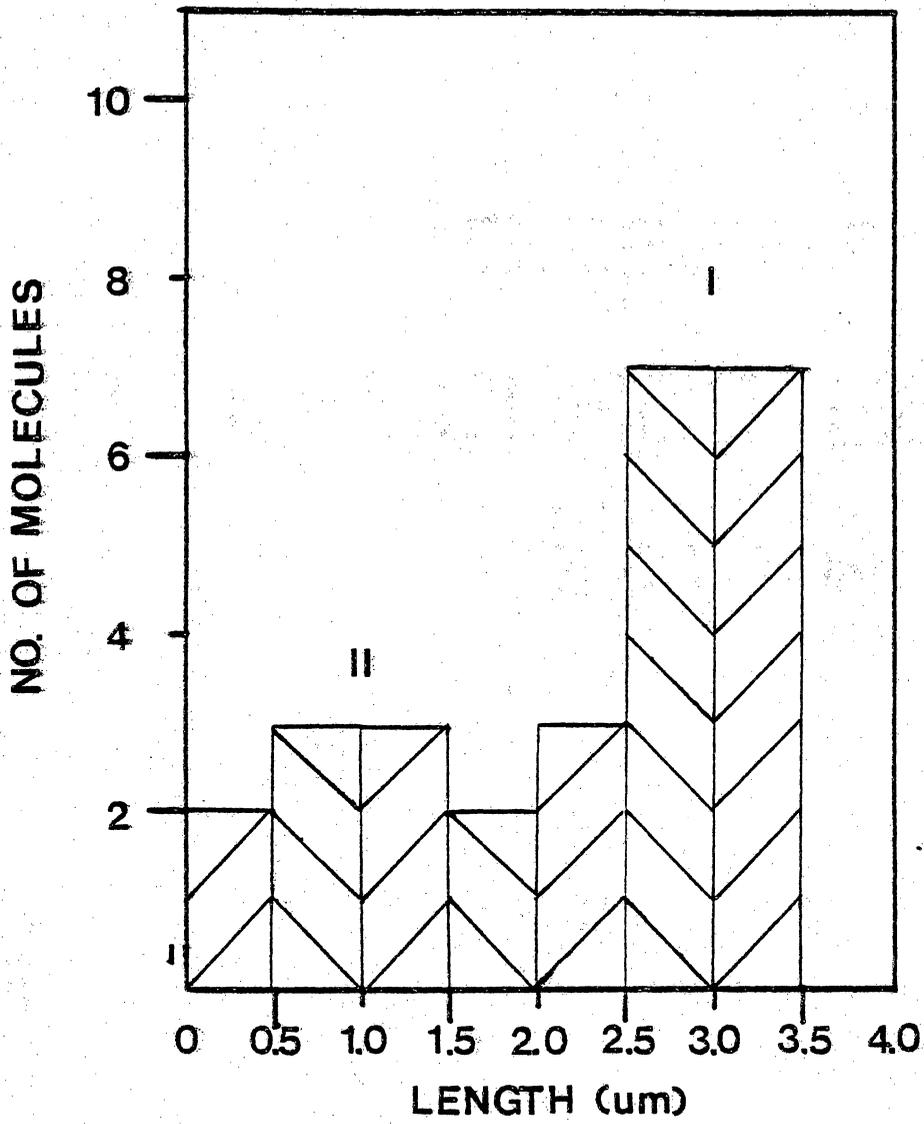


Figure 18. Histogram of length distribution for Prep I, fraction 12.

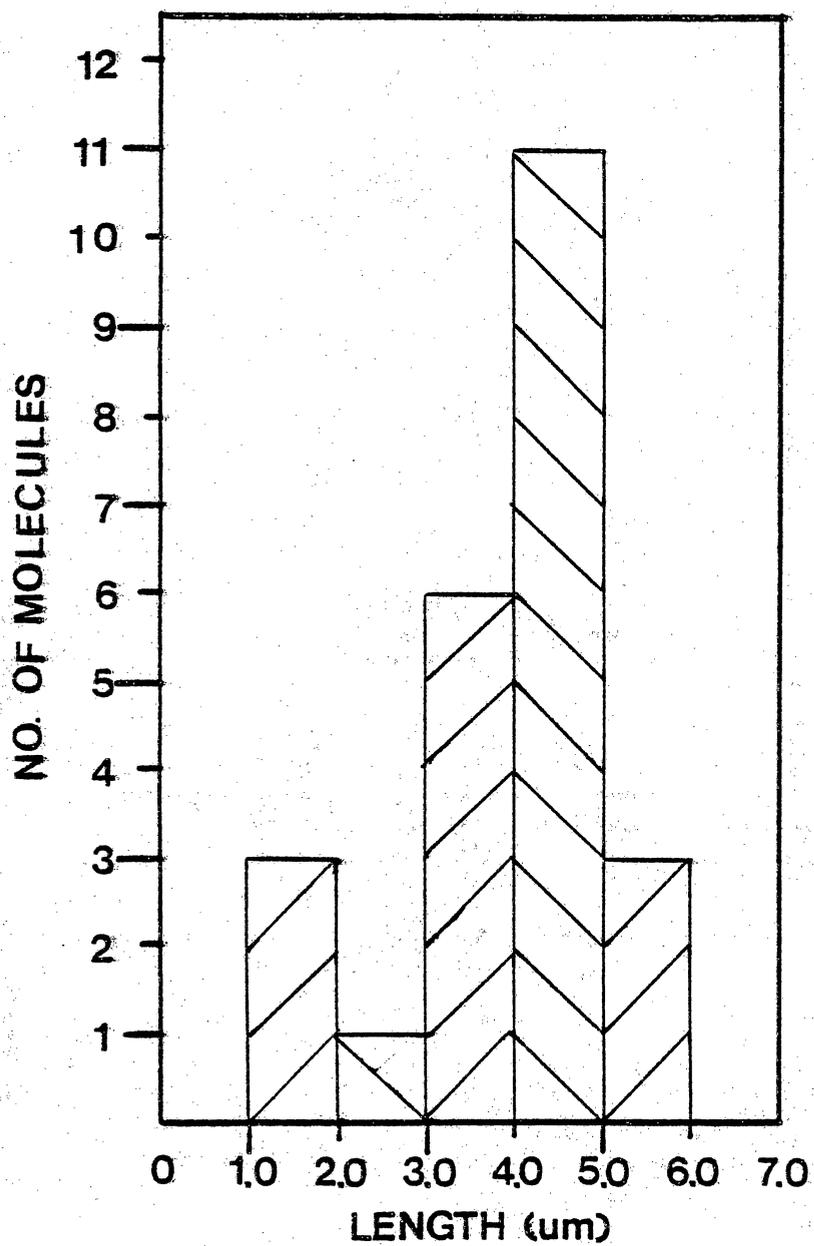


Figure 19. Histogram of length distribution for Prep II, fraction 8.

Table 2. Length Distribution of BPV RF DNA  
Purified on Neutral Sucrose Gradients

| Preparation | Fraction No.      | Population I                          |     | Population II            |    |
|-------------|-------------------|---------------------------------------|-----|--------------------------|----|
|             |                   | Length ( $\mu\text{m}$ ) <sup>a</sup> | n   | Length ( $\mu\text{m}$ ) | n  |
| I           | 10                | 3.99 $\pm$ .07                        | 15  | 1.83 $\pm$ .23           | 6  |
|             | 12                | 2.99 $\pm$ .06                        | 14  | 1.01 $\pm$ .14           | 10 |
|             | 14                | 1.69 $\pm$ .03                        | 44  | --                       |    |
|             | $\Phi\text{X174}$ | 1.63 $\pm$ .01                        | 24  | --                       |    |
| II          | 8                 | 4.02 $\pm$ .24                        | 25  | --                       |    |
|             | 9                 | N.D. <sup>b</sup>                     |     | N.D.                     |    |
|             | 12                | 1.82 $\pm$ 0.039                      | 110 | --                       |    |
|             | $\Phi\text{X174}$ | 1.74 $\pm$ .01                        | 43  | --                       |    |

<sup>a</sup>Mean length  $\pm$  S.E.

<sup>b</sup>Not determined.

measured for  $\Phi$ X174 spread at the same time so those samples in Prep I corresponds to the value given in the literature for this circular molecule (Kay, 1976). Assuming that this 1.63  $\mu\text{m}$  value is the standard or "correct" value and considering that the same lot of  $\Phi$ X174 molecules was used for Prep I and Prep II,  $\Phi$ X174 molecules in Prep II appear to have been elongated by 7% to give a length of 1.74  $\mu\text{m}$ . If one assumes that this length elongation is the same for DNA samples spread at the same time and under the same conditions, it seems logical that the Prep II RF DNA should also be elongated to the same extent. Correcting the 1.82  $\mu\text{m}$  measurement for 7% stretching, yields a value of 1.69  $\mu\text{m}$  which corresponds to the length of DNA of Fraction 14, Prep I.

The lengths of molecules from Fraction 12 and 10 of Prep I approach calculated values for dimer and trimer RF DNA respectively, based upon 1.69  $\mu\text{m}$  length. A similar observation can be made with RF DNA from Fraction 8 of Prep II in that the length approaches the calculated value for dimer DNA based upon the 1.82  $\mu\text{m}$  measurement.

Measurements could not be made upon Fraction 9, Prep II because the DNA failed to spread. The reason for this is unclear. BPV virion single-stranded DNA also failed to spread even though a number experimental parameters were changed systematically. These included changing spreading procedures, use of parlodion, formvar and carbon-filmed grids, use of glass to store grids instead of plastic to prevent the build up of a static charge, and glow discharged or U.V. irradiated grids.

Another method for calculating the length of DNA is the determination of the mode length or the average length of those molecules occur-

ring at the highest frequency. A comparison of the mode and mean lengths for BPV RF DNA is shown in Table 3. The mode length of the RF DNA from fraction 14, Prep I is 1.66  $\mu\text{m}$  and the mode length for fraction 12, Prep II is 1.70  $\mu\text{m}$ . The mode lengths for the molecules are slightly lower than the mean lengths. The mode length of  $\Phi\text{X174}$ , Prep I appears slightly larger than the mean length for the same molecules. The mode and mean lengths for  $\Phi\text{X174}$ , Prep II are the same.

#### 4.3.2 Molecular Weight

Electron microscopy can be used to estimate the molecules using the equation  $M = M'L$  where  $M$  is the molecular weight,  $M'$  as the molar linear density and  $L$  is the contour length (Lang, 1970). The value for  $M'$  is determined using a DNA of a known molecular weight determined from independent experimental procedures. For  $\Phi\text{X174}$  RF II DNA, the molecular weight is  $3.6 \times 10^6$  d with contour length of 1.63  $\mu\text{m}$  (Kay, 1976),  $M'$  is therefore,  $2.2 \times 10^6$  d/ $\mu\text{m}$ . If one assumes a similar base composition, substitution of  $M'$  and the contour length 1.69  $\mu\text{m}$  for BPV RF DNA into the equation yields a molecular weight of  $3.7 \times 10^6$  d.

Molecular weight can also be determined using the mode length values. For  $\Phi\text{X174}$  DNA with a molecular weight of  $3.6 \times 10^6$  d (Kay, 1976) and a mode length of 1.65  $\mu\text{m}$ ,  $M'$  is  $2.18 \times 10^6$  d/ $\mu\text{m}$ . Substitution of  $M'$  and the mode length of 1.66  $\mu\text{m}$  for BPV RF DNA yields a molecular weight of  $3.6 \times 10^6$  d.

#### 4.3.3 Estimation of Errors

Pincushion distortion in lens systems results from spherical aber-

Table 3. Comparison of Mode Length to Mean Length of RF DNA

| Preparation | Fraction No. | Mode Length<br>( $\mu\text{m}$ ) <sup>a</sup> | Mean Length<br>( $\mu\text{m}$ ) <sup>a</sup> |
|-------------|--------------|---|---|
| I           | 14           | 1.66 $\pm$ .01                                | 1.69 $\pm$ .03                                |
|             | $\Phi$ X174  | 1.65 $\pm$ .01                                | 1.63 $\pm$ .01                                |
| II          | 12           | 1.70 $\pm$ .01                                | 1.82 $\pm$ .039                               |
|             | $\Phi$ X174  | 1.74 $\pm$ .004                               | 1.74 $\pm$ .01                                |

<sup>a</sup>Length  $\pm$  S.E.

rations and can never be completely eliminated. When these aberrations occur, the peripheral rays of light or the electron beam are distorted in relationship to those passing through the center of the lens. This results in a magnification difference between the periphery and the center of the field. Distortion of this type in a well-maintained electron microscope at higher magnifications (above 5000x) should, however, be negligible (Meek, 1976). Pincushion distortion at 20,000x in the Jeol 100C electron microscope could not be detected by the method described in Section 3.6.3. Pincushion distortion in the Omega enlarger was 1.9%.

Another source of error is the measurement of the tracings of the nucleic acid molecules. This error was 0.5% for measurements performed with the Kelsh 600-A digitizer.

The accuracy of the grating replica is another source of error. A replica containing 54,864 lines/inch (Fullam) was used in these studies. The grating lines could not be resolved by light microscopy. Therefore, a value of 1.3% as determined by Lang (1970) for the same type of grating replica was used.

#### 4.4 Isopycnic Sedimentation

Determination of bouyant density of nucleic acids has become a standard characterization procedure. The gradient purified Hirt RF DNA has a bouyant density in neutral CsCl of  $1.689 \text{ g/cm}^3$  (see Figure 20). This value corresponds to the  $1.688 \text{ g/cm}^3$  value obtained by Parris (1975) for Hirt supernatant DNA isolated from BPV infected cells.

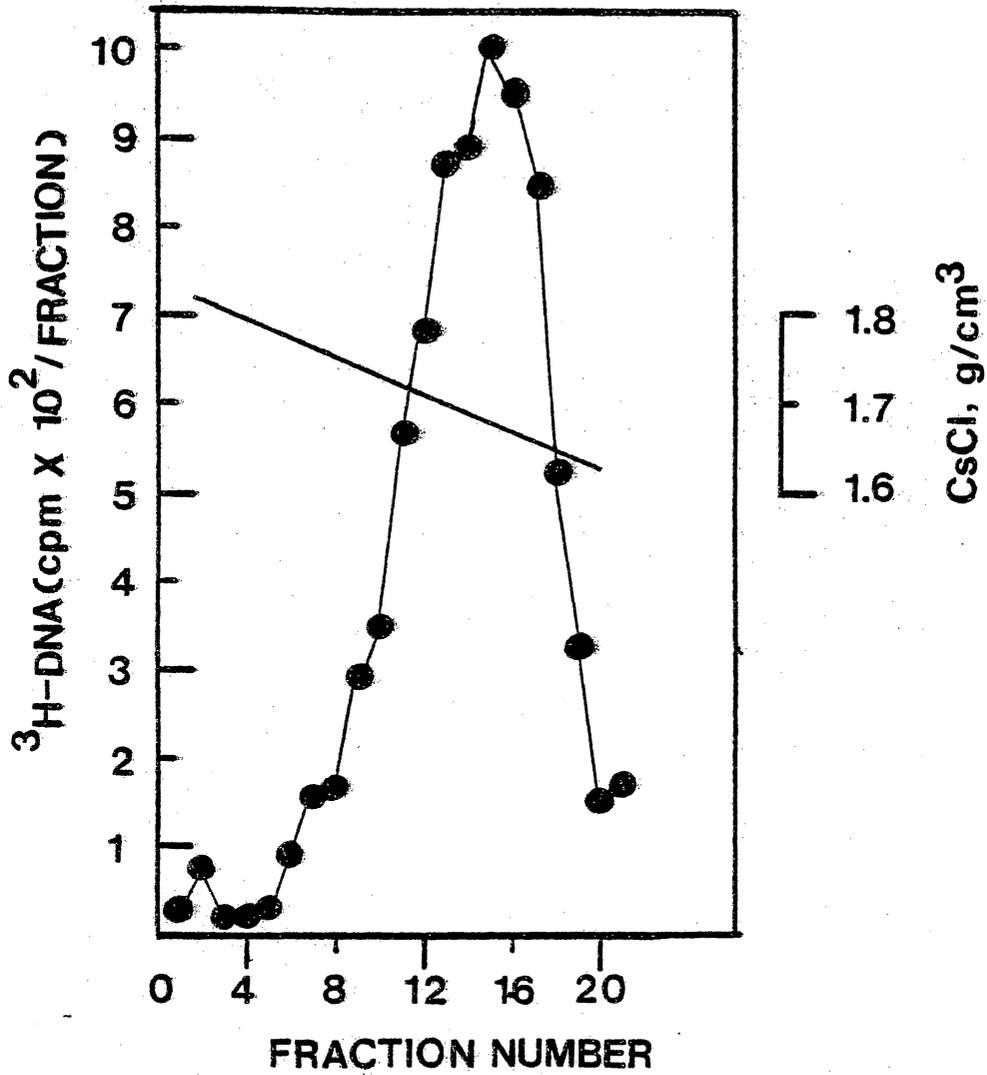


Figure 20. Neutral CsCl gradient profile purified BPV RF DNA.

#### 4.5 Velocity Sedimentation

It was of interest to determine the sedimentation characteristics on neutral and alkaline sucrose of the purified Hirt RF DNA. Figure 21 shows the gradient profiles obtained from these studies.

The neutral gradient contained 1 M NaCl. Under high salt conditions, such as this, single-stranded DNA would be expected to sediment faster than double-stranded DNA due to the high ionic strength. BPV single-stranded DNA marker appears to sediment slightly faster (Figure 21b; see arrows) than purified Hirt RF DNA suggesting that the RF DNA is double-stranded.

Under alkaline conditions, double-stranded DNA is denatured. A double-stranded RF covalently linked at one end, as proposed in the Berns and Hauswirth model (see Section 2.6; Figure 1) should become twice as long under alkaline conditions and would be expected to sediment faster than a single stranded marker. The peak of purified Hirt RF DNA does appear to sediment slightly faster than BPV single-stranded marker DNA. It is also interesting to note the shoulder on the main peak of RF DNA that is in a position corresponding to that of the single stranded marker. This shoulder appears to trail somewhat toward the top of the tube.

McEwen (1967) has described a method for estimating the sedimentation coefficient of nucleic acids from sucrose gradients, in which the rate of sedimentation varies directly with the difference in density between the particle and solution, and inversely with the viscosity of the solution.

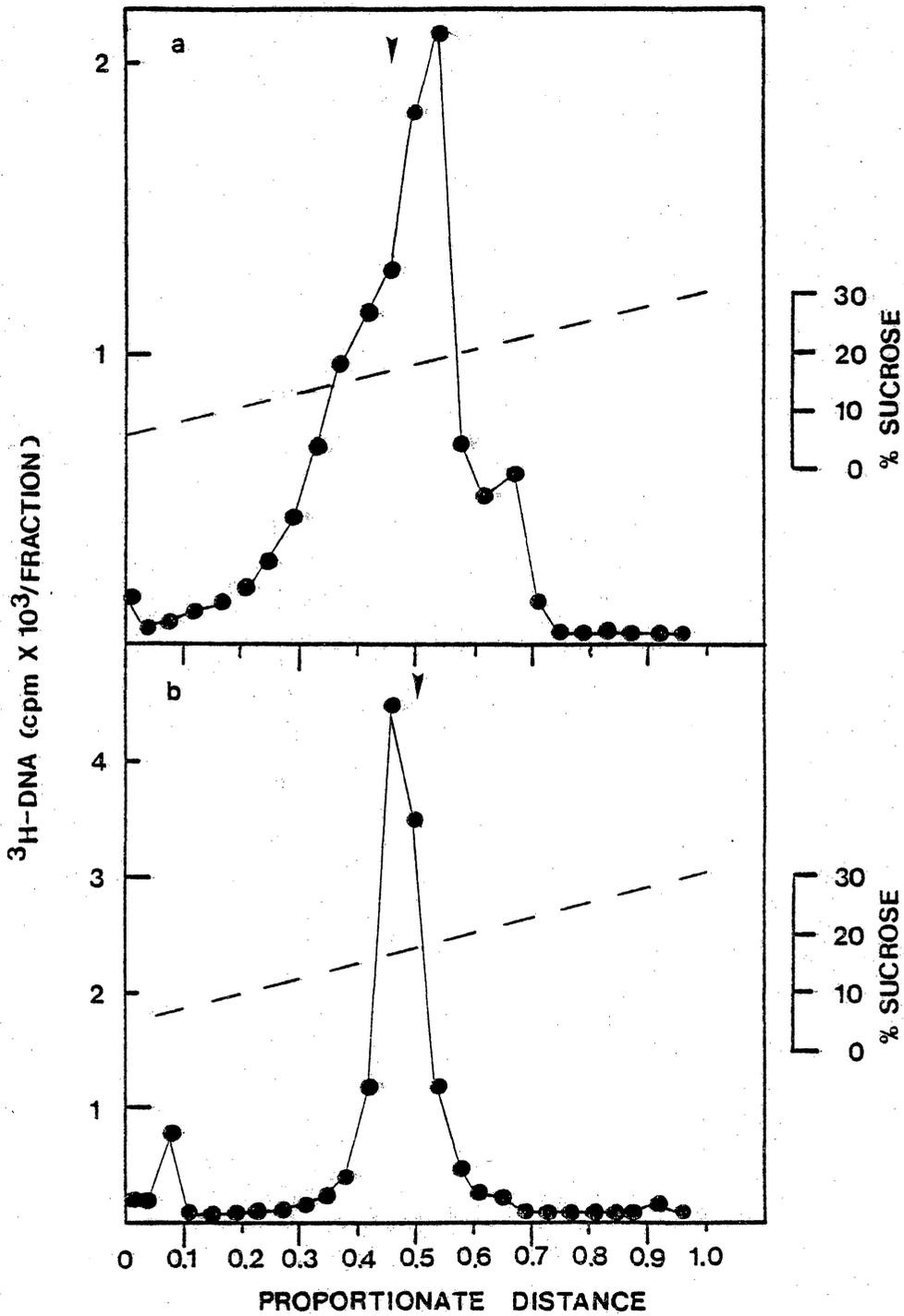


Figure 21. Neutral and alkaline sucrose gradient profiles of purified BPV RF DNA. Arrows indicate position of marker BPV Peak I virion DNA. a) alkaline gradient. b) neutral gradient.

For the neutral sucrose gradient, under the conditions described in Section 3.5.3 and assuming that the gradient is linear, the peak of radioactivity sedimented to a sucrose concentration of 19% sucrose as determined from Figure 21a. This corresponds to a S value of 16. Substitution of 16 S into the equation  $S_{20w}^0 = 0.0882 M^{0.346}$  derived by Studier (1965) for molecular weight estimation, where M is the molecular weight, yields  $3.43 \times 10^6$  d as the weight of purified Hirt RF DNA.

#### 4.6 Hybridization

In order to ensure that the purified Hirt RF DNA was viral specific, hybridization studies were performed. Hybridization techniques have been used successfully to demonstrate base sequence homology among various viral nucleic acids (Green, et. al., 1969). Table 4 shows that 85 to 89% of the purified Hirt RF DNA hybridized to double-stranded BPV Peak II DNA under the experimental conditions described in Section 3.9. BPV Peak II DNA is thought to represent annealed plus and minus strands of BPV virion DNA (Saemundsen, 1978; See Section 2.4). Between 20 and 23% of the purified Hirt RF DNA is shown to hybridize with BFS cell DNA. These results suggest that purified Hirt RF DNA is indeed viral specific but has some cellular DNA contamination.

Table 4. DNA-DNA Hybridization

| Input DNA                                 | <u>Immobilized DNA</u><br>( $\mu\text{g}/\text{filter}$ ) | <u>Bound DNA</u> <sup>a</sup><br>(cpm) (% of input) |          |
|---|---|---|----------|
| BPV RF <sup>3</sup> H-DNA<br>(15,010 cpm) | BPV Peak II<br>(2 $\mu\text{g}$ )                         | 13384<br>13024                                      | 89<br>85 |
| BPV RF <sup>3</sup> H-DNA<br>(15,010 cpm) | Cell DNA<br>(2 $\mu\text{g}$ )                            | 3485<br>3144  | 23<br>20 |

<sup>a</sup>Duplicate determinations

## 5. Discussion

### 5.1 Comparison of G-HCl and Hirt Methods for Isolation of RF DNA

The results obtained from pulse-chase experiments with BPV-infected cells show that there was a decrease with time in the amount of radio-labeled DNA in the double-stranded region of the guanidine-HCl gradients and a concomittant increase in the amount of radiolabeled DNA found in the single-stranded region. These data indicate that there are molecules within the double-stranded DNA region which are involved, in some manner, in the synthesis or evolution of the molecules in the single-stranded DNA region. Similar results were reported by Lavelle and Li (1977) for KRV-infected cells. These molecules could not be detected in gradients of mock-infected cells suggesting that the DNA isolated from BPV-infected cells was viral specific. The broad double-stranded DNA region in these guanidine hydrochloride gradients indicate that there is some heterogeneity among the molecules found within this region. The presence of this heterogeneity is further supported by the results from velocity sedimentation analysis in which the double-stranded RF DNA was distributed throughout the gradient in the neutral gradients. A portion of this RF DNA did cosediment with single-stranded BPV marker in alkaline sucrose indicating that the DNA is similar in size. There is, however, a leading edge of the RF DNA peak suggesting, again, some heterogeneity among the molecules. The most conclusive evidence for heterogeneity is the variety of lengths present in the electron micrographs of this

double-stranded RF DNA of the original guanidine hydrochloride gradients. It is interesting to observe that the double stranded structure of these molecules is confirmed since they appeared to spread by the Kleinschmidt aqueous technique.

Observation of spikes of radioactivity in gradients from mock-infected cells plus the presence of viscous material adhering to the sides of the centrifuge tubes used for sedimentation suggest that cellular DNA may be present in these gradients and also in gradients of infected cells. This cellular contamination may be a contributing source to the heterogeneity observed in infected cells. The possibility that the heterogeneity is due to incomplete pieces of viral DNA cannot be excluded. Hybridization studies would be necessary to confirm either alternative.

A similar heterogeneity was initially observed with the Hirt method; however, further purification separated the Hirt supernatant DNA to such an extent that characterization could be performed. Hydroxyapatite chromatography of Hirt DNA yielded a single peak of radiolabeled DNA eluting at a NaP concentration that falls within the range of the concentrations eluting double-stranded DNA indicating that the Hirt DNA is double-stranded. The length variation of this double-stranded DNA observed by electron microscopy again strongly indicates the heterogeneity within the sample. This was also seen in RF DNA isolated by the guanidine hydrochloride method. Cellular contamination is also suggested.

Purification by velocity sedimentation in neutral sucrose, which separates DNA on the basis of size, shape and density, resolved a major

sharp peak of RF DNA. This technique appeared to separate the Hirt DNA to some extent in that there is a minor broad peak of radio-labeled DNA sedimenting slightly slower than the major RF DNA peak. This is in the region of the gradient where one would expect small DNA molecules to band. Electron microscopy of molecules within this region showed that these molecules were, indeed, considerably shorter than those in the main peak (data not shown). The origin of these small molecules is unclear, as is the source of heterogeneity in the guanidine hydrochloride-isolated RF molecules. Hybridization studies are needed to resolve this question of origin.

Hybridization of the purified RF DNA from the main peak of DNA in the neutral sucrose gradient showed that the purified Hirt RF DNA molecules were 85-89% virus specific. This is comparable to the 80% obtained by Green et. al. (1969) for adenovirus DNA. DNA hybridization under similar conditions showed that the bound DNA was not due to non-specific binding since the purified Hirt RF DNA probe did not adhere to blank filters and that the BPV Peak II DNA and BFS cell DNA were present on the filters.

A small amount of cellular DNA was present in the purified Hirt RF DNA which must be kept in mind when looking at results from other experiments. I feel confident, however, that the results obtained reflect the characteristics of the BPV RF DNA. The presence of this cellular DNA within the purified Hirt RF DNA sample may be indicative of the source of heterogeneity seen in the results of experiments. In addition, the Hirt procedure seems better suited for studies with BPV-infected cells

than does the G-HCl method, since some of the heterogeneity could be eliminated through further purification.

## 5.2 Contour Length Measurements

The mean contour length of purified Hirt RF DNA, Prep I was 1.69  $\mu\text{m}$ . This should be the length of the monomeric form. Molecules whose length approached calculated values for dimer and trimer molecules were also observed. These results are consistent with results obtained by Siegl and Gautschi (1976) for Lu III RF DNA. The Lu III monomer had a contour length of 1.55  $\mu\text{m}$  while longer molecules of 4.7  $\mu\text{m}$  were also observed. Singer and Rhode reported similar results for H-1 RF DNA. The contour length of the H-1 monomer was 1.53  $\mu\text{m}$  and the dimer was 3.10  $\mu\text{m}$ . BPV RF DNA appears to be somewhat longer than LU III or H-1 RF DNA. Comparisons of this type are somewhat difficult to make since these investigators used different spreading procedures. The affect of ionic strength on contour length has been discussed in Section 2.7.

Several branched molecules (RI) were also observed in preparations of BPV RF DNA. Length measurements were not performed because of the small sample size. These molecules appear somewhat shorter than the BPV RF DNA. This might reflect a problem with the spreading procedure rather than a true length discrepancy since problems were also encountered with spreading of single-stranded BPV virion DNA. Siegle and Gautschi (1976) and Singer and Rhode (1977) also observed branched RI. They are also postulated in the Berns and Hauswirth (1978) model for parvovirus replication.

There appeared to be a length discrepancy between the two different

BPV RF DNA preparations that were measured. The molecules from Prep II were 7% longer than those from Prep I as determined by measurement and length comparison of  $\Phi$ X174 RF II DNA. This apparent elongation is possible with some spreading techniques due to ionic strength of the hypophase as described by Lang et. al. (1967; see Section 2.7.8). Since distilled water from stock was used, it is possible that the ionic strength may vary from day to day which could cause such an increase in length to take place. The humidity also seems to play an important role in the spreading of nucleic acids for visualization. An interesting observation that I noted is that the DNA spreads better on days which have more moisture in the air than very dry days. For example double-stranded DNA spreads better during the humid summer than in the winter. I also find it spreads better on rainy days than dry sunny days. The humidity has an effect upon the static charges in the air which may in turn affect the interaction of the carbon film on the grids and the protein-DNA film. The result of this interaction could, of course, be reflected in the contour length of the DNA molecules spread on different days. There is also a slight possibility that there was an error made in determination of magnification. This seems unlikely since three photographs were taken of the grating replica at each calibration check.

Histograms of the lengths of the BPV RF DNA showed a normal distribution of molecules which suggests a single population of molecules. There does appear to be some length heterogeneity since the graphs are somewhat broad especially that of Prep II, Fraction 12. Preliminary results with agarose gel electrophoresis also demonstrate this length

heterogeneity in that a broad band trailing toward the longer length region was observed. The preparative neutral sucrose gradients used in the purification of the RF molecules appear to have separated some of the different sized molecules from Hirt supernatants, but some heterogeneity still exists within the samples.

Histograms of the other fractions from Prep I and Prep II are somewhat more complex. Two populations of molecules with different lengths appear to be present in these samples. Population I consists of molecules whose lengths approach that of dimers and trimers. Population II contains molecules with lengths similar to that of the monomer BPV RF DNA. The origin of Population II is unclear, however, it may result from nicks in the dimer and trimer forms of the RF DNA which break the molecules into monomer-like molecules. Hybridization studies are needed to confirm the viral specificity of the molecules from Population I and II. Firm conclusions as to the true existence of two size populations are also difficult due to the small sample size.

Pincushion distortion in the electron microscope could not be detected by the method described in Section 3.6.3. This is not surprising since a relatively high magnification was used to view the grating replica. Insertion of a small objective aperture, as used in this study, also helps to reduce the affects of pincushion distortion (Meek, 1976). The area of the total field which is actually photographed in the Jeol 100C electron microscope is the center portion. Pincushion distortion is greatest at the periphery of the field and is highly reduced, even at low magnifications, in the center (Meek, 1976).

The greatest source of error stemmed from pincushion distortion within the Omega enlarger and the accuracy of the grating replica. Most of the molecules traced with the Omega Pro-Lab enlarger were, again, those more toward the center of the field than the periphery. The value of 1.3% used for the error in the grating replica may or may not be a reasonable estimate. A better method is needed to measure this parameter than that described in Section 3.6.3.

It is difficult to compare these errors in measurement of contour length for the other autonomous parvoviruses. Values for these factors which will influence the contour length were not reported by those investigators.

The mode length of the BPV RF DNA was 1.66  $\mu\text{m}$  and represents the average length value of molecules occurring at the highest frequency. This is in contrast to the mean length determination which is the average length of all the molecules observed. The use of the mode length, exclusively can lead to a biased representation of the data since all the molecules that are observed are not included in length calculations.

### 5.3 Molecular Weight Determinations Based on Contour Lengths and S-Value

A value of 1.689  $\text{g}/\text{cm}^3$  was obtained for the bouyant density of BPV RF DNA. This agrees with the value obtained by Parris (1975) for BPV Hirt supernatant DNA. It is also similar to the value obtained by Saemundsen (1978; 1.706  $\text{g}/\text{cm}^3$ ) for Peak II BPV virion DNA, which is thought to represent annealed plus and minus strands.

The estimated molecular weight of BPV RF DNA as determined by

electron microscopy is  $3.7 \times 10^6$  d. This value is based upon the mean contour length of BPV RF DNA and is close to twice the value for BPV single-stranded virion DNA of  $1.7 \times 10^6$  d (Saemundsen, 1978). The molecular weight of BPV RF DNA based upon the mode length is  $3.6 \times 10^6$  d.

BPV RF DNA appears to behave as expected when sedimented in both neutral and alkaline gradients. The small shoulder on the main peak of RF DNA as seen in the alkaline sucrose gradient (see Figure 20) appears to cosediment with the single-stranded BPV virion DNA marker indicating that these molecules are the same size. BPV RF DNA that is covalently linked at one end as specified in the Berns & Hauswirth model (Section 2.6; Figure 1) should be denatured and sediment as a dimer. The presence of this denatured unit length BPV RF DNA may reflect a double-stranded RF molecule that has been nicked to produce monomer length molecules plus complementary strands of various sizes which could account for the trailing shoulder. Nicked molecules have been detected by agarose gel electrophoresis for MVM RF DNA (Ward and Dadachanji, 1978). The authors suggested that this nicking is due to the actual replication process of the virus within the infected cell and is consistent with present replication models.

The reason that the single-stranded DNA marker and the BPV RF DNA are not separated to the extent that they are in a guanidine HCl gradient is unclear. Perhaps the longer centrifuge tubes used in the G-HCl gradient and, of course, the different conditions under which the sedimentation occurred had some influence in the banding of the different DNA species. Whether these factors would cause such a dramatic difference in separation remains to be seen.

The value of 16S for the sedimentation coefficient of BPV RF DNA is a reasonable estimate and is similar to the S value obtained by Siegl and Gautschi (1976) of 14.7S for Lu III RF DNA and a value of 17S obtained by Rhode (1974) for HI RF DNA.

Several factors may have an affect on the estimation of sedimentation coefficient as it was described in Section 4.5. First, the gradient was assumed to be linear and the sucrose concentration determined by extrapolation to a line representing the sucrose gradient. Any concentration discrepancy would not have been detected without actual measurement, and this was not performed. Second, the calculations are based upon weight-weight solutions of sucrose (McEwen, 1967). Solutions for these studies were weight-volume solutions which may cause some errors in reading the tables used in the calculations. Even though these factors may have caused some error in the determination, I feel that the value of 16S obtained is reasonable estimate.

The molecular weight of  $3.4 \times 10^6$ d obtained using the S-value of 16 and its substitution into Studier's (1965) equation for determination of molecular weight agrees reasonably well with the  $3.6 \times 10^6$ d or  $3.7 \times 10^6$ d values obtained from electron microscopy. The slight difference, again, may reflect a small error in the determination of the sedimentation coefficient. The  $3.4 \times 10^6$ d molecular weight is twice the weight of single-stranded BPV virion DNA (Saemundsen, 1978).

#### 5.4 Concluding Remarks

The following conclusion can be made based upon the results described:

- 1 - The guanidine-hydrochloride method and the Hirt procedure are effective methods for the isolation of replicative forms from BPV-infected cells, however, the Hirt procedure seems better suited for studies with BPV.
- 2 - The BPV RF DNA has a mean contour length of 1.69  $\mu\text{m}$  and hence a molecular weight of  $3.7 \times 10^6 \text{d}$ . The mode contour length is 1.66  $\mu\text{m}$  which yields a molecular weight of  $3.6 \times 10^6 \text{d}$ .
- 3 - Two size populations were observed in the fractions sedimenting in front of the main peak of Prep I:
  - a) Population I contained molecules that were significantly longer than the predominant RF DNA species. These molecules approach calculated values for dimer and trimer replicative forms.
  - b) Population II contained molecules that had mean lengths similar to the predominant RF DNA species.
- 4 - BPV RF DNA has a bouyant density of  $1.689 \text{ g/cm}^3$  in neutral CsCl.
- 5 - BPV RF DNA has an estimated sedimentation coefficient of 16S in neutral sucrose.

The origin of the heterogeneity in the RF DNA is unclear. Several lines of evidence suggest that it is due to cellular DNA contamination, however the idea that the heterogeneity is of viral origin cannot be overlooked. Siegl (1978) noted that the relative number of monomer and dimer length RF molecules of the parvovirus Lu III varied with the cell type. He also observed short, double-stranded DNA in Hirt supernatants

from HeLa cells infected with Lu III. RF molecules in Hirt supernatants of Lu III-infected NB cells, however, were more homogeneous in size. Since the autonomous parvoviruses are dependent upon a cellular function(s) for their replication, perhaps the source of heterogeneity is the in degree of competency of the cells to synthesize and process parvovirus DNA.

Perhaps the lack of a certain enzyme(s), or the small amount of it within a particular cell type could lead to the formation of incomplete DNA. The existence of defective Lu III virions containing less than unit length DNA have been described (Muller et. al., 1978). The formation of these virions was said to be favored under different conditions such as host-cell system, multiplicity of infection and passage history of the cells. The cell type seemed to have greatest influence on the formation of these defective particles.

It would be interesting to study the BPV RF DNA isolated from different cell types capable of propogating the virus to see if the amount of heterogeneity remained constant. Studies of this type could also aid in explaining the packaging errors observed for BPV (Saemundsen, 1978) that was also dependent upon cell type.

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7. APPENDIX.

Dulbecco's Salt Solution

Ingredients per liter:

|  |         |       |
|--|---------|-------|
| Sodium Chloride.....                     | 8       | g     |
| Disodium Phosphate.H <sub>2</sub> O..... | 0.12    | g     |
| Potassium Chloride.....                  | 0.2     | g     |
| Magnesium Chloride.H <sub>2</sub> O..... | 0.1     | g     |
| Monopotassium Phosphate.....             | 0.2     | g     |
| Calcium Chloride.....                    | 0.1     | g     |
| Phenol Red.....                          | 0.005   | g     |
| Streptomycin.....                        | 0.1     | g     |
| Penicillin.....                          | 100,000 | units |

Saline A

Ingredients per liter:

|                         |         |       |
|-------------------------|---------|-------|
| Sodium Chloride.....    | 8       | g     |
| Potassium Chloride..... | 0.4     | g     |
| Sodium Bicarbonate..... | 0.35    | g     |
| Glucose.....            | 1       | g     |
| Phenol Red.....         | 0.02    | g     |
| Streptomycin.....       | 0.1     | g     |
| Penicillin.....         | 100,000 | units |

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# ELECTRON MICROSCOPY OF BOVINE PARVOVIRUS

## DNA REPLICATION INTERMEDIATES

by

Rebecca B. Young

### (ABSTRACT)

Several low molecular weight DNA species have been isolated from autonomous parvovirus-infected cells that could not be demonstrated in mock-infected cells. The DNA species were termed replicative forms and intermediates. The purpose of this study was to isolate and characterize the replicative forms from bovine parvovirus (BPV)-infected cells.

Low molecular weight DNA was isolated from BPV-infected cells by the guanidine hydrochloride method and the Hirt procedure. Heterogeneity was present in DNA samples isolated by both procedures; however the Hirt procedure seemed better suited for studies with BPV-infected cells since some of the heterogeneity could be eliminated by further purification by hydroxyapatite chromatography and sedimentation through neutral sucrose gradients.

Characterization of the purified Hirt BPV RF DNA by electron microscopy showed that the RF DNA was linear and had a mean length of 1.69  $\mu\text{m}$  or a mode length of 1.66  $\mu\text{m}$ . Several branches molecules were also observed, however length measurements were not performed due to the small sample size. The molecular weight of the RF DNA based upon

the mean length was  $3.7 \times 10^6$  d and  $3.6 \times 10^6$  d when based upon the mode length. Molecules approaching dimer and trimer length were also observed.

Sedimentation analysis of the BPV RF DNA showed that the DNA had a bouyant density of  $1.689 \text{ g/cm}^3$  in neutral CsCl and an estimated sedimentation coefficient of 16S in neutral sucrose gradients. The molecular weight of the RF DNA based upon the sedimentation coefficient was  $3.4 \times 10^6$  d.

Hybridization studies showed that the RF DNA was of viral origin, but contained some cellular DNA contamination. The cellular DNA contamination could be the source of the heterogeneity observed.