REPLICATION OF BOVINE PARVOVIRUS

by

Deborah Sue Parris

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APPROVED:

R. C. Bates, Chairman

C. W. Heald

E. R. Stout

N. R. Krieg

S. A. Tolin

R. A. Paterson

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Blacksburg, Virginia

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1. Introduction

The parvoviruses are icosahedral viruses ranging in size from 18 to 21 nm (Tinsley, 1973). The first of these viruses to be isolated was rat virus (RV, Kilham and Olivier, 1959). Since that time, parvoviruses have been isolated from a variety of animal species. Among the nondefective parvoviruses, which replicate autonomously without the aid of a helper virus, are RV, H-1 virus, Lu III virus, and bovine parvovirus. The defective parvoviruses, the adeno-associated viruses, require the presence of a helper adenovirus in the same cell for replication and maturation to occur.

The genome of the parvoviruses consists of single stranded DNA with a molecular weight of 1.35 to 1.7×10^6 daltons (Rose, 1974), and only linear forms of this DNA have been isolated. The bacterial virus, ØX 174, contains single stranded DNA of approximately the same size. The replication of ØX 174 has served as a model for parvovirus DNA replication, although ØX 174 DNA is circular. During ØX 174 replication, double stranded, covalently closed and open circular replicative forms have been isolated (Sinsheimer *et al.*, 1968). Double stranded replicative forms have also been isolated from defective and nondefective parvovirus-infected cells although no covalently

closed circular forms have been found. However, the double stranded replicative form, as well as single stranded DNA of the adeno-associated viruses can exist in a hydrogen-bonded circular configuration due to self-complementary terminal sequences (Carter _et al_., 1972).

The small genome of the parvoviruses is sufficient to code for only 2 or 3 proteins. Unless the 2 or 3 structural proteins reported for these viruses have enzymatic functions, all of the enzymes necessary for replication to occur must be provided by the host cell. In fact, the replication of the nondefective parvoviruses has been shown to depend upon the physiological state of the host cell. It has been found that these viruses replicated poorly or not at all in stationary, noncycling cells. In general, parvoviruses require actively dividing cells in which to replicate, and optimum replication occurs when S phase cells are infected. However, the factor provided by these cells which enable parvovirus replication has not been elucidated.

Until recently, most of the research concerning the kinetics of replication of the parvoviruses was performed in asynchronous cells. The lack of synchronized cell systems in studies concerning the effect of parvovirus replication on macromolecular synthesis has led to results which are inconsistent. Because of the cell cycle dependence of these viruses, the use of a

well-defined synchronized cell population for viral replication is necessary to yield reproducible information on the kinetics of replication of nondefective parvoviruses, such as bovine parvovirus, and the effects of parvovirus replication on host macromolecular synthesis.

This study was initiated to characterize the replication of bovine parvovirus in a highly synchronized cell system. The specific objectives of this research were:

1. To determine an effective means of synchronizing cells able to support bovine parvovirus replication.
2. To characterize the kinetics of DNA, RNA, and protein synthesis in the synchronized cells.
3. To determine the kinetics of replication of bovine parvovirus in synchronized cells.
4. To determine the effect of bovine parvovirus replication on macromolecular synthesis in infected synchronized cells.
5. To determine the extent of bovine parvovirus replication in cells unable to initiate cellular DNA synthesis.
6. To characterize the double stranded replicative forms of bovine parvovirus DNA isolated from infected synchronized cells.

2. Review of Literature

2.1. The Mitotic Cycle of Mammalian Cells

The mitotic cell cycle is divided into four parts--M, G_1, S, and G_2 phases. Mitosis, or M phase, occupies a brief period in the total cell cycle and is the only stage in which RNA and protein synthesis are absent (Prescott, 1968). During the next stage, G_1, at least some of the events which prepare the cell for replication of its DNA occur (Prescott, 1968; Stein and Baserga, 1972; Baserga, 1969). The DNA synthetic period, S phase, then ensues and is characterized by the ability of the cell to incorporate radioactive thymidine (Prescott, 1968). Following S phase, the cell prepares for mitosis during the G_2 period.

Many viruses, such as the nondefective parvoviruses, the oncornaviruses, SV-40, polyoma virus, and equine abortion virus (Rose, 1974; Pages _et al._, 1973; Basilico and Marin, 1966; Humphries and Temin, 1974; Lawrence, 1971), require cells in a particular stage of the cell cycle in order to replicate or to initiate transformation. An understanding of the events which occur during the stages of the cell cycle may serve to elucidate the critical events associated with the cell cycle dependence of these viruses. These stages of the cell cycle can only be studied

accurately if highly synchronous populations of cells are used. Among the methods most often used for obtaining synchronized cells are mitotic selection, serum stimulation, nutritional deficiencies, and the application of metabolic inhibitors such as thymidine (TdR) and hydroxyurea (HU).

2.2. Methods for Obtaining Synchronized Cells

2.2.1. Mitotic Selection

Cells grown in monolayers display a reduced affinity for the substrate surface during the period of mitosis. At that time, cells become rounded and can be detached from the substrate by shaking. Upon reseeding, these cells progress through G_1 and subsequently synthesize DNA synchronously (Terasima and Tolmach, 1963). Although this method avoids the use of stimulators or inhibitors which may alter the normal state of the cells, the yield of synchronous cells is small due to the small proportion of cells in M phase at any given time in randomly growing populations (Mitchison, 1971). The proportion of cells in M phase can be increased using the reversible metaphase inhibitor, colcemid. However, the addition of colcemid may alter the cells' normal physiology (Mitchison, 1971).

Since cells become rounded only during mitosis which occupies 30 to 60 min, cells selected by the detachment procedure enter G_1 in a highly synchronized fashion (Terasima and Tolmach, 1963). However, since G_1 is the

most variable phase of the cell cycle (Prescott, 1968), the high degree of synchrony is lost by S phase (Pages *et al.*, 1973). Thus, cells synchronized at the end of G_1 or during early S would be preferable for studies requiring highly synchronized S or G_2 phase cells.

2.2.2. Serum Stimulation

Serum provides factors upon which the growth of normal cells, such as 3T3 cells, depend. The saturation density achieved by these cells is, in fact, proportional to the concentration of serum used (Schor and Rozengurt, 1973). Griffiths (1972) showed that the addition of whole medium containing fresh serum to stationary human fetal lung cells stimulated the cells to synthesize DNA and protein and to divide. Addition of serum alone provided less stimulation. This phenomenon could best be explained by the observation that amino acid uptake, which was reduced by cell crowding, was stimulated by serum. Griffiths concluded that serum had the primary function of stimulating DNA synthesis and a secondary function of stimulating amino acid uptake.

Addition of serum is an effective means of inducing cells to undergo DNA synthesis synchronously. Using stationary human diploid lung fibroblasts, Ellem and Mironescu (1972) found that 50 to 60% (v/v) produced maximum stimulation of DNA synthesis. DNA synthesis

occurred in a parasynchronous fashion in 65 to 80% of the cells beginning at 12 hr after addition of serum. Stationary cells exposed to serum for less than 7 hr did not initiate DNA synthesis. However, if the serum was removed after 7 hr, DNA synthesis was initiated although initiation occurred in fewer cells than if serum remained the entire 12 hr. Crystalline bovine serum albumin substituted to some extent for fetal calf serum. However, because the concentration of albumin in medium containing 20% serum (0.6%) produced the same stimulation as 2% albumin, albumin was shown to account for only one-third of the stimulatory activity of serum (Ellem and Mironescu, 1972).

2.2.3. Amino Acid Deficiency

Although serum may induce synchrony in a variety of cell types, addition of serum alone to Chinese hamster ovary (CHO) cells which had become stationary in F-10 medium did not stimulate the cells to synthesize DNA. However, addition of complete F-10 medium or spent F-10 medium to which isoleucine and glutamine had been added stimulated the cells to synthesize DNA and to divide (Ley and Tobey, 1970). If CHO, mouse L, or BHK-21 cells were allowed to reach stationary phase due to depletion of other amino acids, subsequent addition of these allowed the cells to resume cell cycle traverse but not in synchrony (Ley and Tobey, 1970; Tobey and Ley, 1971).

F-10 medium prepared without isoleucine was not completely devoid of isoleucine due to amino acid release from the breakdown of serum proteins (Tobey and Ley, 1971). It was proposed that sufficient isoleucine was available in isoleucine deficient medium to allow cell cycle traverse to G_1 phase, but not enough to allow the cells to synthesize DNA (Tobey and Ley, 1971; Enger and Tobey, 1972). This hypothesis was supported by the finding that uridine incorporation dropped to 73% of its initial value only after cells had been exposed 30 hr to isoleucine deficient medium. Protein was also shown to be synthesized in cells deficient in isoleucine (Enger and Tobey, 1972).

Amino acid deficiencies other than isoleucine can result in accumulation of cells at one stage of the cell cycle. For example, mouse LM cells were synchronized in F-10 medium deficient in tryptophan. Addition of tryptophan to stationary cells seeded in this medium stimulated cells to synthesize DNA within 6 hr (Brunner, 1973). However, complete tryptophan starvation did result in less synchrony than when cells were exposed to deficient medium, possibly due to the inability of cells to synthesize proteins required for movement into S. Furthermore, this cell type could not be synchronized using isoleucine or leucine deficiency (Brunner, 1973). It appears that many cell types may be synchronized by the use of medium deficient in one or more amino acids. However, the

particular deficiency allowing such synchrony may vary depending on the cell type employed.

2.2.4. Excess Thymidine (TdR)

Cells can be induced to synthesize DNA synchronously by blockage of DNA replication with antimetabolites such as TdR and HU. However, only those events which are closely associated with DNA replication are synchronized in this way because the synthesis of RNA, proteins, lipids, and carbohydrates is maintained during the block (Mueller and Kajiwara, 1969; Mitchison, 1971). Nevertheless, much information has been obtained concerning events required for the initiation and maintenance of DNA replication using these inhibitors.

The most commonly used DNA synthesis inhibitor is excess TdR. Although TdR is a normal metabolite of cells, its addition in 2 to 10 mM quantities inhibits DNA replication by feedback inhibition of the deoxycytidine pathway by means of its binding to ribonucleotide reductase (Xeros, 1962; Bjursell and Reichard, 1973).

Cells exposed to a single treatment of TdR can be induced to synthesize DNA almost immediately after the addition of deoxycytidine or after removal of the medium containing TdR (Galavazi *et al.*, 1966). However, a small population of the cells are not synchronized since cells in S phase at the time of addition are not able to proceed

through the cell cycle while those in other stages of the cell cycle will proceed to late G_1 or early S phase before becoming inhibited. Galavazi _et al_. further showed that those cells labeled with ^{3}H-TdR immediately before application of the TdR block, and therefore in S phase at the time of the block, accounted for the fraction of cells not synchronized by a single TdR treatment. It was found that application of a second block after all cells had proceeded through S phase produced optimum synchrony upon reversal (Galavazi _et al_., 1966) resulting in 80 to 90% of the cells entering S phase between 1.5 and 3 hr following removal of the second TdR block (Bootsma _et al_., 1964).

Although it was initially assumed that TdR prevented cells from entering S (Mitchison, 1971), it has been found that CHO cells treated with TdR accumulate at some point in S phase based on the DNA content of the cells (Tobey and Crissman, 1972) and on the continued incorporation of DNA precursors into the DNA of S phase cells treated with TdR (Bjursell and Reichard, 1973). On the other hand, it was demonstrated that excess TdR reduced the rate of DNA synthesis in S phase cells (Bjursell and Reichard, 1973). Thus excess TdR is useful in studies requiring cells with a high degree of synchrony in S phase. However, since DNA synthesis does occur at low levels in the presence of TdR, the use of inhibitors preventing entry into S phase would be more desirable in studies regarding factors required for initiation of DNA replication.

2.2.5. Hydroxyurea (HU)

HU has been shown to be an effective synchronizing agent for a variety of cell lines (Sinclair, 1965; Pfeiffer and Tolmach, 1967; Kim *et al.*, 1967; Adams and Lindsay, 1967; Plagemann *et al.*, 1974). Concentrations of HU completely inhibitory for DNA synthesis have little or no effect on RNA and protein synthesis (Pfeiffer and Tolmach, 1967; Young and Hodas, 1964; Pollak and Rosenkranz, 1967) thus allowing cell cycle traverse. Following reversal of the HU block by washing, cells begin to synthesize DNA immediately and divide in synchrony (Tobey and Crissman, 1972).

Furthermore, HU has been shown to synchronize cells at the G_1/S border without allowing cells to enter S phase. These cells not only have the DNA content of G_1 phase cells, but require a longer period of time before division following removal of HU than cells synchronized with excess TdR confirming the earlier phase in which inhibition occurs (Tobey and Crissman, 1972).

The means by which HU inhibits DNA replication are not clear. It has been shown that HU inhibits the enzyme ribonucleotide reductase *in vitro* (Brockman *et al.*, 1970). Pollak and Rosenkranz (1967) suggested that the mechanism of action may be more complex *in vivo* because DNA synthesis in BHK-21 cells seeded in medium containing HU and all four deoxyribonucleotide triphosphates was inhibited to the

same extent as cells seeded in HU alone. On the other hand, all four deoxyribonucleosides reversed HU inhibition of mouse L cells (Adams and Lindsay, 1967). These authors concluded that HU acted at the level of inhibition of ribonucleotide reductase. Discrepancies between these sets of data may be due to differences in the cell types used or to the fact that extremely high concentrations of deoxyribonucleosides were required in the latter study for reversal of the HU block. High concentrations of deoxyadenosine and deoxyguanosine have been reported to reverse HU inhibition in Novikoff rat hepatoma cells (Plagemann and Erbe, 1974). In CHO cells, HU has been shown to reduce the accumulation of dATP only, while reduction of the other 3 nucleosides continued to occur producing increased levels of dGTP, dTTP, and dCTP during the HU block (Walters et al., 1973). The complex allosteric regulation of the enzyme ribonucleotide reductase by nucleosides and nucleotides (Moore and Hurlbert, 1966) may explain the differences observed in the various cell types. Addition of HU to cells with differences in the intracellular pools of these compounds may induce different effects on the final pool sizes in the cells following treatment. If differences in pool size did result from HU treatment, it would be expected that nucleosides or nucleotides required to reverse the block would vary with the cell type.

The cytotoxicity of HU for cells also depends on the cell type. CHO cells in S phase at the time of addition of HU are selectively killed (Sinclair, 1965; Sinclair, 1967; Barranco and Novak, 1974). However, Pfeiffer and Tolmach (1967) showed that HU was not toxic to S phase HeLa cells. In addition, HeLa cells remained viabile and could be synchronized after 16 hr of exposure to HU (Pfeiffer and Tolmach, 1967; Kim et al., 1967), while there was a rapid loss of viability of CHO cells 12 hr after exposure to HU (Tobey and Crissman, 1972). There was also a high rate of survival and synchrony produced in mouse L cells after treatment with 2 mM HU for 18 hr (Adams and Lindsay, 1967), and primary rabbit kidney cells remained viable after 70 hr in HU (Adams et al., 1966). Thus with respect to HeLa and mouse L cells, extended exposure to HU does not result in a state of irreversible unbalanced growth. Synchronization of rabbit kidney cells was not attempted so that further investigations are necessary to determine if HU treatment results in irreversible unbalanced growth of these cells.

2.3. Periodicity of Events Occurring During the Cell Cycle

2.3.1. Fluctuation of Enzyme Levels

Enzyme level oscillations have been shown to be characteristic of normal mammalian fibroblasts (Klevecz and Kapp, 1973). Thymidine kinase and deoxycytidine

deaminase have been shown to be the only two enzymes examined which are present in S phase but not also in G_1 (Stein and Baserga, 1972). Furthermore, these enzymes together with thymidylate kinases and ribonucleotide reductase all show a marked increase at the beginning of S phase and a marked decrease at the end of S (Watson, 1971). Since inhibition of protein synthesis at the end of G_1 prevents cells from entering S phase, the transition from G_1 to S may be due to the synthesis of a particular protein (Prescott, 1968). The correlation in the time of increase of these enzymes and that of the initiation of DNA synthesis suggests that one or more of these may be the initiating factor. However, evidence has been obtained which indicates that these enzymes are probably not involved in the regulation of initiation of DNA synthesis because i) in regenerating liver, the enzyme levels increase prior to DNA synthesis but remain high and ii) in mouse embryo cells, they increase following initiation of DNA replication and increase in accordance with the rate of DNA synthesis (Watson, 1971). Although there is a close association between the synthesis of these enzymes and the synthesis of cellular DNA, synthesis of the enzymes may require DNA synthesis rather than initiate it.

The levels of DNA polymerase have been examined during the cell cycle of synchronized HeLa cells (Spadari and Weissbach, 1974). The level of DNA polymerase β, the

low molecular weight polymerase associated exclusively with the nucleus, was shown not to vary during the cell cycle. However, the high molecular weight DNA polymerase found in the nucleus and in the cytoplasm, DNA polymerase α, increases during S but reaches maximum levels following the initiation of DNA synthesis. The only DNA polymerase shown to fluctuate in a pattern similar to DNA synthesis is DNA polymerase γ. This polymerase efficiently copies polyribonucleotide strands but its role in cellular DNA synthesis remains obscure (Spadari and Weissbach, 1974; Bollum, 1975).

2.3.2. Fluctuation in Levels of Nucleic Acid Precursors

Fluctuations in the intracellular content of nucleotide precursors of DNA may be involved in the regulation of initiation of DNA synthesis. The pool sizes of dATP, dGTP, dTTP, and dCTP were examined during the cell cycle of CHO cells synchronized by mitotic selection or isoleucine deficiency. Although it was found that all pool levels increased as cells moved into S phase (Walters *et al.*, 1973; Skoog *et al.*, 1973), maximum levels of dATP, dGTP, and dTTP were achieved after the peak in DNA synthesis. Because the dCTP pool reached maximum levels coincident with the maximum rate of DNA synthesis, it was proposed that the level of the dCTP pool governed the initiation and rate of DNA synthesis (Skoog *et al.*, 1973).

Using a double TdR block on the same cell type, Bjursell and Reichard (1973) showed that the dCTP pool decreased to 10% of the control value in the presence of TdR while the pool sizes of dTTP, dGTP, and dATP increased. Cells treated with TdR in S phase rapidly decreased their rate of synthesis of DNA although 100% of the cells incorporated radioactive TdR into DNA. The addition of deoxycytidine increased the pool of dCTP to near normal levels and reversed the inhibition of the rate of DNA synthesis. Since cells were capable of synthesizing DNA even with the reduced level of dCTP present, it was concluded that the dCTP pool might have a regulatory role in the rate of DNA synthesis rather than in its initiation (Bjursell and Reichard, 1973). On the basis of this data alone, it cannot be ruled out that dCTP may be a factor regulating initiation. Although dCTP was present in low quantities, this level may have been sufficient to initiate DNA synthesis.

Walters et al. (1973) showed that HU applied to CHO cells inhibited DNA synthesis. During HU treatment of G_1 cells, the pool sizes of dTTP, dCTP, and dGTP increased in a manner similar to that in control G_1 cells. Only dATP remained at the baseline level observed during mitosis, with the level abruptly increasing following removal of HU. The authors concluded that the pool of dATP regulated initiation. These results are in contrast to those obtained

by Bjursell and Reichard (1973) who found that dATP increased in the presence of TdR, while dCTP was reduced. Walters *et al.* (1973) did show that at 1 hr after HU removal, dCTP levels increased, while the high level of dATP achieved immediately after HU removal was reduced. The differences in pool sizes of dCTP and dATP before DNA synthesis may reflect the use of two different inhibitors of DNA synthesis. However, the increase in dCTP 1 hr after HU removal is consistent with its role in the regulation of the rate of DNA synthesis shown by Bjursell and Reichard (1973).

Inhibition of DNA synthesis occurred in Novikoff rat hepatoma cells treated with HU. This inhibition was reversed by the addition of high concentrations of deoxyadenosine and deoxyguanosine (Plagemann and Erbe, 1974). These authors concluded that the inhibition of DNA synthesis (and of its initiation) was due to depletion of dATP and dGTP pools. One should view results obtained using such high concentrations of deoxynucleosides with caution. For example, Moore and Hurlbert (1966) have demonstrated that complex allosteric regulation of the enzyme ribonucleotide reductase exists. Inhibition of this enzyme has been reported by many authors to be the mode of action of HU (Young and Hodas, 1964; Wright and Lewis, 1974; Adams and Lindsay, 1964; and Adams *et al.*, 1966). Reversal of the HU block and the subsequent initiation of DNA synthesis by

deoxyadenosine and deoxyguanosine may have been due to allosteric regulation of the ribonucleotide reductase by the deoxyribonucleosides rather than to a simple increase in dATP and dGTP pool levels. Therefore, further studies are needed to elucidate those factors contributing to the initiation and maintenance of DNA synthesis.

2.4. General Properties of Parvoviruses

2.4.1. Classification

In 1959, Kilham and Olivier isolated and characterized viruses in rats from metastasizing sarcomas and a transplantable leukemia. These isolates of rat virus (RV) were shown to be resistant to ether and to hemagglutinate guinea pig erythrocytes. Subsequent isolations of viruses with similar characteristics were made from human cells, including H-1 and H-3 virus isolated from transplantable human tumors (Toolan *et al.*, 1960; Dalldorf, 1960), and HB and HT virus isolated from human placentas (Toolan, 1964). Mayor and Melnick (1966) established these viruses as a distinct group, the picodnavirus group, based on their 20 nm size, their ability to produce intranuclear inclusions, their DNA content, and their high buoyant density of 1.40 to 1.43 g/cm^3. The International Committee for Nomenclature of Viruses has since renamed this group the parvoviruses (Wildy, 1971).

Parvoviruses have also been isolated from calves (Abinanti and Warfield, 1961; Bates _et al_., 1972), adenovirus stocks (Atchison _et al_., 1965; Hoggan _et al_., 1966), hogs (Mayr _et al_., 1968), dogs (Binn _et al_., 1970), cats (Johnson, 1965), birds (Dutta and Pomeroy, 1967), and from a variety of cell lines (Hallauer _et al_., 1971).

Parvoviruses can be divided into two groups based on their ability to replicate autonomously (Melnick, 1971). The nondefective parvoviruses replicate in cells without the aid of a helper virus. Viruses within this group include RV, H-1, feline panleucopenia virus (FPV), porcine parvovirus (PPV), bovine parvovirus (BPV), minute virus of canines (MVC), and minute virus of mice (MVM) (Rose, 1974). The defective adeno-associated viruses (AAV) of human, simian, bovine, avian, and canine origin have been shown to require a helper adenovirus present in the cells for replication and maturation to occur (Rose, 1974).

2.4.2. Morphology and Size

The parvoviruses are very small icosahedral particles (Rose, 1974). Various members of the parvovirus group have been reported to range in size from 15 to 30 nm. The actual variation in size may be less since the size of particles examined electron microscopically may be due to the orientation of the particle or to the method of staining. Hoggan (1971) showed that 1% uranyl acetate caused the

parvoviruses to appear 12 to 15% larger than those stained with 2% phosphotungstic acid. A mean diameter range of 18 to 21 nm has subsequently been reported (Tinsley, 1973).

2.4.3. Stability

Parvoviruses are stable in the presence of ether and thus do not contain a lipid envelope. Although the stability of parvoviruses to heat varies to some extent, most are stable at 56 C for 30 min (Rose, 1974).

2.4.4. Hemagglutination

With the exception of MVC, all the nondefective parvoviruses examined are capable of agglutinating erythrocytes. Of the defective parvoviruses, only AAV-4 has the property of hemagglutination (HA) (Tinsley, 1973; Rose, 1974). The species of erythrocytes agglutinated has been used as a distinguishing characteristic of the members within the parvovirus group (Toolan, 1968).

2.4.5. Chemical Composition

2.4.5.1. Nucleic Acid

Intranuclear inclusions produced by the parvoviruses H-1, H-3, and RV, stained positively with the Feulgen stain suggesting that these viruses contained DNA. Acridine orange staining of the virions provided evidence that the DNA genome was single stranded (Mayor and Melnick, 1966). Later the single stranded nature of the genome was

confirmed by techniques such as sedimentation in alkaline sucrose and CsCl gradients, thermal denaturation, base composition, and digestion with single strand specific nucleases (Siegl, 1973; Salzman and Jori, 1970; Salzman _et al_., 1971; Rose and Koczot, 1971). The molecular weight of the single stranded genome of parvoviruses ranges from 1.35 to 1.7 x 10^6 daltons (Rose, 1974).

The single stranded nature of the genome of RV has been studied in great detail. Upon isolation of DNA with a buoyant density of 1.71 to 1.72 g/cm^3 from purified virions, Salzman and Jori (1970) found the base composition to be 26.7% A, 30.8% T, 20% G, and 22.5% C. Since the adenine content did not equal the thymine content nor did the guanine residues equal the cytosine residues, it was concluded that the DNA was not double stranded. Further evidence of a single stranded genome was obtained by reacting RV DNA with formaldehyde. Formaldehyde increases the absorbance of single stranded DNA at 260 nm by reacting with free amino groups. Intact RV virions showed a 13 to 16% increase in absorbance after reaction with formaldehyde while native double stranded DNA showed less than 2% increase. If the double stranded DNA were denatured to single strands, these single strands increased 10% in absorbance after reaction with formaldehyde.

RV also showed thermal melting characteristics unlike those observed for double stranded genomes (Salzman and Jori,

1970). Only a gradual increase in absorbance at 260 nm occurred between 50 C and 100 C and at 100 C there was only a 12.8% increase. Following rapid cooling, no residual hyperchromicity remained. These authors concluded that the lack of a sharp melting curve and the low residual hyperchromicity of RV DNA indicated that its genome was single stranded.

In further studies RV DNA was shown to be linear by electron microscopic examination and susceptibility to digestion with _E. coli_ exonuclease I. The circular genome of ØX 174 was not susceptible to exonuclease digestion, and when prepared in the same way as RV DNA, 80 to 90% of the DNA molecules appeared circular by electron microscopic examination (Salzman _et al._, 1971).

DNA isolated from Lu III virions was also shown to be single stranded (Siegl, 1973). Viral DNA was not eluted from a benzoylated naphtoylated DEAE Cellulose (BND-Cellulose) column under conditions required to elute double stranded DNA. Since the rate of digestion of Lu III DNA with exonuclease from _Verongia aerophoba_ occurred at the same rate as for known single stranded DNA, the genome may be linear. However, Siegl did not use ØX 174 as a control to determine if the method of extraction would be vigorous enough to nick circular DNA.

When DNA was extracted from purified AAV virions using standard procedures, the DNA exhibited characteristics

of double stranded molecules (Rose *et al.*, 1966; Parks *et al.*, 1967). However when the DNA was extracted with low ionic strength buffers, it was shown to be single stranded and could hybridize to form double stranded molecules (Mayor, *et al.*, 1969). Further evidence of the ability of AAV DNA preparations to form duplexes was independently shown by Rose *et al.* (1969). When heavy (2-bromodeoxyuridine-substituted) DNA from AAV was annealed with light (unsubstituted) viral DNA molecules with hybrid densities were detected. DNA at hybrid densities contained one strand of heavy and one strand of light DNA. Thus it was demonstrated that AAV virus preparations contained complementary plus and minus strands of DNA encapsidated into separate virions.

It was first suggested by Carter *et al.* (1972) that single stranded AAV DNA contained self-complementary sequences. On hydroxylapatite columns, 56% of intact minus strands remained bound at ionic strengths sufficient to elute single stranded DNA. However, sonic treatment of minus strands decreased by 50% the proportion of DNA eluted as double strands, indicating that many of the complementary sequences had been released. Furthermore, destruction of hydrogen bonds by heat decreased the amount of DNA binding as double stranded to 8%.

Evidence that the complementary sequences of AAV DNA were at or near the ends of the molecules was produced when denatured duplex AAV was shown to form stable, hydrogen

bonded circles upon reannealing (Koczot et al., 1973). The number of nucleotides involved in the self-annealed sequences was shown to be 400 to 500 bases determined by the amount of material resistant to single strand specific, S_1 nuclease (Koczot et al., 1973), and 1.5% of the genome based on electron microscopic examination (Berns and Kelly, 1974). Interestingly, the DNA of adenovirus which serves as a helper in the replication of AAV also possesses an inverted terminal sequence (Koczot et al., 1973).

2.4.5.2. Protein

The capsids of both defective and nondefective parvoviruses contain 2 or 3 polypeptides, the combined molecular weights of which exceed the coding capacity of the genome (Rose, 1974). H-1 virus grown in HE cells or in unsynchronized NB cells contains 3 distinct polypeptides by polyacrylamide gel electrophoresis. However, H-1 grown in synchronized cells contained only 2 capsid polypeptides whose molecular weights were 92,000 (VP 1) and 72,000 (VP 2') daltons. VP 2' was converted to a form which comigrated with VP 2 (69,000 daltons) isolated from unsynchronized NB cells. It appears that only 2 peptides are contained in the capsid of H-1 virus and that the third reflects an artifact (Kongsvik et al., 1974; Kongsvik and Toolan, 1972).

Gautschi and Siegl (1973) also found that only two polypeptides with molecular weights of 62,000 and 75,000 daltons were essential parts of infectious Lu III virions. However, a third polypeptide was always isolated from virus with buoyant densities of 1.35 and 1.37 g/cm^3. Thus they speculated that the third peptide was of host origin. FPV was shown to have 2 polypeptides. However, an additional minor peptide was present in some preparations (Johnson *et al*., 1974).

Johnson and Hoggan (1973) demonstrated 3 polypeptides in purified BPV and AAV. More studies are necessary to determine if one or more of these peptides is an artifact of the purification or extraction procedure or if these viruses differ from the previously mentioned parvoviruses in having an additional polypeptide.

2.4.6. Density and Sedimentation

The parvoviruses have been reported to have relatively high buoyant densities in CsCl ranging from 1.38 to 1.47 g/cm^3 (Tinsley, 1973; Rose, 1974). However, several parvoviruses have been shown to also contain particles banding at lower densities in CsCl. Three peaks of HA activity were observed for RV preparations centrifuged in CsCl--1.41, 1.38, and 1.36 g/cm^3. The particles banding at 1.41 g/cm^3 contained 80 to 90% of the infectivity associated with the RV preparation, while the

1.38 g/cm^3 band contained a majority of empty capsids. The lightest band contained mostly cellular debris (Salzman and Jori, 1970).

Lu III virus was also shown to contain particles banding at three densities in CsCl. This virus appears to be more dense than RV with HA activity observed at 1.444, 1.415, and 1.355 g/cm^3. All bands contained virions with at least some DNA since ^{3}H-TdR, which was used to label the virus before purification, was found at all densities. Although the ratio of HA activity to ^{3}H-TdR was comparable in the 2 most dense bands, a much higher HA to ^{3}H-DNA ratio was observed for the lightest band. Although virus with a buoyant density of 1.355 g/cm^3 contained some DNA, most of the HA activity was associated with empty or partially empty capsids. Interestingly, the DNA contained in virions banding at 1.444 and 1.415 g/cm^3 showed similar sedimentation properties (Siegl, 1973).

The buoyant densities of particles of FPV and mink enteritis virus (MEV) were comparable banding at 4 densities--1.44, 1.41, 1.36 and 1.31 g/cm^3. The proportion of complete to empty capsids was further shown to vary depending on the technique used for purification (Johnson *et al.*, 1974).

BPV was detected in three distinct bands in CsCl with buoyant densities of 1.30, 1.35, and 1.42 g/cm^3 observed. Although particles associated with the three densities

contained both HA activity and infectivity, the highest ratio of infectivity to HA activity was observed in the band whose density was 1.42 g/cm^3. In addition, a fourth band at 1.46 g/cm^3 was occasionally observed (Bates, 1972).

Most parvoviruses examined to date have sedimentation rates of 110 to 125 S. The rodent viruses RV, MVM, and H-1 have all been shown to sediment at 110 S while the defective parvoviruses AAV-1, AAV-2, AAV-4, have sedimentation rates of 125, 125, and 137 S, respectively (Rose, 1974).

2.4.7. Affinity of Parvoviruses for Cellular Membranes

The parvoviruses H-1 (Hampton, 1970), RV (Salzman *et al*., 1972), Lu III (Siegl and Gautschi, 1973b) and BPV (Bates and Storz, 1973) were shown to remain associated with cellular material, appearing in the extracellular medium only late during their replication cycles. Even extracellular Lu III virus remained associated to a large extent with cells and cellular debris (Siegl and Gautschi, 1973b). Titers of H-1 virus in stock preparations were shown to increase if the pH of H-1-infected cell preparations was brought to 8.0 before low speed centrifugation. In electron micrographs, clumps of H-1 virus were attached to cellular material at pH's of 7.5-7.9 but very little attachment occurred at pH 8.0. Further, the clumps were not affected by DNase treatment but were released following addition of the nonionic detergent, NP-40, which destroys

membranes. Thus, it was shown that at lower pH's, H-1 virus remains adsorbed to cytoplasmic membrane material and is lost in the pellet following low speed centrifugation (Gierthy et al., 1974).

2.5. Parvovirus-like Viruses

The bacterial virus ØX 174 was first shown to contain single stranded DNA in 1959. Although ØX 174 shares many characteristics in common with the animal parvoviruses including stability to heat and ether, a diameter of 25 nm, and a buoyant density of 1.43 g/cm^3 (Tinsley, 1973; Rose, 1974; Sinsheimer, 1959), it has been classified separately (Lwoff and Tournier, 1966).

The insect virus of Galleria mellonella, densonucleosis virus (DNV), also is characterized by properties including a diameter of 21 to 23 nm and a buoyant density of 1.44 g/cm^3. However, DNV does appear to contain more morphological subunits in its capsid than animal parvoviruses and has been placed into the group Densoviridae (Lwoff and Tournier, 1971).

2.6. Candidate Parvoviruses

Although slightly larger than most parvoviruses, the 27 nm virus associated with hepatitis A may be a member of the parvovirus group. Like parvoviruses, it has a buoyant density of 1.4 g/cm^3 and is resistant to ether and heat (Feinstone et al., 1974). The Norwalk Agent, shown to

have caused at least one outbreak of human nonbacterial gastroenteritis, is also 27 nm in diameter and appears parvovirus-like (Kapikian _et_ _al_., 1972). Another virus, 22 nm in diameter, has been isolated from the feces of human volunteers with and without gastroenteritis. Although this virus has not yet been shown to be the etiologic agent of gastroenteritis, its buoyant density of 1.38 g/cm^3 resembles that of the parvovirus, PPV, which was assayed in the same manner (Paver _et_ _al_., 1974). To date, a successful cell culture system for propagating these candidate parvoviruses has not been found. Because most methods used to fully characterize viruses require large quantities of purified virus grown under _in_ _vitro_ conditions, further characterization of these viruses requires the discovery of such a cell culture system which will allow replication of these viruses to occur.

2.7. Dependence of Nondefective Parvoviruses on the Physiological State of the Host Cell

During experimental inoculations of RV into suckling hamsters, Margolis and Kilham (1965) found that RV selectively injured germinal tissues, such as ondoblastic tissue and the cerebellum, resulting in tooth dysplasia and intractable cerebellar ataxia. In the germinal tissues of the cerebellum, the virus was capable of replication and of causing severe destruction of cells,

although no activity was evident in the adjacent Purkinje cell layer. The cells in the latter tissue are stationary while those in the germinal tissues are characterized by a high level of mitotic activity. In fact, these authors observed that the single feature common to all sites of viral involvement was the presence of populations of dividing cells. Margolis and Kilham thus hypothesized that the dividing cell was the primary target of RV. However, not all dividing cells were susceptible to active infection with RV. The mucosa of the gastrointestinal tract and the hematopoetic tissues, both containing numerous cells with mitotic activity, were not involved in RV replication.

In 1971, Tennant suggested that the events required by RV and present in actively dividing cells were associated with S phase. The highest efficiency of infection, based on the percentage of cells synthesizing viral specific antigen, was obtained if cells were infected 12 hr after stimulation with serum. At that time, cells were actively synthesizing DNA. Cells infected with RV 10 hr after serum was added were labeled with ^{3}H-TdR during adsorption. Following adsorption, the label was removed and the cells were incubated for 12 hr to allow the appearance of viral specific antigen. Simultaneous examination by immunofluorescence and autoradiography revealed that 95% of the cells containing viral specific antigens also incorporated

^{3}H-TdR at the time of infection. However, not all ^{3}H-labeled cells contained viral antigens. Tennant concluded that the increased efficiency of RV replication in cultures active in DNA synthesis was related to some event in S phase (Tennant, 1971).

Further investigations of RV infections in vitro confirmed the requirement by RV for cellular synthetic processes. Rat embryo cells exposed to low doses of ultraviolet (u.v.) light and X-irradiation did not support replication of RV. Similar irradiation had no effect on the replication of DNA-containing viruses such as herpes simplex virus (HSV), polyoma virus, SV-40, adenovirus, and pseudorabies virus. Further, 5-fluorouracil, which inhibits DNA, RNA, and protein synthesis in these cells, also inhibited RV though the replication of pseudorabies virus was unaffected (Tennant and Hand, 1970).

Similar results were obtained for in vivo infections of hamsters with RV (Lipton and Johnson, 1972). Control hamsters were injected with ^{3}H-TdR at 4 days after birth and the tissues prepared for autoradiography. Examination of hamsters inoculated with RV revealed that the distribution of cellular sites susceptible to RV infection corresponded to the distribution of TdR uptake. Again, although all cell types with viral antigen incorporated TdR in the control, not all cells incorporating TdR were susceptible

to RV. Therefore, DNA synthesis and mitotic activity alone did not determine the susceptibility of cells to RV *in vivo*.

PPV replication was also shown to depend on the cellular physiological state. PPV replicated at lower than optimum temperatures, although cell multiplication rate declined with temperature. However, there was a corresponding delay in the appearance of infectious virus and viral hemagglutinins at 33 C and 28 C. At 40 C replication was initiated quickly but soon declined. Furthermore, cells grown at this temperature showed a fast onset of multiplication which subsequently declined. Thus the kinetics of replication of PPV were dependent upon the kinetics of cell multiplication. Since PPV replication was initiated in all cells before maximum mitotic activity was achieved, it was proposed that some stages in DNA synthesis provided essential factors for PPV replication (Bachman, 1972).

Indirect evidence that FPV also has a predilection for cells in active mitosis was demonstrated by Johnson (1967). Immunofluorescence revealed that nuclei containing viral antigens were usually found in paired cells (presumably daughter cells) rather than single cells. In addition, efficiency of antigen production increased as cell density and density-dependent inhibition of DNA synthesis decreased. Replication of MVM was also shown to be density-dependent with the largest plaques obtained in plates with the lowest

density of cells (Tattersall, 1972).

Using synchronized populations of cells, it was possible to determine more precisely that portion of the cell cycle required for initiation of parvovirus replication. Synthesis of H-1 viral hemagglutinins was greatest 24 hr postinfection (p.i.) if cells synchronized in S phase by a double amethopterin block were infected during the first hour of S phase. If infection of cells was delayed until 4 hr into S, only half as much HA activity was observed at 24 hr p.i., and if delayed an additional 8 hr to the time of late S or early G_2 phase, little HA activity was observed. These results, together with the fact that DNA synthesis in these cells occurred at maximum rates 3 hr into S, led to the conclusion that the viral DNA was synthesized using the DNA synthetic system of the host cell (Hampton, 1970).

The minimum latent period of Lu III virus was also shown to occur if cells were infected during early S phase. In fact, a strict dependence on events occurring during S phase was found. Synchronized cells infected at the beginning of S phase and 3.5 hr later began to synthesize detectable viral specific proteins within the nucleus at the same time in the cell cycle. However, specific fluorescence was delayed in cells infected later than 3.5 hr into S phase. These results demonstrated that cellular events necessary for completion of Lu III replication occur

between mid S and the completion of S phase (Siegl and Gautschi, 1973a).

Completion of replicative events can best be determined by the appearance of HA activity or infectious progeny virus. Rhode (1973) showed that cells synchronized by serum stimulation produced HA activity at a time coinciding with the decline of cellular DNA synthesis if cells were infected at the beginning of S. Rhode concluded that S phase (i.e. DNA synthesis) alone was not sufficient for initiation of HA production. Rather, the fact that cells initiated DNA synthesis at least 5 hr before the viral DNA synthesis required for HA production (HA-DNA synthesis) suggested that a particular event occurring within S phase was required.

Curiously, cells infected with parvoviruses during late S or G_2 initiate viral replication before the subsequent S phase. Siegl and Gautschi (1973a) showed that the kinetics of replication of Lu III virus in cells infected during late S and G_2 resembled closely those observed in asynchronous cell cultures. Further, although stationary rat embryo cells supported very little replication of RV, cells infected 24 hr after serum stimulation (G_2 phase) produced some FA positive cells 12 hr p.i. (Tennant et al., 1969). Although the percentage of FA positive cells was less than that observed for cells infected at the beginning

of S, production of viral antigen was initiated before the subsequent period of DNA synthesis.

Explanation for this leaky dependency remains unclear. However, this situation could result from the existence of a factor required for parvovirus replication during all or most stages of the cell cycle in dividing cells. The level of this factor could be produced in a larger quantity during a particular time during S phase (i.e. mid or late S). Further studies are necessary to elucidate the exact mechanism required for initiation of parvovirus replication.

2.8. Kinetics of Replication of the DNA of Bacteriophage ØX 174

Although ØX 174 has been placed into a distinct group separate from the animal parvoviruses (Lwoff and Tourier, 1966), the presence of a single stranded DNA genome similar to genomes of the animal parvoviruses dictates that replication of ØX 174 DNA may be similar to that for parvovirus DNA. Furthermore, the events occurring during replication of DNA can be more easily studied in well-characterized bacterial hosts than in mammalian cells whose regulation remains unclear. Therefore, because the replication of ØX 174 DNA has been studied in great detail while little is known of the replication of parvovirus DNA, it is appropriate that ØX 174 replication serve as a model for the replication of the DNA of animal parvoviruses.

Sinsheimer _et_ _al_. (1968) showed that upon entry into the bacterial cell, the circular single stranded DNA genome of ØX 174 is converted to a double stranded replicative form (RF) using preexisting host enzymes. Both covalently closed and open circular forms can be found at this time. The RF subsequently becomes associated with a preexisting site on the membrane. This association is necessary in order for further replication to occur. The number of sites is limited, however, reducing the number of RF's which can be replicated in a given host.

Semiconservative replication of the attached RF then ensues. The parental viral strand (plus) was shown to remain associated with the membrane site since labeled infecting DNA was never recovered from progeny virus at low multiplicities of infection (Sinsheimer _et_ _al_., 1968). Replication of the parental RF then takes place by means of the rolling circle mechanism of DNA replication to produce progeny RF's utilizing both host and viral encoded enzymes (Davis _et_ _al_., 1973). These daughter RF's which are replicated until 12 min p.i. are released into the cytoplasm (Sinsheimer _et_ _al_., 1968).

Following this phase of replication, host DNA synthesis ceases together with the cessation of net RF synthesis. Synthesis of progeny single stranded DNA subsequently begins by means of the rolling circle model using the daughter RF's as templates (Davis _et_ _al_., 1973). In order

for single stranded DNA to be produced, progeny RF's must be in the open circular form. The single stranded DNA produced is immediately encapsidated, since free single strands of DNA have never been found in ØX 174-infected cells (Sinsheimer *et al.*, 1968).

2.9. Kinetics of Replication of Animal Parvoviruses

2.9.1. Nondefective Parvoviruses

Most of the nondefective parvoviruses show similar kinetics of formation of viral DNA, viral antigens, and infectious progeny. Although cultural conditions for separate studies differ with respect to the degree of synchrony of the cells, the stage of the cell cycle in which cells are infected, and the multiplicity of infection, the latent periods observed before appearance of viral progeny are between 6 and 16 hr after infection. In general, viral DNA synthesis was shown to occur immediately before synthesis of infectious virus.

Of the nondefective parvoviruses, the type species RV has been studied in the greatest detail. Cells infected with RV at a multiplicity of 4.7 plaque forming units (PFU)/cell showed increasing titers of infectious virus at 12 to 14 hr p.i. The titer subsequently increased exponentially reaching maximum levels at 23 hr p.i. of approximately 200 times the level observed during the latent period (Salzman *et al.*, 1972).

The relatively long latent period observed in this study may reflect the use of stationary cells for infection. Heavily monolayered cells were infected and subsequently incubated in medium containing only 5% serum. Although some stimulation of DNA synthesis in mock-infected cells was shown (Salzman *et al.*, 1972), the level was not representative of synchronized cells. In fact, under these conditions only 30% of the cells became infected (Salzman and White, 1973). Furthermore, the latent period for replication of RV was indeed shorter. Using a multiplicity of 10, Salzman *et al.* (1972) reported a latent period of only 6 hr.

It was concluded that RV DNA synthesis was initiated by 8 hr p.i. because total DNA synthesis in cells infected with the lower multiplicity increased at that time. Thus viral DNA was synthesized 4 hr before the appearance of viral progeny in these cells. Since 5-fluorodeoxyuridine (FUdR) reduced the final titer of RV if added at any time prior to 23 hr p.i., viral DNA synthesis appeared to be required for optimum replication during the entire replication cycle (Salzman *et al.* 1972). However, the use of FUdR inhibits cellular DNA synthesis and thus the reduction of viral replication might have been due to an indirect inhibition of cellular synthesis.

Due to the close correlation in the time of production of infectious progeny and the time of DNA synthesis

determined above, it is likely that progeny DNA synthesis was measured rather than the initiation of synthesis of double stranded forms. The initiation of viral DNA synthesis was more precisely determined by extraction of low molecular weight DNA using the Hirt procedure (Salzman and White, 1973). By infecting cells with RV whose single stranded DNA was labeled with ^{3}H-TdR, it was found that radioactive DNA sedimented at two rates in neutral sucrose density gradients beginning at 60 min p.i. Because the slower moving band of radioactive DNA sedimented with purified RV single stranded DNA, the faster moving band was assumed to be a double stranded form. Evidence supporting the double stranded nature of this band included the movement of radioactivity to a denser region upon denaturation in alkaline CsCl and the fact that the sedimentation rate of 14.5 S suggested a molecular weight twice that of single stranded RV DNA. The double stranded form was hypothesized by these authors to be an early replicative form.

RV-hybridizable RNA was shown to increase at 2 hr p.i. (Salzman and Redler, 1974). At that time double stranded viral DNA replication had been initiated (Salzman and White, 1973) but progeny DNA was not produced until 6 hr after viral specific RNA was shown to increase (Salzman et al., 1972). These authors suggest that the lag in synthesis of progeny DNA and viral RNA may imply that the

RNA codes for an enzyme or a structural protein required for the initiation of progeny DNA synthesis (Salzman _et al._, 1972). However, the RNA detected was not necessarily functional mRNA since total and not polysomal RNA was extracted. In addition, these authors used RV viral DNA as a probe for RNA hybridization. Although it is possible that RV RNA is transcribed from the viral strand, ØX 174 RNA and AAV RNA are transcribed from the complementary minus strand (Hayashi _et al._, 1963; Rose and Koczot, 1971).

The latent period observed for Lu III virus replication was shown to vary depending on the time of infection. Cells infected with 10 tissue culture infective doses $(TCID)_{50}$/cell at the beginning of S phase or at 3.5 hr into S produced intranuclear viral antigens 10 hr into S phase. A longer latent period was observed for asynchronous cells and cells infected in late S or G_2 (Siegl and Gautschi, 1973a). The minimum latent period of 6.5 hr observed for Lu III compares well with the 6 hr latent period for RV at a comparable high multiplicity of infection. Following the latent period, Lu III virus-specific intranuclear antigen increased synchronously and reached maximum levels at 14 hr p.i. if cells were infected at 3.5 hr into S.

At various times postinfection, Lu III-infected cells were labeled for 2 hr with ^{3}H-TdR and harvested at 24 hr p.i. Viral specific DNA synthesis was then determined by the level of ^{3}H-TdR incorporated into purified virus.

Using this procedure, viral DNA synthesis preceded intranuclear antigen formation by 2 hr. Furthermore, inhibition of DNA synthesis with FUdR, cytosine arabinoside (ara C), or mitomycin C at 6 hr p.i. resulted in inhibition of intracellular antigen. However as pointed out by these authors, it cannot be determined by this experiment whether the inhibition of antigen formation reflects the inhibition of cellular DNA synthesis required for viral replication or the inhibition of viral DNA synthesis (Siegl and Gautschi, 1973b).

In asynchronous cells, H-1 viral antigen does not increase until 12 to 16 hr p.i. (Hampton, 1970). However, in cultures synchronized with high concentrations of serum and infected during early S, synthesis of hemagglutinins and intranuclear antigens was detected at 10 hr p.i. (Rhode, 1973). Viral DNA extracted by the Hirt procedure to separate it from cellular DNA was detected at 6 to 8 hr p.i. (Rhode, 1974). The time of appearance of the DNA precedes antigen formation by at least 2 hr but occurs 5 to 7 hr after the initiation of cellular DNA synthesis--that is, during late S phase (Rhode, 1973).

Ara C added to H-1-infected cells up to 8 hr p.i. completely inhibited HA production. However, HA production became resistant to ara C if the inhibitor was added after 8 hr. Thus DNA synthesis upon which subsequent HA production is dependent (HA-DNA synthesis) was initiated at 8 hr p.i.

The possibility that ara C inhibited HA production indirectly by inhibition of viral DNA synthesis is reduced by the fact that HA-DNA synthesis began at approximately the same time as Hirt-extracted viral DNA (Rhode, 1974). Nevertheless, HA-DNA synthesis and viral DNA synthesis may not be the same event because the possibility remains that by 8 hr into S, the function associated with DNA synthesis and required for subsequent viral DNA replication has already been expressed and is not subject to inhibition by ara C.

The latent periods of PPV and BPV have been reported to be 12 and 16 hr respectively (Bachman, 1972; Bates and Storz, 1973). However, under the suboptimum conditions of cultivation of cells at 33 C and 28 C, progeny PPV was not observed until 16 and 26 hr p.i. (Bachman, 1972). Because the above latent periods were determined in asynchronous cells, the time required for progeny virus production may be less.

2.9.2. Defective Parvoviruses

Replication of the defective parvoviruses and that of the nondefective parvoviruses is similar. However, they differ in that the initiation of replicative events of the defective parvoviruses requires helper functions provided by adenoviruses or herpesviruses while nondefective parvovirus replication depends upon the physiological state of the host cell.

Separate helper functions of adenovirus were shown to be provided for AAV DNA and RNA synthesis (Carter *et al.*, 1973). When cells were infected simultaneously with adenovirus and AAV, AAV DNA synthesis began at 6.5 to 7.5 hr p.i. although AAV specific RNA did not appear until 9-10 hr p.i., and infectious progeny did not increase until 17 hr p.i. (Carter *et al.*, 1973; Rose, 1974). The initiation of AAV RNA synthesis coincided with a rapid increase in adenovirus specific RNA (Carter *et al.*, 1973). If cells were infected with AAV 10 hr after adenovirus infection, both AAV DNA and RNA synthesis were initiated simultaneously at 3 to 4 hr p.i. with the latent period reduced to 4 to 6 hr (Carter *et al.*, 1973; Rose, 1974). Thus, in the simultaneous infection, adenovirus provided helper functions for AAV DNA synthesis prior to those required for AAV RNA synthesis. When adenovirus infected cells were superinfected with AAV, functions required for both AAV DNA and RNA synthesis had been provided. Therefore, initiation of AAV DNA and RNA synthesis occurred at the same time.

Completion of AAV replicative events, including RNA synthesis, antigen formation, and production of infectious progeny, appears to require viral DNA synthesis. In the presence of FUdR, AAV RNA was not initiated (Carter *et al.*, 1973); and ara C added before 6 hr p.i., prevented formation of AAV progeny (Drake *et al.*, 1974). However AAV specific

antigens, RNA, and infectious DNA, do not require synthesis of helper virus DNA since these are produced at the nonpermissive temperatures for DNA replication of temperature sensitive mutants of adenovirus and HSV (Mayor and Ratner, 1973; Drake et al., 1974). Production of infectious AAV virions at nonpermissive temperatures has also been reported using a temperature sensitive mutant of adenovirus unable to synthesize adenovirus DNA and completely deficient in adenovirus late protein synthesis (Straus et al., 1974). Thus viral DNA synthesis inhibited by ara C and FUdR but required for subsequent AAV replication does not appear to be adenovirus DNA synthesis, but rather AAV DNA synthesis. Although the ts mutants do not synthesize any detectable helper virus DNA, some events associated with adenovirus DNA replication may occur and at least one of these events may be required for AAV replication. This hypothesis would explain the finding that both adenovirus DNA and AAV DNA are synthesized concomitantly in simultaneous infections (Carter et al., 1973).

HSV provides helper functions needed for AAV RNA, DNA, and antigen synthesis. However, complete virions are not synthesized with HSV as a helper virus, but are produced with adenovirus as a helper. Therefore, one of the helper functions provided by adenoviruses may be assembly (Carter et al., 1973). Further investigations

are necessary before those functions provided by the helper viruses can be determined.

2.10. Properties of DNA Produced During Infection with Parvovirus

During the replication of ØX 174, covalently closed circular and open circular forms of double stranded DNA have been isolated and have been shown to play a role in the replication of the single stranded DNA genome (Fiers and Sinsheimer, 1962; Sinsheimer *et al.*, 1968). Double stranded viral DNA forms have also been isolated from cells infected with RV, H-1 virus, MVM, and AAV.

RV DNA extracted by the Hirt procedure exhibited a faster rate of sedimentation in neutral sucrose gradients than single stranded RV DNA, although in alkaline sucrose, its sedimentation was the same. In addition, the form eluted as double stranded DNA from hydroxylapatite had a molecular weight twice that of single stranded RV DNA (Salzman and White, 1973). It was postulated that this double stranded DNA was a functional replicative form of RV.

Double stranded RF forms have also been reported for H-1 virus (Rhode, 1974). These double strands had a buoyant density of 1.705 g/cm^3 in neutral CsCl and were shown to contain viral DNA by competition hybridization with H-1 DNA. Although ØX 174 produces covalently closed

circular RF's, sedimentation in the presence of the intercalating agent, ethidium bromide, produced only a minute amount of DNA at the high buoyant density characteristic of covalently closed circles. Thus, the isolated RF of H-1 is linear or is in an open circular configuration.

Tattersall _et al_. (1973) showed that 20 to 40% of the double stranded DNA of MVM was resistant to exonuclease and partially resistant to S_1 nuclease. Although these results are consistent with the presence of covalently closed circular forms, no DNA was found to sediment at the rate expected for these forms. The resistance to nuclease could be explained if spontaneous intramolecular renaturation occurred and no 3'-hydroxyl terminus was exposed.

AAV-4 double stranded DNA isolated by the Hirt procedure had a buoyant density of 1.699 to 1.710 g/cm^3 in CsCl, and circular forms were not seen upon electron microscopic examination. In addition, the DNA was shown not to be infectious (Hadidi _et al_., 1973). Infectious DNA has been isolated from AAV-4 infected cells at nonpermissive temperatures using a temperature sensitive mutant of HSV-1 as helper (Mayor _et al_., 1974). The discrepancy in infectivity of AAV-4 DNA most likely reflects poor adsorption of DNA to the indicator cells in the former case with more optimum conditions used in the latter. Although infectious, the AAV-4 DNA made at nonpermissive temperatures for the replication of HSV DNA had a density of 1.68 g/cm^3

while that made at permissive temperatures had a buoyant density of 1.72 g/cm^3. AAV-1 double stranded DNA has also been shown to be infectious (Boucher *et al.*, 1971).

2.11. Properties of Viral RNA Transcribed During Parvovirus Infections

Viral specific RNA isolated from 8 to 24 hr p.i. represented only 0.5 to 1% of the total RNA isolated from RV-infected cells. This RNA had a base composition complementary to the RV virion DNA and hybridized specifically to this DNA. Therefore, Salzman and Redler (1974) concluded that RNA was transcribed from the infecting plus strand of viral DNA. However, total RNA rather than messenger RNA was isolated in these studies and hybridization to negative DNA strands was not attempted.

Viral specific RNA labeled at 5 to 6 hr p.i. showed a sedimentation profile of predominantly an 18 S species (7.0×10^5 daltons) representing 40 to 50% of the RV genome and a minor peak of 26 S (1.7×10^6 daltons) representing the entire genome. A similar profile was obtained after a 16 hr labeling period so it was concluded that cleavage of the 26 S species to the 18 S species did not occur (Salzman and Redler, 1974).

Evidence has been presented that AAV RNA is transcribed from the complementary minus strand rather than the viral DNA strand. The minus strand is thymine rich and has a higher buoyant density than the plus strand when

5-bromodeoxyuridine is substituted for thymine bases. RNA isolated during AAV infections was shown to hybridize to the heavy, minus strand only. Furthermore, the base composition of AAV RNA was similar to that of the infecting plus strand (Rose and Koczot, 1971). In subsequent studies, Carter et al. (1972) showed that AAV DNA-DNA hybridization was inhibited 35 to 40% by prior incubation with AAV RNA. Therefore, since only the minus strand of DNA was complementary to RNA, 70 to 80% of the minus strand was transcribed.

The sedimentation velocity of AAV specific RNA produced at 4 hr p.i. was demonstrated under denaturing conditions to be 20 S corresponding to a molecular weight of 0.9 to 1.0×10^6 daltons (Carter, 1974). This molecular weight represents 65 to 74% of the genome transcribed, calculated using hybridization techniques (Carter et al., 1972; Carter and Rose, 1974). Similar degrees of transcription for the AAV genome with HSV used as a helper are likely since AAV RNA produced during coinfection with HSV showed the same strand specificity, hybridization inhibition, and velocity sedimentation as that produced in the presence of helper adenoviruses (Carter and Rose, 1972).

As shown for RV RNA, no posttranscriptional cleavage was observed for AAV RNA (Carter and Rose, 1974). Both nuclear and polysomal AAV RNA's were similar in size. Moreover, pulse labeled 20 S nuclear species could be

chased without cleavage to the cytoplasm in the absence of radioactive label. As a control, these techniques were shown to allow the detection of cleavage of adenovirus RNA.

2.12. Effect of Parvovirus Replication on Host Cell Macromolecular Synthesis

There is little agreement on whether parvovirus replication increases or decreases DNA, RNA, and protein synthesis. Rat embryo cells infected with RV at a multiplicity of 10 PFU/cell immediately before the addition of fresh serum showed a decrease in the rate of ^{3}H-TdR incorporation by 5 hr p.i. with respect to the controls. Furthermore, under these culture conditions, control cells began to synthesize DNA at 10 hr p.i., although the rate in infected cells remained low at that time. Not all cells were prevented from entering S phase since autoradiographic analysis revealed incorporation of ^{3}H-TdR to the same extent as control cells even though the multiplicity of infection was high enough to allow all cells to become infected with RV (Tennant, 1971). In synchronized cells the rate of total DNA synthesis was also shown to decrease early. However, between 8 to 23 hr p.i., levels in infected cells surpassed those in control cultures (Salzman *et al.*, 1972). The higher level of total DNA synthesis was probably due to synthesis of viral DNA in cells not actively synthesizing cellular DNA.

The rate of ^{3}H-TdR incorporation in cells infected with H-1 virus was not significantly reduced during the first 24 hr after infection (Hampton, 1970). However, at later times there was a marked inhibition in the rate of TdR incorporation. Autoradiographic analysis of infected cells revealed that the decreased rate was due to a decrease in the rate per cell (due to a lower density of labeling) rather than a decrease in the number of cells synthesizing DNA (Fong et al., 1970).

The total rate of DNA synthesis in rapidly dividing monolayers of cells infected with MVM was not altered through 4 hr p.i. (Tattersall, 1972). A slight depression in ^{3}H-TdR incorporation was observed in suspension cultures between 10 and 20 hr p.i. (Tattersall et al., 1973). Although DNA synthesis is depressed to some extent within cells infected with MVM, the degree of inhibition appears to be less than that observed with H-1 virus or RV.

RNA synthesis is not reduced in RV-infected cells. Tennant (1971) reported that uridine incorporation in cells infected with RV was virtually unaffected with respect to controls. The total rate of RNA synthesis in RV-infected cells was reported by Salzman et al. (1972) to decrease until 2 hr p.i. However, after that time, the total rate of uridine incorporation remained the same or higher than that in control cells. In contrast, hybridization assays of host specific RNA showed a progressive inhibition of

cellular RNA after RV infection even in the presence of control levels of RNA synthesis (Salzman and Redler, 1974). It seems that the maintenance of a high level of total RNA synthesis in infected cells may reflect synthesis of viral specific RNA species.

H-1 virus decreases the rate of uridine incorporation in infected cells only after 20 hr p.i. At that time the level of incorporation was only 50% of control values while that at 70 hr p.i. was only 7% of control rates. No difference in soluble pools of uridine for infected and uninfected cells was observed through 63 hr p.i. Thus, the decrease in rate did not reflect a reduced rate of uridine transport. Further, fewer cells were incorporating ^{3}H-uridine later after infection indicating that the decrease in RNA synthetic rate was due to the number of cells capable of synthesizing RNA (Fong et al., 1970).

Protein synthesis in RV-infected cells was initially depressed as was RNA synthesis, but subsequently increased and was maintained at 75 to 100% of the control rate throughout the 23 hr replication cycle (Salzman et al., 1972). Total protein synthesis was shown to be affected very little until 36 hr p.i. when cytopathic effects (CPE) became evident (Tennant, 1971). However, these latter cells were infected 10 hr before S phase in serum stimulated cells and the length of time required before CPE was evident may reflect these suboptimum conditions.

Cellular mitosis has been shown to be inhibited after infection for a variety of parvoviruses including Lu III, RV, H-1, and MVM. The mitotic activity of cells infected during early S with Lu III virus was completely suppressed. If cells were infected at later times during S and G_2, less reduction in the mitotic index of infected cells was observed. In fact, if cells were infected as late as G_2, cells divided normally (Siegl and Gautschi, 1973a). Reduction in mitotic activity may not be due solely to replication leading to infectious virus since infection of cells in late S with Lu III virus inactivated with ultraviolet light produced a comparable reduction in the mitotic index. UV inactivated virus inoculated in early S delayed mitosis by 3 hr and the mitotic wave was delayed by several hours when cells were infected with intact Lu III during late S.

RV produced a rapid inhibition of mitosis rendering all cells unable to divide as early as 2 hr p.i., although less than 30% of the cells actively synthesized virus as determined by immunofluorescence (Tennant, 1971). Because cells continue to become actively infected with RV, Tennant concluded that the cells must continue to cycle and that the block in mitosis may be temporary. However, Lu III virus has been shown to initiate replication at times after S phase (Siegl and Gautschi, 1973a). Thus it

is possible that cells blocked from entering mitosis could initiate replication of RV without cycling.

The total number of NB cells one day after infection with H-1 virus was less than the number in uninfected controls. From these results alone, Fong et al. (1970) concluded NB cells did not divide after infection with H-1 virus. These results could also be explained by the sloughing of infected cells due to CPE or to a delay in mitotic activity. Hampton (1970) reported that infection of cells in S phase or late G_2 with H-1 produced a 50 to 75% inhibition of mitosis although the method used for this determination was not stated. Thus, although there is circumstantial evidence that H-1 virus inhibits mitosis to some extent, no conclusive evidence has been presented. BPV may not cause permanent inhibition of mitosis since binucleated cells which were also FA positive were often observed (Bates, 1972). Whether the differences observed for the effects of the various parvoviruses on the synthesis of DNA, RNA, and protein, and on mitosis reflect differences in the viruses or differences in culture conditions will require further investigation.

3. Materials and Methods

3.1. Preparation of Cell Cultures

Bovine fetal spleen (BFS) cell cultures were prepared from spleens of 5 to 7 month bovine fetuses by the method of Youngner (1954). Cells were routinely maintained in Eagle's minimum essential medium (MEM) supplemented with 10% lamb serum and were subcultured every 6 to 7 days. Only those cells in the tenth to twentieth passages were used.

3.2. Synchronization of Cells

BFS cells were synchronized by seeding 1 ml of cell suspension into Leighton tubes or 5 ml into plastic petri dishes (60 x 15 mm) at a concentration of 1 to 1.6×10^5 cells per ml of synchronization medium. Synchronization medium, prepared immediately before use, consisted of MEM containing 10% serum and varying concentrations of HU. Cells were released from the HU block by washing at the appropriate time.

3.3. Propagation of Bovine Parvovirus

Stock pools of bovine parvovirus (BPV) originally obtained from the isolates of Abinanti and Warfield (1961) were prepared by seeding 2.75×10^6 BFS cells into 250 ml plastic flasks (Falcon), and inoculating cells with BPV

18 to 24 hr after seeding. Occasionally, stock virus was prepared from HU-synchronized cells by inoculating cells immediately after release from the HU block. Following an adsorption period of 30 min to 1 hr, MEM containing 10% lamb serum was added to each flask. Cells were harvested 48 to 72 hr later when 95 to 100% of the cells exhibited CPE. Inoculated flasks were stored at -20 C and frozen and thawed three times before the material was combined. The medium containing virus and cell debris was subsequently centrifuged at 500 x g. The resulting supernatant was stored at -20 C and titrated by plaque assay before use.

3.4. Inactivation of Bovine Parvovirus Infectivity by Ultraviolet Irradiation

Infectivity of BPV stock preparations, as demonstrated by plaque assay, was completely inactivated by exposure of 1 ml of virus in open petri dishes (60 x 15 mm) to a 254 nm ultraviolet light (19.5 watts) for 5 min at a distance of 2.5 cm.

3.5. Purification of Bovine Parvovirus

3.5.1. Procedure for Obtaining Purified Virions

BFS cells synchronized with 2 mM HU were infected with a multiplicity of infection (m.o.i.) of 10 to 20 PFU of BPV/cell immediately after release from the HU block. Following a 2 to 3 hr adsorption period, the unadsorbed

virus was removed and MEM containing 10% lamb serum was added. At 36 hr p.i. when cultures exhibited 90 to 100% CPE, medium was collected. Those cells remaining attached to the glass were removed by treatment with trypsin-versene solution and combined with the medium. The following steps were followed to collect and purify virus from infected cells.

1. The medium containing infected cells was centrifuged at 10,000 x *g* in an SS-34 rotor (Sorvall) for 15 min at 4 C.
2. The cell pellets were resuspended in tris buffered saline (TBS; 0.05 M Tris, 0.1 M NaCl, pH 8.0) and stored at -20 C until the remainder of the purification procedure was initiated.
3. Following thawing, cells were sonicated at an output of 83 watts using a Virtis Virsonic Cell Disrupter until the suspension appeared opalescent (15 to 20 sec).
4. The sonicated cellular material was layered over a 30 ml discontinuous 10-30% sucrose gradient containing either 0.2% sodium docecyl sulfate (SDS) or 0.5% sodium dodecyl sarcosinate (Sarkosyl).
5. Gradients were centrifuged at 63,000 x *g* for 3 hr at 4 C (Sarkosyl) or at 15 C (SDS) in an SW 25.1 rotor (Beckman).

6. The top 5 ml was discarded from each gradient and the remaining supernatant centrifuged at 114,000 x *g* for 5 hr at 4 C in a type 50.1 rotor (Beckman) to sediment the virus.
7. The pellet was resuspended in TBS and sonicated as before.
8. One-half ml of sonicate was layered over 4.5 ml CsCl at a density of 1.42 g/cm^3 and centrifuged at 114,000 x *g* at 4 C for 24 hr.
9. Gradients were fractionated by bottom puncture and fractions with a density of 1.40 to 1.43 g/cm^3 were combined and brought to 1.42 g/cm^3 with CsCl. Occasionally, fractions with a density of 1.38 to 1.34 g/cm^3 or 1.30 to 1.33 g/cm^3 were combined. The density of this material was made 1.35 g/cm^3 or 1.32 g/cm^3 respectively using CsCl. All CsCl gradients were then centrifuged at 114,000 x *g* at 4 C for 48 hr. Densities were calculated from refractive indices measured with a Bausch and Lomb refractometer.
10. Gradients were fractionated as before and material sedimenting at a density of approximately 1.42, 1.35, and 1.32 g/cm^3 was collected, dialyzed overnight against phosphate buffered saline (PBS, 0.01 M KPO_4, 0.15 M NaCl, pH 7.2), and stored at -20 C.

3.5.2. Negative Staining and Electron Microscopy

Dialyzed preparations of purified BPV were placed on 400-mesh copper grids coated with parlodion and carbon. The grids were subsequently washed with distilled water and stained 1 min with 1% uranyl acetate.

Preparations of BPV in CsCl examined prior to dialysis were added to grids placed film side up over 1% Ionagar. The CsCl was allowed to be adsorbed into the agar and the grids washed in distilled water and stained 1 min with 1% uranyl acetate. Grids were examined using a JEOL JEM 100B electron microscope.

3.6. Procedures to Determine Extent of Replication of Bovine Parvovirus

3.6.1. Fluorescent Antibody Staining

BFS cells grown on coverslips in Leighton tubes were harvested at various times after infection with BPV. Coverslips were washed briefly in Dulbecco's phosphate buffered saline and fixed 5 min in acetone at -20 C, dried, and stored at -20 C until stained. Prior to staining, coverslips were washed in PBS and dried. Cells were stained 20 min with antiserum prepared in rabbits against purified BPV and conjugated with fluorescein isothiocyanate dye. Conjugated antiserum had previously been adsorbed with bovine spleen powder to remove nonspecific antibodies.

Following staining, coverslips were washed three times in PBS, mounted onto slides with glycerol PBS, and examined with ultraviolet illumination using a Zeiss model RA microscope equipped with exciter filter BG-3 and barrier filters 47 and -65.

3.6.2. Preparation of Samples for Hemagglutination and Infectivity Assays

Petri dish cultures of cells infected with BPV were harvested at various times postinfection by removing the medium, adding 1 or 2 ml of MEM, and scraping cells into plastic tubes. If significant CPE was apparent, the medium was centrifuged at 750 x g and the cell pellet resuspended in MEM and added to the cell layer in the dish before scraping. Samples were stored at -20 C. After thawing, samples were sonicated for 15 sec in a Virsonic Cell Disrupter at an output of 83 watts and assayed for hemagglutinating antigen and infectious virus.

Leighton tube cultures of infected cells were occasionally used but were harvested by freezing and thawing the tubes three times prior to assaying the medium.

3.6.3. Hemagglutination Assay

The titer of hemagglutinating antigens in samples prepared as described was determined using the microtiter method (Sever, 1962). Guinea pig erythrocytes at a

concentration of 0.5% were added to all dilutions and samples were routinely incubated at 25 C for 4 hr before titers were read.

3.6.4. Plaque Assay

BFS cells were seeded into petri dishes (60 x 15 mm) at a concentration of 0.8 to 1.0 x 10^6 cells per dish in MEM with 10% lamb serum. The cell layers were washed and infected 18 to 24 hr later with 0.25 ml of each virus dilution. Virus adsorption was allowed to occur for 30 min at 37 C and the agar overlay was then added. The overlay consisted of MEM, 10% lamb serum, and 1% Ionagar. Following an incubation period of 6 to 7 days at 37 C, plaques were visualized by staining with neutral red or crystal violet.

3.7. Radioisotope Techniques

3.7.1. Labeling with Radioactive Precursors

^{3}H-TdR (18.4 Ci/mmole), ^{3}H-uridine (28 Ci/mmole), and ^{14}C protein hydrolysate (55 mCi/mAtom) were used. The rates of DNA and RNA synthesis were determined by adding ^{3}H-TdR or ^{3}H-uridine directly to the medium to a final concentration of 0.5 µCi/ml for 15 and 60 min respectively. The rate of protein synthesis could not be measured by adding labeled precursors directly to the medium because of the high concentration of amino acids

in MEM. Therefore, before addition of ^{14}C protein hydrolysate, the medium was removed and similar medium without amino acids but containing 3% lamb serum was added. Then ^{14}C protein hydrolysate was added for 1 to 2 hr to a final concentration of 0.1 µCi/ml. The addition of medium without amino acids did not affect the availability of amino acids to the cells since uptake of the ^{14}C protein hydrolysate was linear for at least 2 hr.

3.7.2. Measurement of Rates of DNA, RNA, and Protein Synthesis

Petri dish cultures of cells were assayed for the amount of ^{3}H-uridine and ^{14}C amino acids incorporated into trichloroacetic acid (TCA)-insoluble material by the method of Regan and Chu (1966). TCA-soluble pools were assayed at 4 C by precipitating sonicated cellular material prepared as above in 5% TCA for 10 min and centrifuging at 800 x g. Aliquots from the supernatant were added to vials containing 10 ml Dimilume (Packard) and counted using a Packard Tri-Carb liquid scintillation spectrometer. Counts per minute for both TCA-soluble and insoluble material were standardized on the basis of protein content by the method of Lowry et al. (1951).

Cells which were grown on coverslips and pulse labeled were fixed in 1% $HClO_4$, rinsed in water, and dried. The rate of DNA synthesis was determined by breaking the coverslips labeled with ^{3}H-TdR into liquid scintillation

vials and solubilizing overnight in 0.5 ml Soluene 350 (Packard). Toluene-based scintillation fluid was then added and the samples were counted by liquid scintillation spectrometry.

3.7.3. Autoradiography

Coverslips fixed in acid as described above were mounted on microscope slides with Permount and dipped in NTB-2 photographic emulsion (Kodak). Slides were stored in the dark at 4 C for 2 to 3 days before developing in D-19 (Kodak). Developed slides were stained by the Giemsa method and in all cases, 300 to 500 cells per slide were scored.

3.7.4. Labeling of Virion DNA

BFS cells were synchronized with HU and infected with BPV at an m.o.i. of 10 to 20 PFU/cell immediately after release from the HU block. Following a 3 hr adsorption period, virus was removed and MEM containing 10% lamb serum and 1.5 μCi/ml of ^{3}H-TdR (18.4 Ci/mmole) was added. Infected cells were collected at 36 hr p.i. and BPV containing ^{3}H-TdR labeled DNA was purified as described previously.

3.8. Extraction of Bovine Parvovirus DNA

3.8.1. Extraction from Purified Bovine Parvovirus

BPV DNA was extracted from purified virions with a

buoyant density of 1.42 g/cm^3 by the enzyme digestion treatment described by Salzman and White (1973). Briefly, β-mercaptoethanol was added to purified BPV in 0.05 M Tris, pH 6.8, to a final concentration of 0.3 M. Papain (7 Units/ml) was added and the preparation incubated 16 hr at 37 C. Trypsin (100 µg/ml) was then added and digestion continued another 3 hr followed by a 30 min incubation at 37 C with 2 mg/ml pronase which had been previously self-digested by incubation at 80 C for 1 min and 37 C for 6 hr. SDS was then added to a final concentration of 2% and the preparation held at 25 C for 30 min. The DNA was then extracted twice with phenol and dialyzed against buffer containing 0.05 M Tris, 0.01 M NaCl, and 0.15% Sarkosyl, pH 7.4.

3.8.2. Extraction from Bovine Parvovirus-Infected Cells

When radioactively labeled DNA was prepared, infected or mock-infected cultures were labeled for 30 min to 1 hr with ^{3}H-TdR at a final concentration of 0.5 µCi/ml. Medium containing ^{3}H-TdR was subsequently removed and medium containing only MEM with 10% serum was added. An additional incubation of 1 hr was performed to chase the labeled TdR from soluble pools into DNA. Both radiolabeled and unlabeled cultures were then extracted by the SDS-NaCl-pronase procedure of Hirt (1967) using self-digested pronase at a final concentration of 2 mg/ml. The

supernatant obtained following centrifugation at 17,300 x *g* for 30 min was allowed to reprecipitate at 4 C an additional 8 to 24 hr, and centrifuged at 17,300 x *g* for 30 min at 4 C. The supernatant was collected and stored at -20 C.

3.8.3. Isolation of Double Stranded DNA by Hydroxylapatite Chromatography

3.8.3.1. Column Chromatography

Hydroxylapatite column chromatography was performed using a modification of the method described by Meinke *et al.* (1974). Hirt-extracted DNA preparations were made 8 M with respect to urea and 0.24 M with respect to $NaHPO_4$, pH 6.8. This material was layered over a 4 x 1 cm column of hydroxylapatite (HAP) previously washed with 8 M urea, 0.24 M $NaHPO_4$, pH 6.8. A flow rate of 1 ml/min was maintained with a peristaltic pump and the eluant was monitored at 260 nm in a Gilford 2400 spectrophotometer. The column was washed with 150 ml of 8 M urea, 0.24 M $NaHPO_4$, pH 6.8, until the absorbance at 260 nm was less than 0.1. Sodium phosphate at a concentration of 0.14 M, pH 6.8, was then added to elute single stranded forms of DNA in a total of 100 ml. Double stranded DNA was subsequently eluted with 15 ml of 0.48 M $NaHPO_4$, pH 6.8. Fractions were collected and assayed for protein, RNA, and DNA content by the methods of Lowry *et al.* (1951), Mejbaum (1939), and Burton (1956), respectively.

3.8.3.2. Batch Chromatography

Hirt-extracted DNA (50 ml) was prepared as above and shaken with 2 g HAP for 30 min at 30 C followed by centrifugation at 3500 x *g* in an SS-1 rotor (Sorvall). The HAP pellet was washed twice with 8 M urea, 0.24 M $NaHPO_4$, pH 6.8, followed by three washes in 0.14 M PO_4, pH 6.8. Then 1.2 M $NaHPO_4$, pH 6.8, was added to the HAP and the double stranded DNA isolated in the supernatant after centrifugation for 5 min at 1,000 x *g* in an HB-4 swinging bucket rotor (Sorvall).

3.9. Physical Properties of Bovine Parvovirus DNA

3.9.1. Buoyant Density Determination

The densities of both Hirt and phenol-extracted DNA were determined by layering 0.2 ml of the DNA preparation over 5 ml of CsCl at a density of 1.68 g/cm^3 in cellulose nitrate tubes. Gradients were centrifuged to equilibrium at 114,000 x *g* for 48 hr at 4 C in an SW 50.1 rotor. Gradients were fractionated by bottom puncture and the refractive index of each fraction determined as previously described. From each fraction, 50 µl was placed into liquid scintillation vials containing 5 ml of Dimilume and counted by liquid scintillation spectrometry.

3.9.2. Sedimentation in Neutral Sucrose Gradients

^3H-TdR labeled BPV DNA (0.2 ml) isolated from purified virions was layered over a 5 ml continuous 10 to

30% sucrose gradient in TBS, pH 7.1. Gradients were centrifuged at 4 C in an SW 50.1 rotor (Beckman) at 114,000 x *g* for 15 hr and fractionated by bottom puncture. A 50 µl aliquot was removed from each fraction, added to 5 ml Dimilume in liquid scintillation vials, and counted by liquid scintillation spectrometry.

3.9.3. Kinetics of Thermal Denaturation and Renaturation

HAP-chromatographed double stranded DNA isolated from infected cells, *Escherichia coli* DNA, and *Bacteroides fragilis* DNA, were dialyzed against 0.14 M $NaHPO_4$, pH 6.8 containing 0.1% SDS prior to denaturation. The temperature of the DNA preparations was raised from 65 C to 99 C at a rate of 1 C/min using a Gilford 2527 Thermo-programmer. After the temperature of 99 C was achieved, the preparations were cooled rapidly (10 C/min) to 60 C. Absorbance at 260 nm (A_{260}) was continuously monitored.

3.9.4. Agarose Gel Electrophoresis

Double stranded BPV DNA, obtained by HAP column chromatography, was dialyzed at 4 C against buffer containing 6 mM Tris, 6 mM $MgCl_2$, and 6 mM NaCl, pH 7.2, prior to electrophoresis. ØX 174 DNA and *E. coli* plasmid (PML 21) DNA, dissolved in similar buffer, were the kind gifts of Dr. M. H. Edgell (University of North Carolina, Chapel Hill). Eleven cm gels were prepared immediately before electrophoresis using agarose dissolved in electrophoresis

buffer at a concentration of 1%. Electrophoresis buffer was prepared as described by Edgell *et al*. (1972). Samples were made 10% with respect to sucrose by the addition of an appropriate volume of 40% sucrose in dialysis buffer. Following the addition of electrophoresis buffer to the gels, samples containing 1 to 2 ug of DNA were underlaid directly over the gels. Electrophoresis was carried out for 4 hr at 4 C in a Hoeffer Scientific Instruments electrophoresis apparatus with 3 mamp/gel requiring 90 to 100 volts. Gels were removed and stained at least 1 hr in electrophoresis buffer containing 1 μg/ml ethidium bromide and examined with 300 nm u.v. light.

4. Results

4.1. Synchronization of Bovine Fetal Spleen Cells with Hydroxyurea

4.1.1. Determination of Optimum Conditions

In order to determine the effectiveness of HU as a synchronizing agent for BFS cells, it was first necessary to determine concentrations of HU which would completely inhibit DNA synthesis. In addition, the time required for cells to traverse the cell cycle to the G_1/S border in the presence of HU was elucidated by determining the time of exposure to HU which enabled the greatest percentage of cells to progress into S phase following HU removal.

DNA synthesis, as determined by autoradiography, was not completely inhibited when cells were exposed to concentrations of HU below 0.8 mM for up to 48 hr. However, at concentrations from 0.8 to 4 mM, incorporation of ^{3}H-TdR into acid precipitable material was completely inhibited 24 hr after cells were seeded in synchronization medium. The cells were released from the HU block at intervals after seeding, pulse labeled with ^{3}H-TdR, and prepared for autoradiography and liquid scintillation counting. Examination of autoradiograms revealed that the maximum percentage of cells synthesizing DNA was

obtained following release of cells exposed to these concentrations of HU for 32 hr.

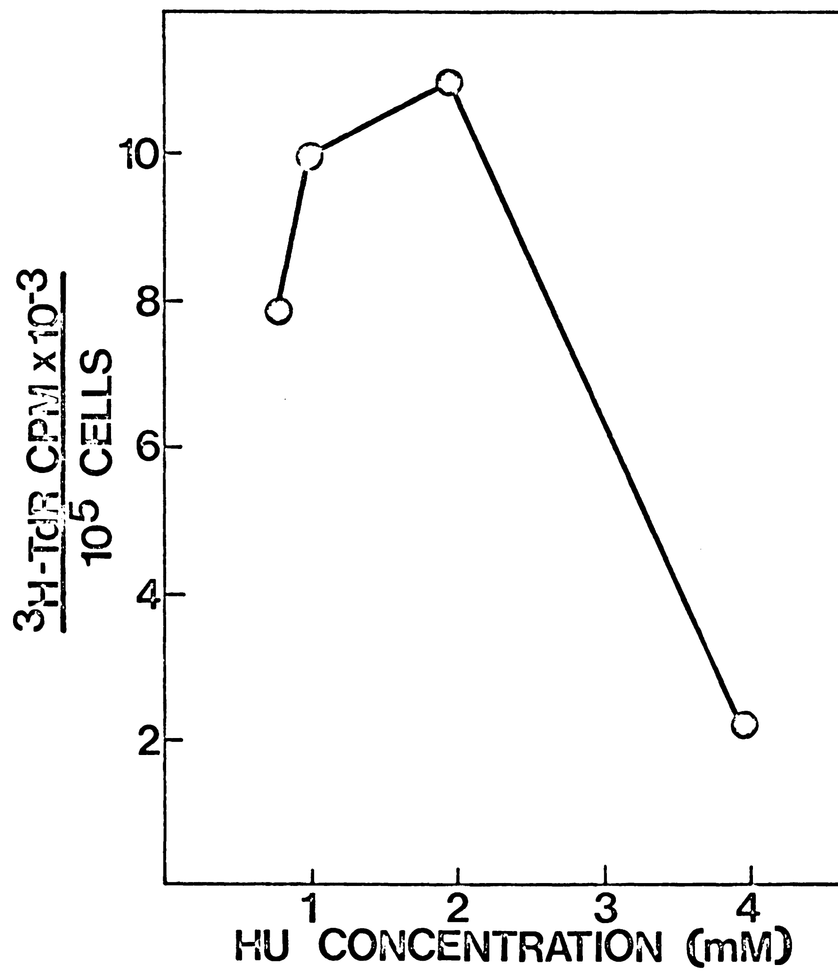
The rate of DNA synthesis following release of cells exposed to 0.8, 1, 2, or 4 mM HU for 32 hr is shown in Figure 1. At 2 hr after release, incorporation of ^{3}H-TdR increased with increasing HU concentrations of up to 2 mM. However, the rate of DNA synthesis in cells exposed to 4 mM HU was severely reduced at this time. Therefore, maximum synchronization of BFS cells was achieved when cells were exposed to 2 mM HU 32 hr before release.

4.1.2. Synchronization of DNA Synthesis

The kinetics of ^{3}H-TdR incorporation in cells synchronized with 2 mM HU for 32 hr was determined by labeling cells before and after release (Fig. 2). Before release from the HU block, DNA synthesis above background levels was not observed. Cells labeled with ^{3}H-TdR immediately after release were synthesizing DNA and within 2 hr, 80-85% of the cells contained significantly labeled nuclei as determined by autoradiography.

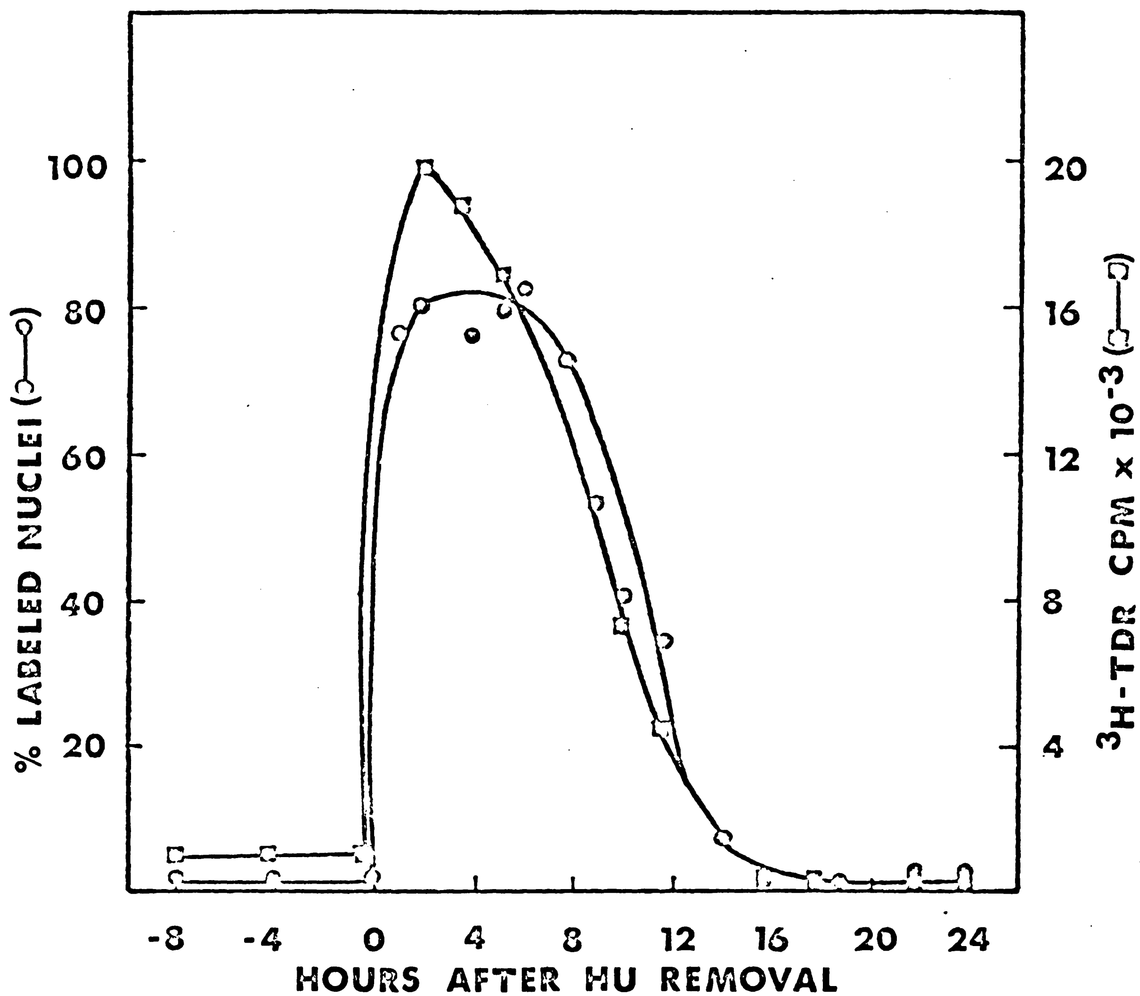
The maximum rate of DNA synthesis was observed 2 to 4 hr after release of cells from HU. At 9 to 10 hr after release, the rate had decreased to 50% of the level observed by 2 hr. By 16 hr, only background levels of DNA synthesis were occurring. Application of a second HU block at 16 hr did not increase the degree of synchronization of BFS cells following release.

Fig. 1. $HClO_4$-insoluble ^{3}H-TdR incorporation in BFS cells 2 hr after release of cells from 32 hr of exposure to various concentrations of HU.



3H-TdR CPM x 10-3 / 10^5 CELLS
10
8
6
4
2
1
2
3
4
HU CONCENTRATION (mM)

Fig. 2. DNA synthetic profile of BFS cells synchronized by exposure to 2 mM HU for 32 hr. Percent of cells incorporating ^{3}H-TdR into DNA as determined by autoradiography (o——o); rate of DNA synthesis (□——□).



100
80
60
40
20
% LABELED NUCLEI
20
16
12
8
4
^{3}H-TDR CPM x 10^{-3}
-8
-4
0
4
8
12
16
20
24
HOURS AFTER HU REMOVAL

4.1.3. Effect of Hydroxyurea Treatment on RNA and Protein Synthesis

To determine the effect of HU treatment on RNA and protein synthesis in BFS cells, cells were seeded in growth medium with or without 2 mM HU. Through at least 20 hr after seeding, the rate of RNA synthesis in HU-treated cells was only 80% of the rate observed in untreated, control cells while the rate of protein synthesis in cells exposed to HU occurred at 100% of the control rate (Fig. 3). Both RNA and protein synthetic rates decreased after 20 hr of exposure to HU. However, the RNA synthetic rate was inhibited to the greater extent, maintaining only 41% of the rate observed in controls at 32 hr while 52% of the control rate of protein synthesis occurred at this time. The parallel decrease in RNA and protein synthetic rates was not caused by a decrease in the transport of precursors into the cells since the TCA-soluble pools of uridine and amino acids were comparable for treated and untreated cells at these times (Table 1).

HU treatment did not result in a state of irreversible unbalanced growth since after release from the HU block RNA and protein synthesis increased reaching maximum levels at 4 hr (Fig. 4). This peak in synthesis corresponded to the time of maximum DNA synthesis in these cells. The RNA and protein synthetic rates subsequently decreased

Fig. 3. Rates of RNA and protein synthesis in cells seeded in medium containing 2 mM HU. Rates are expressed as percent of those in cells seeded in medium without HU. Rate of RNA synthesis (○—○); rate of protein synthesis (●—●).

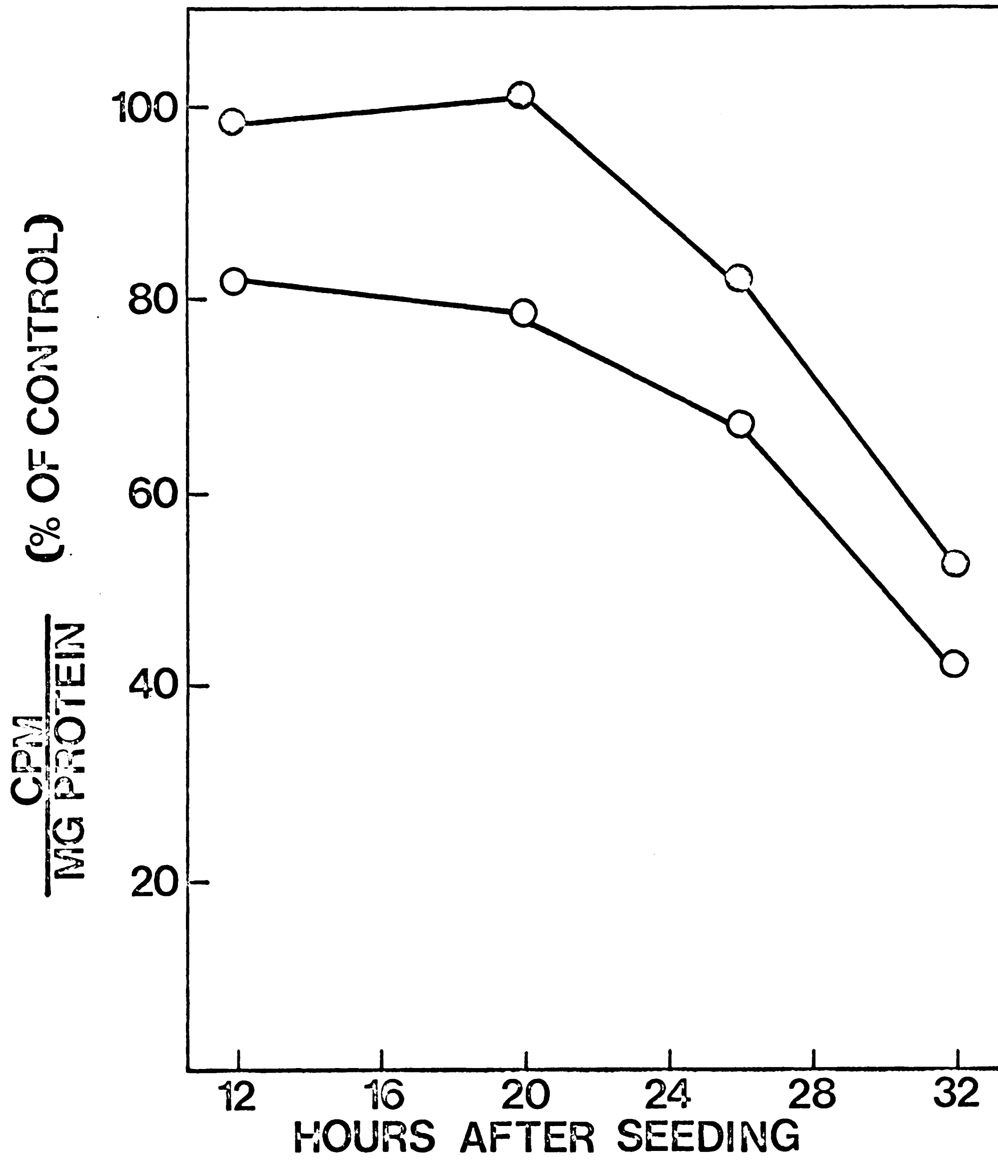

100
80
60
40
20
CPM / MG PROTEIN (% OF CONTROL)
12
16
20
24
28
32
HOURS AFTER SEEDING

Table 1

Effect of HU Treatment on Transport of Uridine and Amino Acids into Intracellular Pools[a]

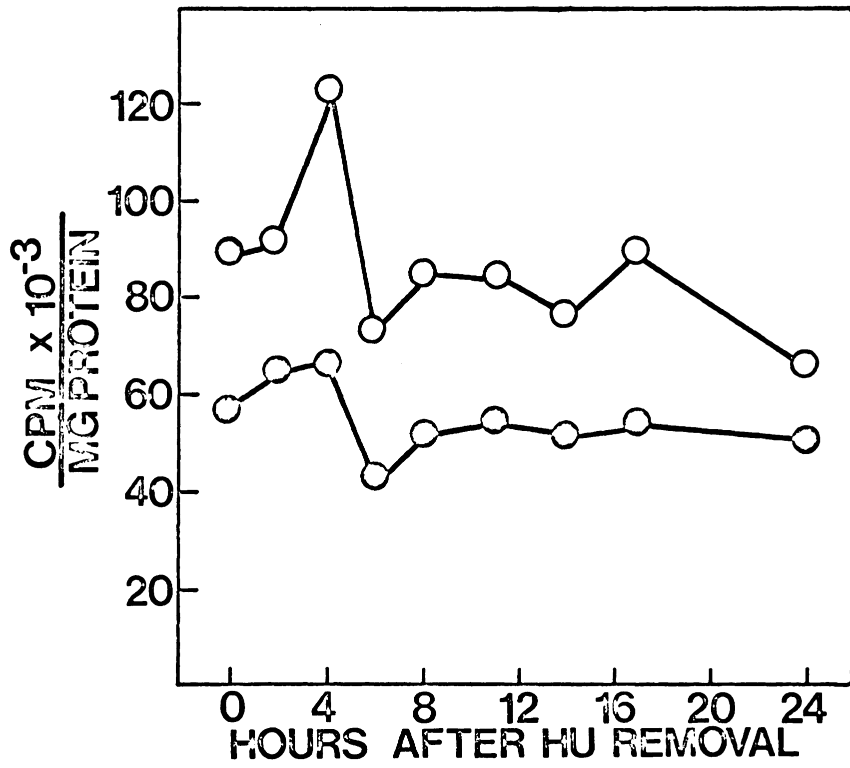
Hours after seeding	Uridine[b] HU treated cells	Uridine[b] Untreated cells	Amino acids[c] HU treated cells	Amino acids[c] Untreated cells
12	405.0	307.4	39.3	32.4
20	415.9	366.0	35.9	33.8
26	453.1	471.0	33.0	36.3
32	337.5	374.4	31.6	28.8

[a]Determined by the level of radioactive precursors in the TCA-soluble fraction.

[b]Levels are expressed as ^{3}H-uridine cpm/mg protein x 10^{-3}.

[c]Levels are expressed as ^{14}C amino acids cpm/mg protein x 10^{-3}.

Fig. 4. Rates of RNA and protein synthesis in BFS cells synchronized with HU. Rate of RNA synthesis (o——o); rate of protein synthesis (●——●).

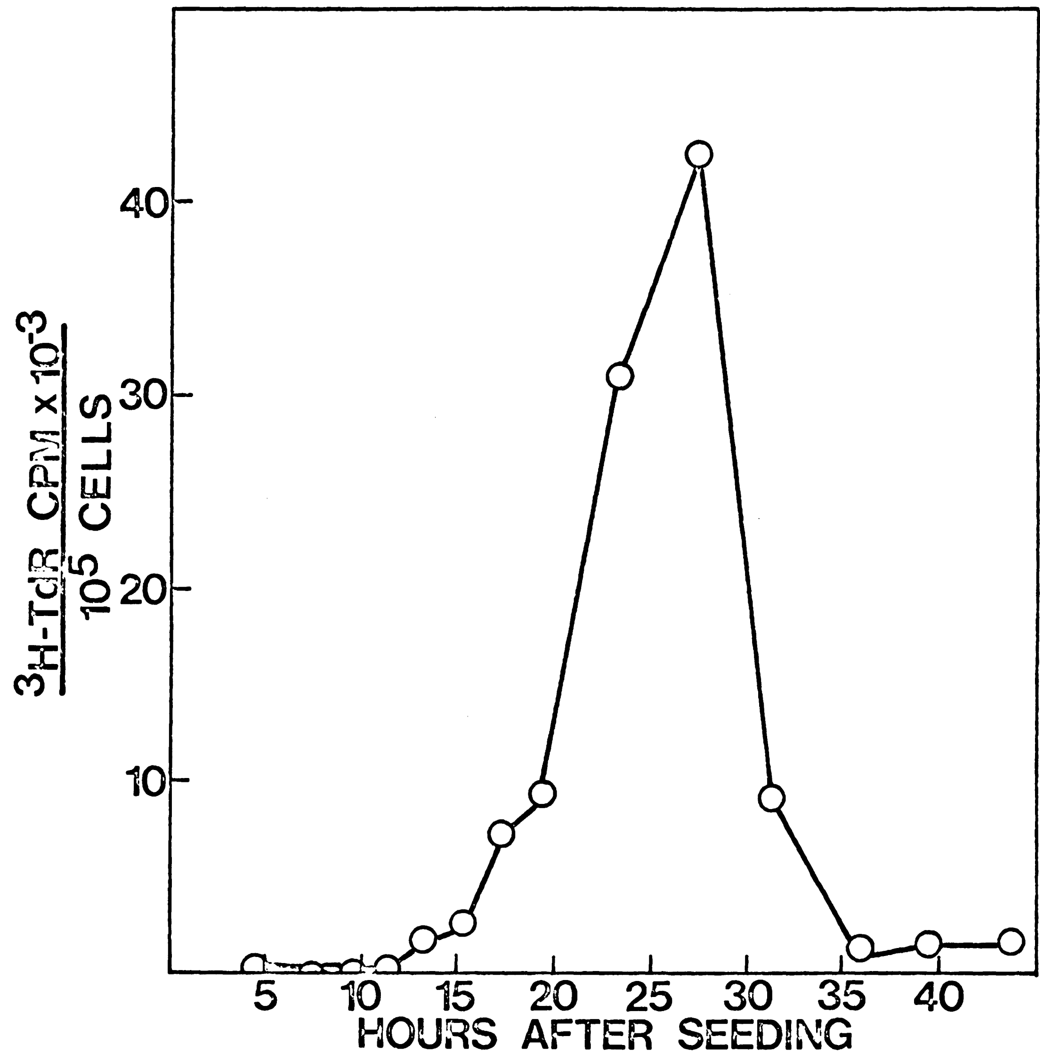

120
100
80
60
40
20
$\frac{CPM \times 10^{-3}}{MG PROTEIN}$
0
4
8
12
16
20
24
HOURS AFTER HU REMOVAL

but were maintained at high levels throughout the 24 hr period observed after release.

4.2. Synchronization of Cells Using Serum Stimulation

The degree of synchrony achieved by serum stimulation of BFS cells was determined by labeling cells with ^{3}H-TdR at various times after seeding cells in MEM containing 10, 20, 30, or 40% lamb serum. Incorporation of ^{3}H-TdR per cell occurred at the greatest rate after cells were exposed to 10% serum. Although concentrations of serum above 10% also stimulated cells to synthesize DNA, the rate of synthesis decreased with increasing serum concentration. In BFS cells seeded in 10% serum, synthesis of DNA did not occur above background levels until after 16 hr (Fig. 5). DNA synthesis occurred over a 20 hr period in these cells with the maximum rate observed 12 hr after its initiation (28 hr after seeding). This represents an S phase 2 times the length of that observed in HU-synchronized cells. In addition, the degree of synchrony obtained in HU-synchronized cells is greater than in serum stimulated cells since only 4 hr is required for the maximum rate of DNA synthesis to occur following initiation of S phase upon HU removal.

Fig. 5. Rate of DNA synthesis in BFS cells synchronized with 10% serum.



^{3}H-TdR CPM x 10^{-3} / 10^5 CELLS
40
30
20
10
5
10
15
20
25
30
35
40
HOURS AFTER SEEDING

4.3. Replication of Bovine Parvovirus in Hydroxyurea-Synchronized Cells

4.3.1. One-Step Growth Curve

BFS cells were infected with BPV at an m.o.i. of 40 PFU/cell immediately after release from HU to determine the kinetics of replication of the virus in HU-synchronized cells. Titers of infectious BPV began to increase after 8 hr p.i. and increased exponentially over the next 6 hr (Fig. 6). At 20 hr p.i., the maximum titer of BPV was reached representing a 3×10^3-fold increase above that observed during the eclipse and equivalent to the production of approximately 46 infectious virions per cell.
A synchronous increase in the number of cells involved in viral replication was demonstrated by the rapid appearance of fluorescent-stainable viral antigen after 8 hr p.i. with more than 85% of the cells exhibiting specific intranuclear fluorescence by 20 hr p.i. Although detectable hemagglutinating antigens did not appear until 10 hr p.i., their titers showed a parallel synchronous increase (Table 2).

BPV DNA was selectively removed from the high molecular weight cellular DNA by the Hirt procedure. The increase in the radioactivity in the Hirt supernatant of infected cells labeled with ^{3}H-TdR at various times p.i. was due to viral DNA since the radioactivity in the supernatant from

Fig. 6. One-step growth curve of BPV in HU-synchronized cells. Titer of infectious progeny virus (o——o); percent fluorescent nuclei (●——●).

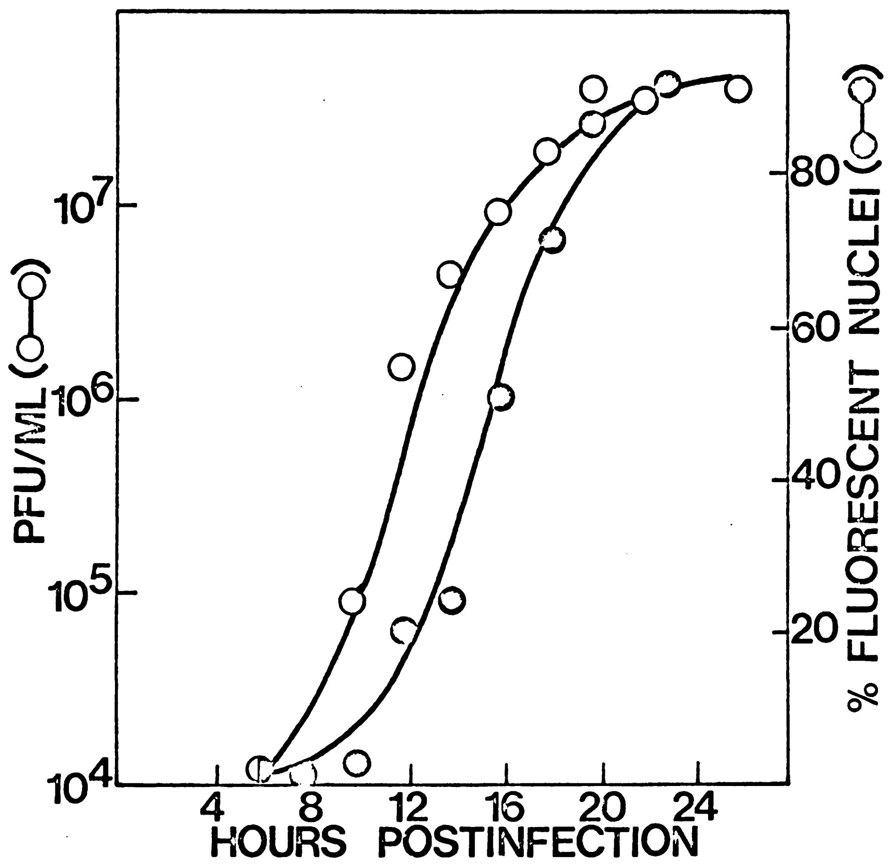

PFU/ML
10^7
10^6
10^5
10^4
4
8
12
16
20
24
HOURS POSTINFECTION
80
60
40
20
% FLUORESCENT NUCLEI

Table 2

Titers of BPV Hemagglutinating Antigen in Infected HU-Synchronized Cells

Hours postinfection	HA titer[a]
2	4
4	4
6	--[b]
8	--
10	2
12	64
16	64
20	1024
24	4096

[a]HA titer is the reciprocal of the highest dilution resulting in complete hemagglutination.

[b]No hemagglutination was noted at a 1:2 dilution.

mock-infected cells remained at a constant low level (Fig. 7). BPV DNA synthesis was detected after 6 hr p.i., preceding synthesis of progeny virus by approximately 2 hr. Subsequently the rate of BPV DNA synthesis increased synchronously in parallel with viral progeny. The maximum rate of DNA synthesis was reached between 14 and 16 hr p.i. Although the synthetic rate of BPV DNA decreased after 16 hr, maturation of virus continued at a reduced rate until 20 hr p.i. Therefore, it is apparent that an interrelationship exists between the time of synthesis of infectious progeny and of viral DNA as well as a relationship between the rates of viral DNA synthesized and the quantity of infectious virus produced.

4.3.2. Effect on Cellular S Phase

In order to determine if infection of cells at the beginning of S phase affected the rate of progression of cells through S, the rate of total DNA synthesis in cells infected with two m.o.i. was compared to the rate in control cells. DNA synthesis in cells infected with BPV at an m.o.i. of 40 PFU/cell occurred at approximately the same rate as in mock-infected controls through 6 hr p.i. (Table 3). At 8 hr, the rate had decreased to background levels in controls corresponding to the movement of cells out of S phase. In contrast, the rate of DNA synthesis in infected cells increased with maximum levels observed between 12 and 16 hr p.i. (Fig. 8).

Fig. 7. Kinetics of replication of BPV in HU-synchronized cells. Titer of infectious progeny virus (○——○); ^{3}H-TdR cpm in mock-infected cell Hirt supernatant (□——□); ^{3}H-TdR cpm in infected cell Hirt supernatant (●——●).

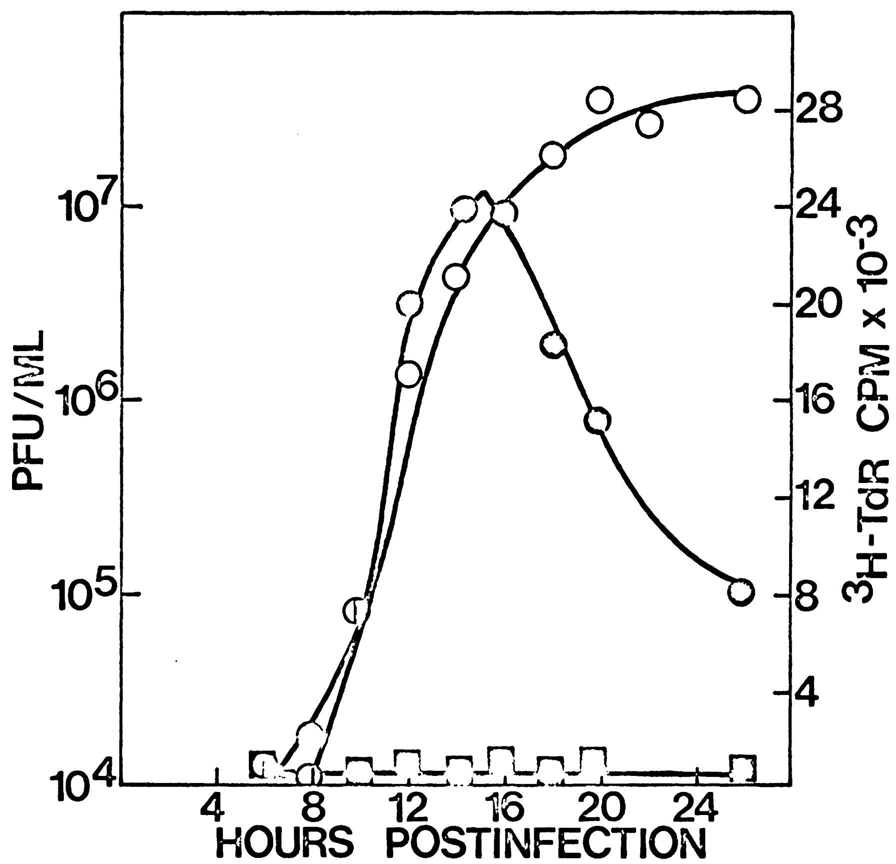

PFU/ML
10^7
10^6
10^5
10^4
^{3}H-TdR CPM x 10^{-3}
28
24
20
16
12
8
4
4
8
12
16
20
24
HOURS POSTINFECTION

Table 3

Effect of BPV Replication

(m.o.i. = 40 PFU/cell) on S phase

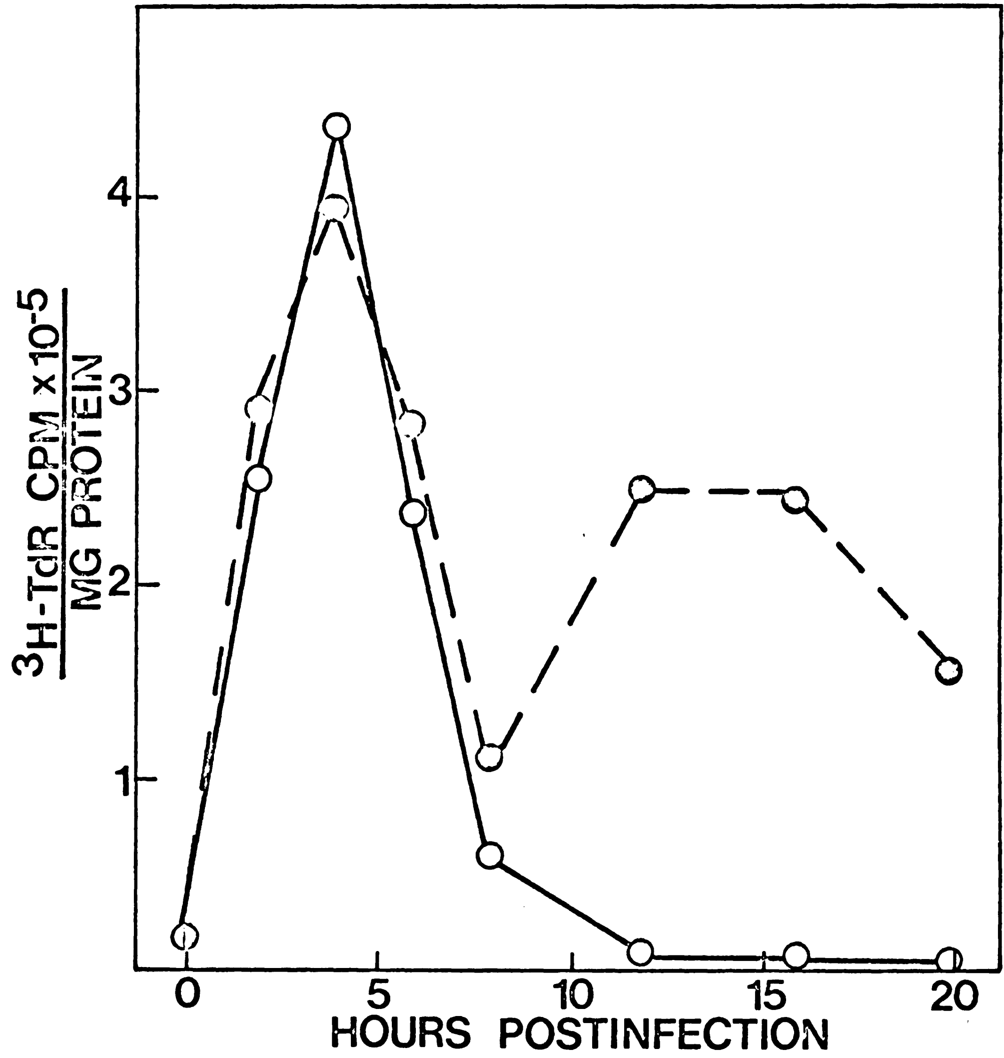
Hours postinfection	Mock-inf. cells[a]	Inf. cells[a]	% of control[b]
0	16	n.d.[c]	n.d.
2	260	290	112
4	440	399	91
6	240	258	107
8	58	113	193

[a]Expressed as TCA-insoluble ^{3}H-TdR cpm/mg protein x 10^{-3}.

[b]Expressed as ^{3}H-TdR incorporation in infected cells ÷ incorporation in mock-infected cells.

[c]Not determined.

Fig. 8. Effect of BPV replication (m.o.i. = 40 PFU/cell) on the rate of DNA synthesis in HU-synchronized cells. Mock-infected cells (o——o); infected cells (•- - -•).



^{3}H-TdR CPM x 10^{-5} / MG PROTEIN
4
3
2
1
0
5
10
15
20
HOURS POSTINFECTION

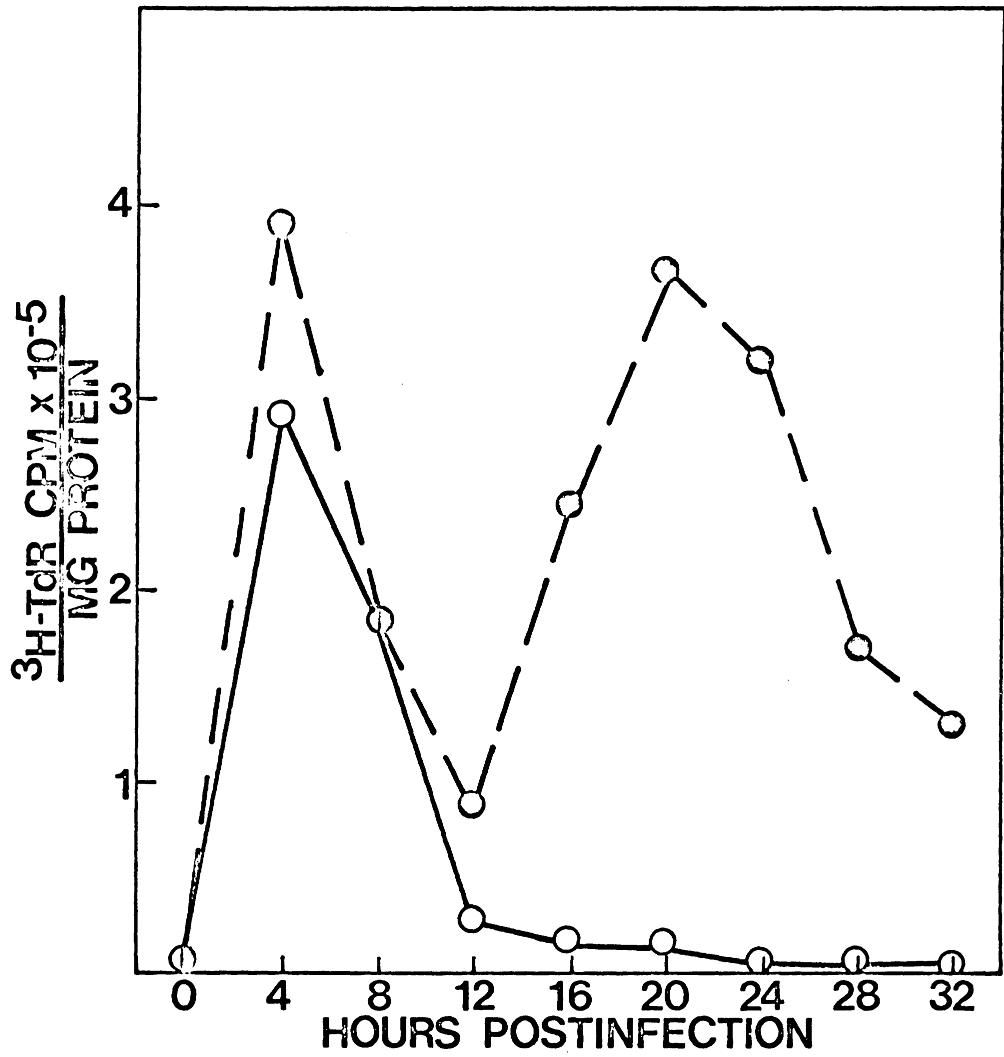
A similar profile of total DNA synthesis was observed for synchronized cells infected with 2 PFU/cell (Fig. 9). However, S phase was extended by 4 hr in these cells since DNA synthesis continued until 12 hr after HU release. Therefore, an increase in DNA synthesis in cells infected at either m.o.i. was evident corresponding to the time when reduced rates of cellular DNA synthesis were occurring.

In order to determine if viral proteins in the inoculum resulted in the observed increase in the rate of total DNA synthesis, DNA synthesis was monitored in cells inoculated with u.v. inactivated virus immediately after release from HU. DNA synthesis occurred in these cells at normal rates throughout S phase although the increase in the rate observed in cells inoculated with infectious virus was not seen (Fig. 10). Instead, DNA synthesis remained at background levels in cells inoculated with u.v. inactivated virus following S phase.

4.3.3. Time of Synthesis of Bovine Parvovirus DNA

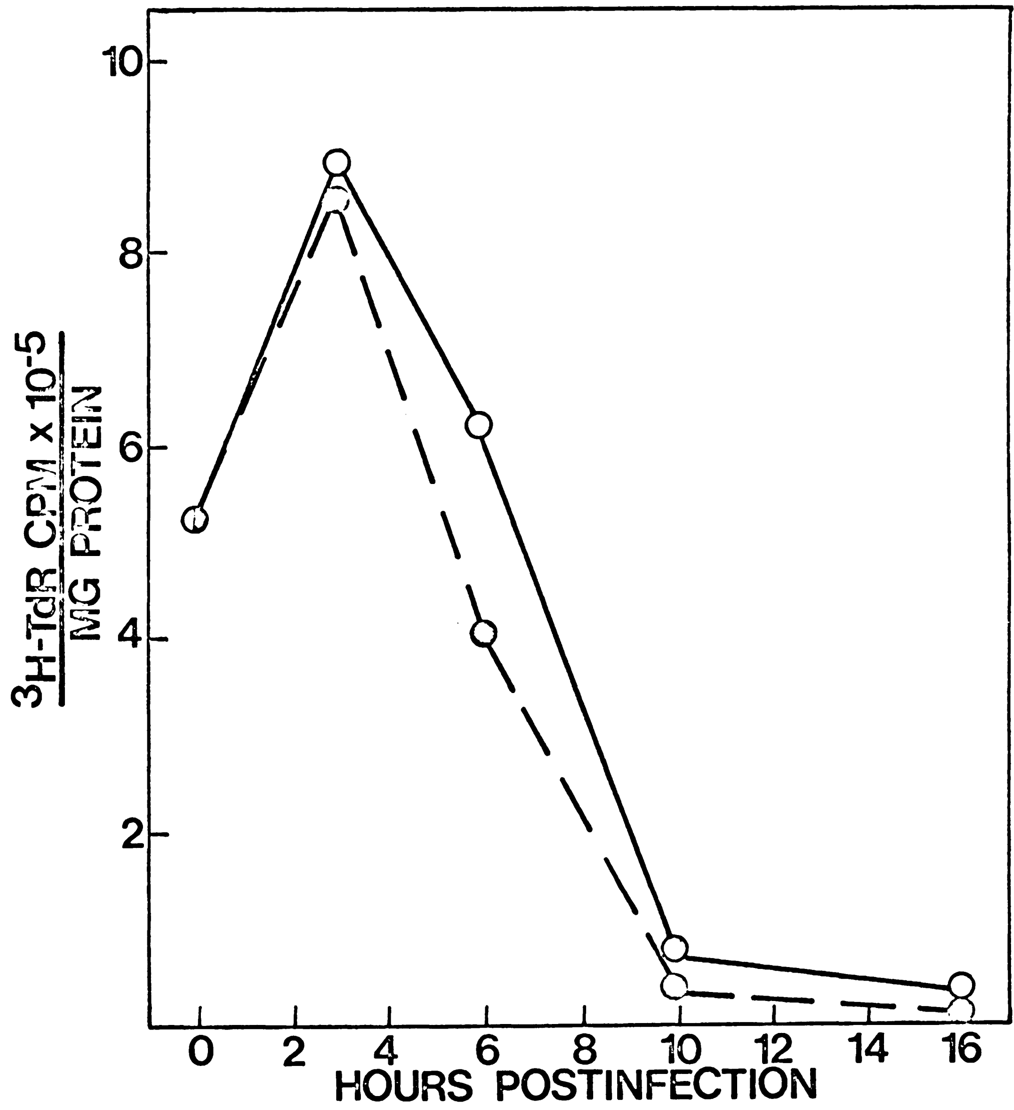
Since cells inoculated at the beginning of S phase with infectious or u.v. inactivated BPV replicate their DNA at normal rates throughout S phase, comparison of the time of synthesis of BPV DNA in infected cells and of cellular DNA in control cells was used to determine the cell cycle dependence of BPV DNA synthesis. Synthesis of

Fig. 9. Effect of BPV replication (m.o.i. = 2 PFU/cell) on the rate of DNA synthesis in HU-synchronized cells. Mock-infected cells (o——o); infected cells (●- - -●).



^{3}H-TdR CPM x 10^{-5} / MG PROTEIN
4
3
2
1
0
4
8
12
16
20
24
28
32
HOURS POSTINFECTION

Fig. 10. Effect of u.v. inactivated BPV on the rate of DNA synthesis in HU-synchronized cells. Mock-infected cells (o——o), cells inoculated with u.v. inactivated BPV (●- - -●).



10
8
6
4
2
^{3}H-TdR CPM x 10^{-5}
MG PROTEIN
0
2
4
6
8
10
12
14
16
HOURS POSTINFECTION

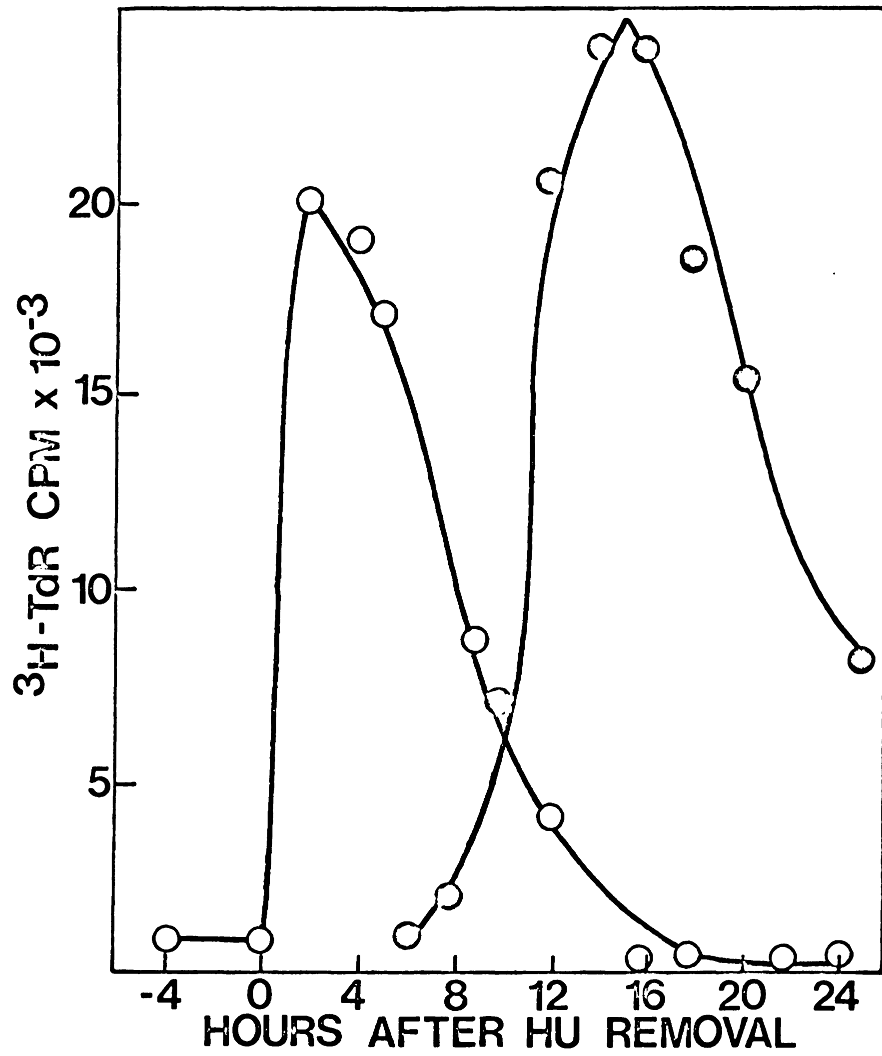
BPV DNA, detected by the increase in radioactivity in the Hirt supernatant, began at 6 hr p.i., although cells were moving out of S phase at that time (Fig. 11). The time when the maximum rate of BPV DNA synthesis occurred corresponded to the time when only background levels of DNA was synthesized in control cells. Thus, initiation of BPV DNA synthesis occurs during late S phase of the cell cycle. Furthermore, continued viral DNA synthesis is not dependent upon concomitant cellular DNA synthesis.

4.3.4. Effect on RNA and Protein Synthesis

Replication of BPV in cells inoculated with 40 PFU/cell at the beginning of S phase affected RNA and protein synthesis to the same extent (Fig. 12). Although the rates in infected cells were initially lower than those in mock-infected control cells, at 6 hr p.i. RNA and protein were synthesized at higher rates than in control cells. However, after 6 hr when CPE became evident, both the absolute and relative rates of synthesis decreased (Table 4). These rates continued to decrease throughout the replication cycle so that by 17 hr p.i., the RNA synthetic rate was only 20% and the protein synthetic rate only 10% of control levels.

When early S phase cells were infected with only 2 PFU/cell, similar effects on RNA and protein synthesis were observed (Table 5). However, the rates of RNA and

Fig. 11. Rate of synthesis of cellular and BPV DNA in HU-synchronized cells. ^{3}H-TdR incorporation in mock-infected cells (o——o); ^{3}H-TdR incorporation in the Hirt supernatant from infected cells (•——•).



20
15
10
5
^{3}H-TdR CPM x 10^{-3}
-4
0
4
8
12
16
20
24
HOURS AFTER HU REMOVAL

Fig. 12. Rates of RNA and protein synthesis in HU-synchronized cells infected with BPV (m.o.i. = 40 PFU/cell). Rates are expressed as percent of those in mock-infected cells. Rate of RNA synthesis (o——o); rate of protein synthesis (●——●).

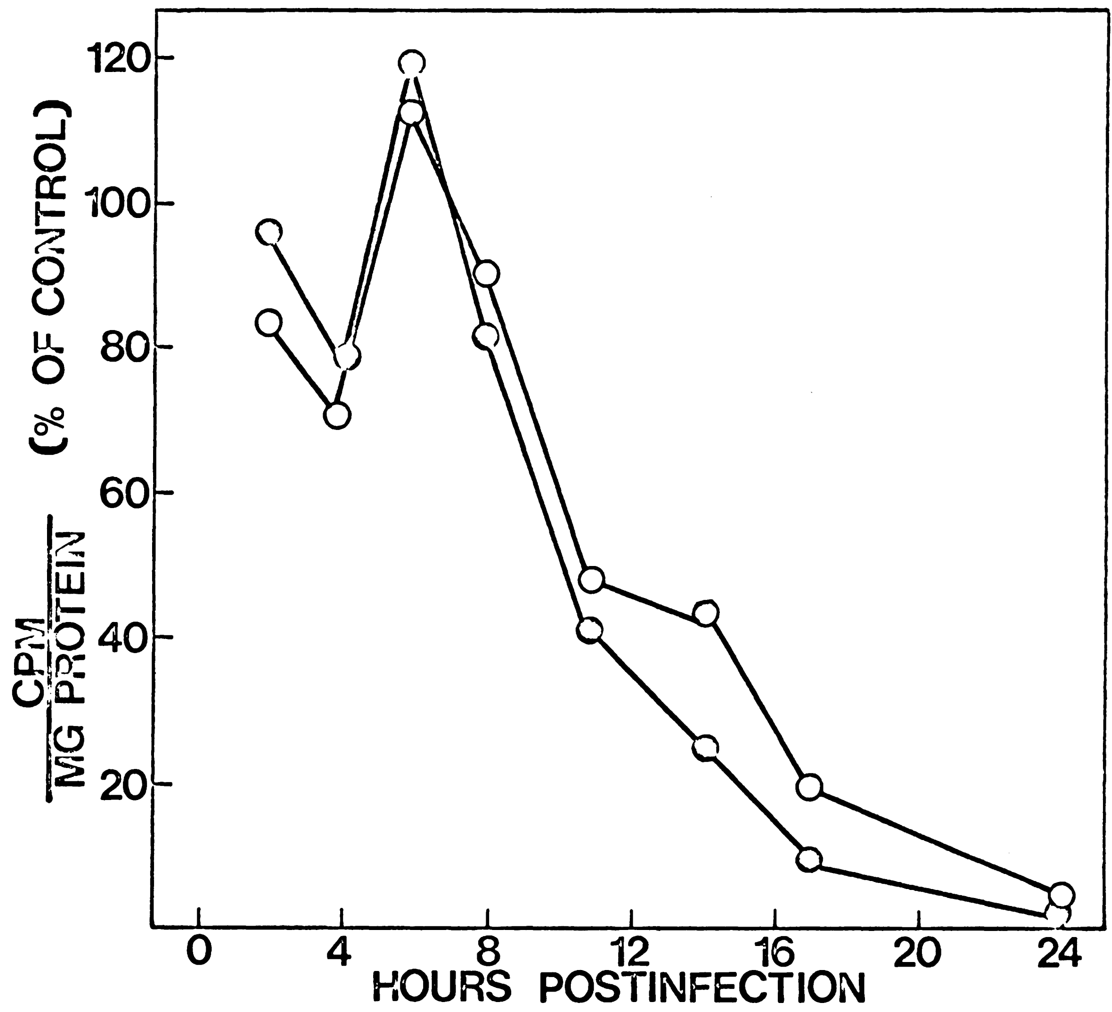

120
100
80
60
40
20
CPM / MG PROTEIN (% OF CONTROL)
0
4
8
12
16
20
24
HOURS POSTINFECTION

Table 4

Effect of BPV Replication (m.o.i. = 40 PFU/cell) on

Rates of RNA and Protein Synthesis in HU-Synchronized Cells

	RNA synthesis[a]			Protein synthesis[b]		
Hours postinfection	Mock-inf. cells	Inf. cells	% of control[c]	Mock-inf. cells	Inf. cells	% of control[d]
2	92.9	78.2	84.2	65.1	62.7	96.3
4	124.0	88.3	71.2	66.7	53.0	79.3
6	74.1	83.2	112.0	43.3	51.6	119.0
8	84.4	76.0	90.0	52.6	43.6	82.9
11	83.0	40.4	48.7	55.1	22.7	41.3
14	77.8	33.8	43.5	52.3	13.8	26.4
17	85.1	17.2	20.2	55.6	5.3	9.5
24	66.5	3.4	5.1	51.1	1.4	2.7

[a] Expressed as TCA-insoluble ^{3}H-uridine cpm/mg protein x 10^{-3}.

[b] Expressed as TCA-insoluble ^{14}C amino acids/mg protein x 10^{-3}.

[c] Expressed as ^{3}H-uridine incorporation in infected cells ÷ incorporation in mock-infected cells.

[d] Expressed as ^{14}C amino acids incorporation in infected cells ÷ incorporation in mock-infected cells.

Table 5

Rates of RNA and Protein Synthesis in HU-Synchronized Cells

Inoculated with BPV at a m.o.i. of 2 PFU/cell or with u.v. Inactivated BPV

Hours postinfection	Cells infected with 2 PFU/cell		Cells inoculated with u.v. inactivated BPV	
	RNA synthesis (% of control)[a]	Protein synthesis (% of control)[b]	RNA synthesis (% of control)[a]	Protein synthesis (% of control)[b]
3	n.d.[c]	n.d.	93	104
4	114	100	n.d.	n.d.
6	n.d.	n.d.	92	91
8	94	93	n.d.	n.d.
10	n.d.	n.d.	125	108
12	85	84	n.d.	n.d.
16	68	62	94	85
20	66	39	n.d.	n.d.
24	56	42	95	81
28	31	30	n.d.	n.d.
32	18	19	n.d.	n.d.

[a] Expressed as ^{3}H-uridine/mg protein incorporated into the TCA-insoluble fraction in infected cells ÷ incorporation in the TCA-insoluble fraction in mock-infected cells.

[b] Expressed as ^{14}C amino acids/mg protein incorporated into the TCA-insoluble fraction of infected cells ÷ incorporation in the TCA-insoluble fraction in mock-infected cells.

[c] Not determined.

protein synthesis in infected cells were not significantly lower than in control cells until 12 hr p.i., 4 hr later than the reduced rates were evident in cells infected with the higher m.o.i. Therefore, it is evident that although the lower m.o.i. causes a delay in the cytotoxic action of replication of BPV, the ultimate effects on macromolecular synthesis are the same.

The decrease in RNA and protein synthesis at both m.o.i. was not due to inhibition by viral proteins in the inoculum since synthetic rates occurred at control levels in cells inoculated with u.v. inactivated BPV (Table 5). In addition, the decrease in RNA and protein synthesis in infected cells did not result from a decrease in uptake of the radioactive precursors since the radioactivity in TCA-soluble material from infected cells was equal to or greater than that in TCA-soluble material from control cells (Table 6).

4.4. Replication of Bovine Parvovirus in the Presence of Hydroxyurea

Although cellular DNA is not synthesized after cells are exposed to 2 mM HU for 32 hr, BPV DNA and infectious virions were synthesized in the continued presence of HU. BFS cells inoculated with 25 PFU/cell after exposure for 32 hr with HU were not released from the HU block but remained in HU throughout the experiment. At 16 hr p.i.,

Table 6

Effect of BPV Replication (m.o.i. = 40 PFU/cell) on Transport of Uridine and Amino Acids into Intracellular Pools[a]

	Uridine[b]		Amino acids[c]	
Hours postinfection	Mock-inf. cells	Inf. cells	Mock-inf. cells	Inf. cells
4	945	913	99	101
8	895	1010	93	85
12	950	1225	88	87
16	868	1065	87	98

[a]Determined by level of radioactivity in the TCA-soluble fraction.

[b]Expressed as ^{3}H-uridine cpm/mg protein x 10^{-3}.

[c]Expressed as ^{14}C amino acids cpm/mg protein x 10^{-3}.

an increase in infectious virus was detected. Synthesis of BPV then increased exponentially reaching a titer of 2.7×10^5 PFU/ml by 36 hr (Fig. 13). Although this represents an increase in titer of more than 10-fold over that observed during the 16 hr eclipse period in these cells, it is 100-fold less than the maximum titer of 3.7×10^7 PFU/ml achieved in cells which had been released from the HU block (Fig. 6). Synthesis of detectable hemagglutinating antigens was also evident in the presence of HU beginning at 24 hr p.i. reaching a maximum titer at 36 hr of 1024 (Table 7).

The rate of DNA synthesis in infected, HU-blocked cells began to increase after 16 hr p.i. relative to that in control cells (Fig. 13). Control cells did not synthesize DNA as determined autoradiographically by the absence of grains over the nucleus, and only background rates of synthesis occurred throughout the time examined. The rate of synthesis of viral DNA extracted by the Hirt procedure also increased at 16 hr p.i. reaching maximum levels between 20 and 24 hr p.i. comparable to the time in which the maximum rate of DNA was synthesized in infected cells (Fig. 13).

To determine if viral synthesis occurred in a few cells able to escape the HU block or in a majority of the cells, the number of cells synthesizing viral antigens was analyzed by immunofluorescence. There was a

Fig. 13. One-step growth curve of BPV in cells continuously exposed to 2 mM HU. Titer of infectious virus (o——o); rate of DNA synthesis in infected cells relative to the rate in mock-infected cells (●——●); ^{3}H-TdR incorporation in the Hirt supernatant of infected cells (□- - -□).

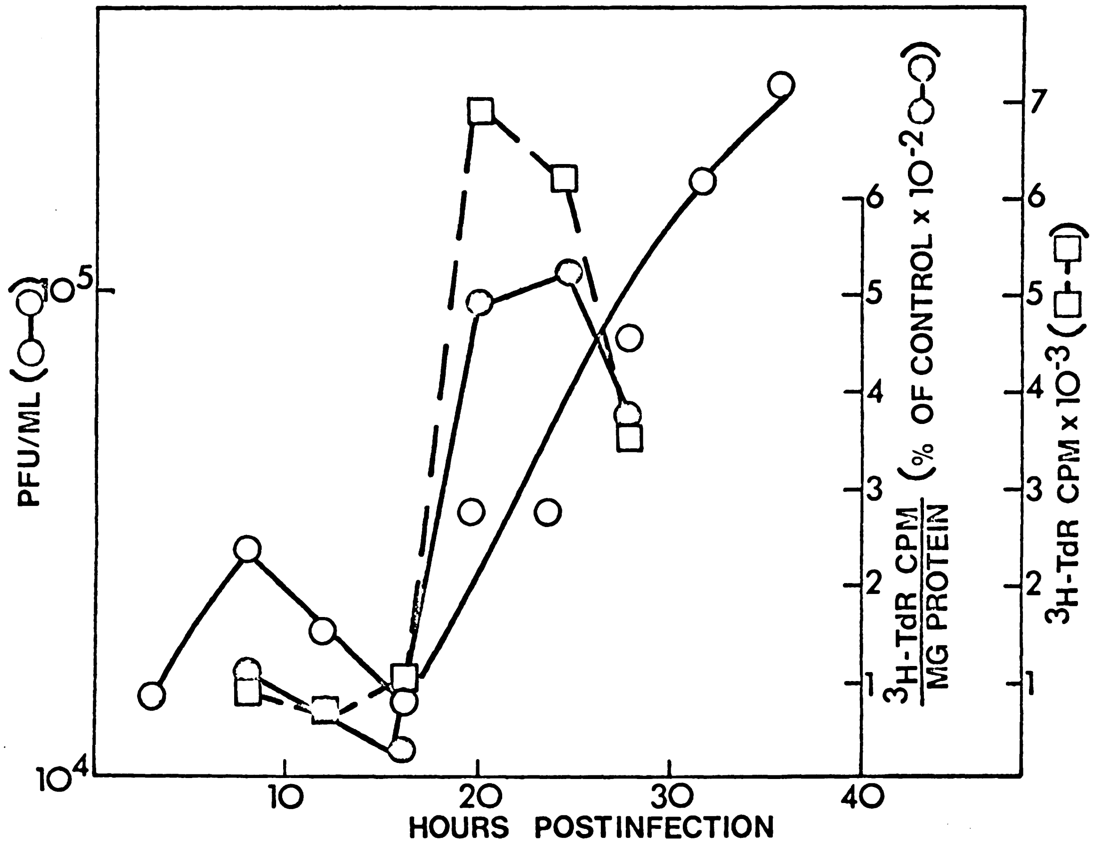

PFU/ML (○—○)
10^5
10^4
10
20
30
40
HOURS POSTINFECTION
^{3}H-TdR CPM / MG PROTEIN (% OF CONTROL x 10^{-2} ○-○)
1
2
3
4
5
6
7
^{3}H-TdR CPM x 10^{-3} (□--□)

Table 7

Hemagglutination Titers and Percent Cells Synthesizing Viral Antigens[a] in BPV-Infected Cells Exposed Continuously to 2 mM Hydroxyurea

Hours postinfection	HA titer[b]	% fluorescent nuclei
3	2	0
8	--[c]	2
12	--	5
16	--	3
20	--	21
24	32	29
28	128	56
32	1024	57
36	1024	75

[a]Determined by immunofluorescence.

[b]HA titer is the reciprocal of the highest dilution resulting in complete hemagglutination.

[c]No hemagglutination was noted at a 1:2 dilution.

synchronous increase in the number of cells involved in viral replication with significant percentages of nuclei exhibiting specific fluorescence after 16 hr p.i. (Table 7). At 36 hr p.i. when maximum virus titers were detected, 75% of the nuclei contained viral specific antigens.

BPV was also synthesized in cells continuously exposed to concentrations of HU up to 8 mM. However, at 36 hr p.i., the HA titers in cells exposed to greater than 3 mM HU were 128, or approximately one tenth the level in cells exposed to 2 or 3 mM HU (Table 8).

4.5. Physical Properties of Bovine Parvovirus DNA

4.5.1. Buoyant Density of Hirt-Extracted DNA

To determine some of the characteristics of DNA collected in the Hirt supernatant, infected cell DNA was labeled with ^{3}H-TdR at 14 hr p.i. when viral DNA synthesis was maximum. Mock-infected cell DNA was labeled at 4 hr p.i. when cellular DNA was synthesized at maximum rates or at 14 hr p.i. Following isopycnic centrifugation, no significant radioactivity occurred at any CsCl density with material extracted from mock-infected cells regardless of the time in which radioactive label was added (Fig. 14a). However, isopycnic banding of DNA from infected cells revealed a peak of DNA located at a density of 1.688 g/cm^3 (Fig. 14b). Therefore, the BPV DNA isolated by the Hirt

Table 8

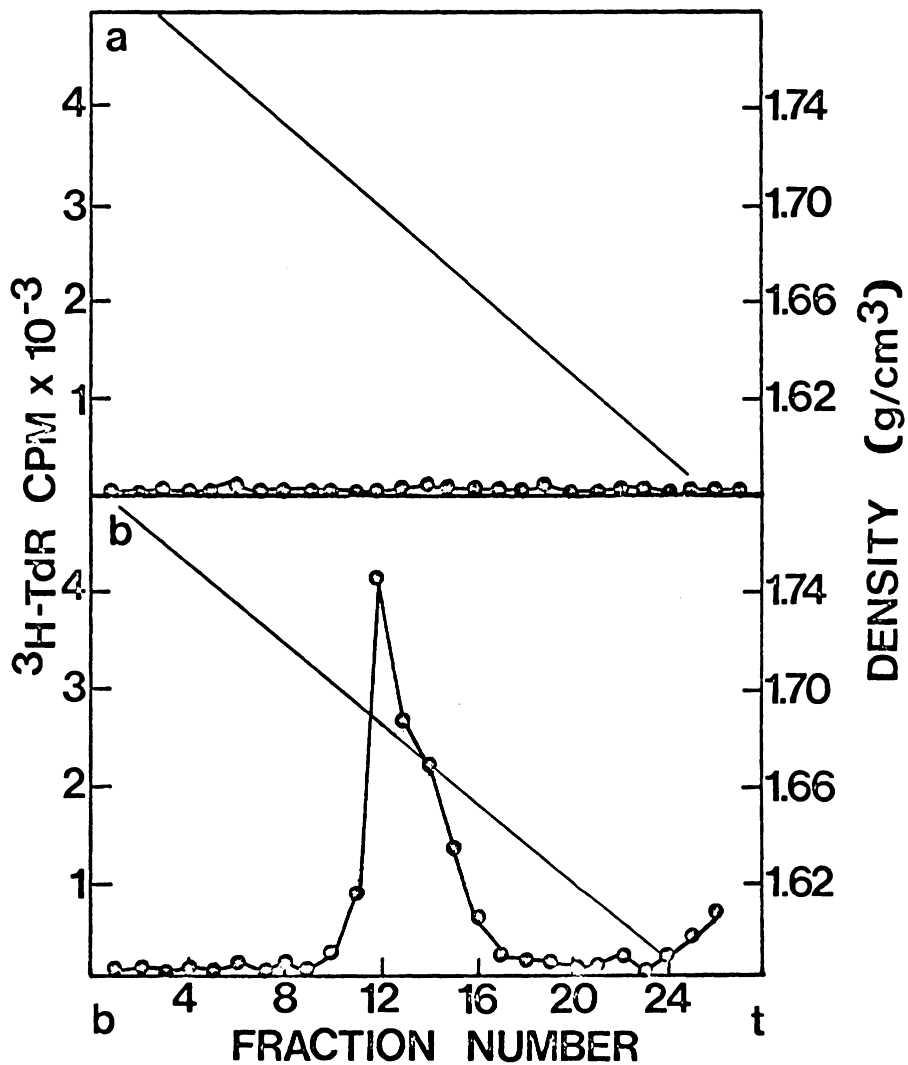
Hemagglutination Titers[a] in BPV-Infected Cells Exposed Continuously to Hydroxyurea

Concentration of HU (mM)	HA titer[b]
2	1024
3	1024
4	128
6	128
8	128

[a]Titers were determined at 36 hr p.i.

[b]HA titer is the reciprocal of the highest dilution resulting in complete hemagglutination.

Fig. 14. Density of Hirt-extracted DNA in equilibrium CsCl gradients. a. Mock-infected cells; b. Infected cells.


a
b
4
3
2
1
1.74
1.70
1.66
1.62
3H-TdR CPM x 10-3
DENSITY (g/cm3)
b
4
8
12
16
20
24
t
FRACTION NUMBER

procedure contained little or no contaminating cellular DNA and was relatively homogeneous.

4.5.2. Buoyant Density and Sedimentation Profile of DNA Extracted from Purified Bovine Parvovirus

BPV banded by isopycnic centrifugation in CsCl and having a density of 1.42 g/cm^3 contained a predominance of complete virions (Fig. 15). Single stranded DNA isolated by enzymatic treatment of ^{3}H-TdR labeled virions purified in this way produced a heterogeneous pattern when centrifuged in CsCl (Fig. 16). Major peaks of radioactivity were located at 1.720 and 1.665 g/cm^3. In addition to the wide range in density observed in CsCl, a heterogeneous sedimentation pattern resulted after centrifugation of the single stranded material in neutral sucrose (Fig. 17).

4.5.3. Kinetics of Thermal Denaturation and Renaturation Of Double Stranded Bovine Parvovirus DNA

The rate of thermal denaturation of double stranded BPV DNA, isolated by HAP column chromatography, was determined and compared to that of linear double stranded DNA from *Bacteroides fragilis*. Although the initial A_{260} values for BPV and *B. fragilis* DNA were 0.307 and 0.401 respectively, Figure 18 indicates both as containing the same initial A_{260} for ease of comparison of changes in absorbance. At 99 C, the A_{260} of *B. fragilis* DNA had

Fig. 15. Electron photomicrograph of purified BPV ($1.42\ g/cm^3$) negatively stained with uranyl acetate. Magnification x 80,000.

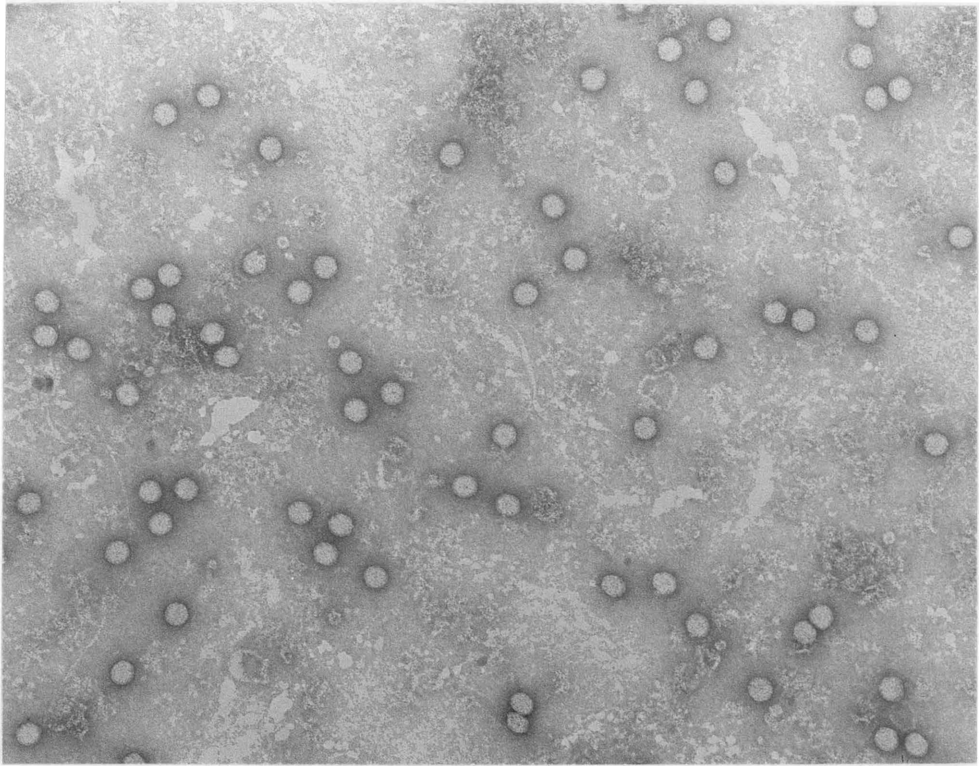


Fig. 16. Sedimentation in CsCl of DNA extracted from purified BPV.

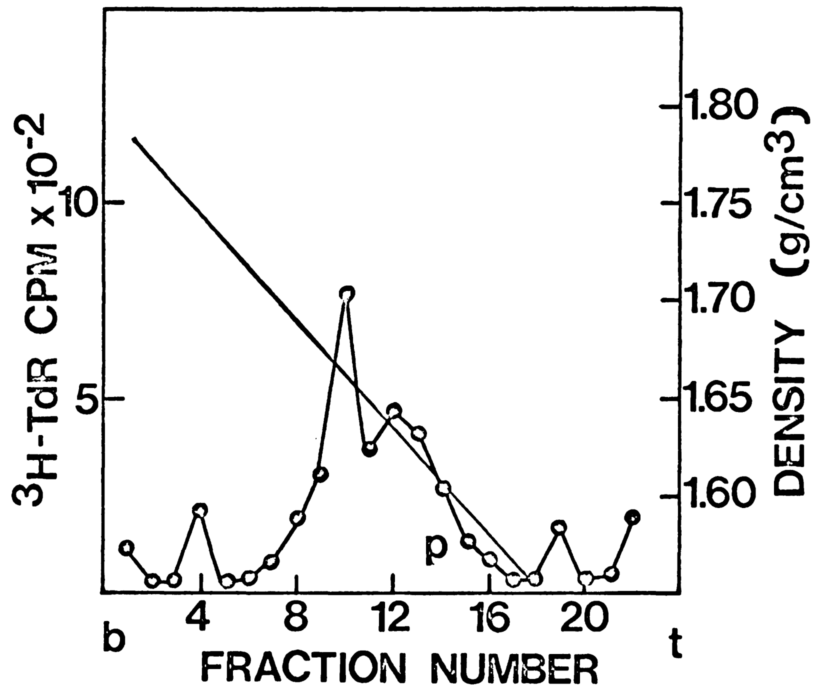

^{3}H-TdR CPM x 10^{-2}
10
5
1.80
1.75
1.70
1.65
1.60
DENSITY (g/cm^{3})
p
4
8
12
16
20
b
t
FRACTION NUMBER

Fig. 17. Sedimentation in neutral sucrose of DNA extracted from purified BPV.

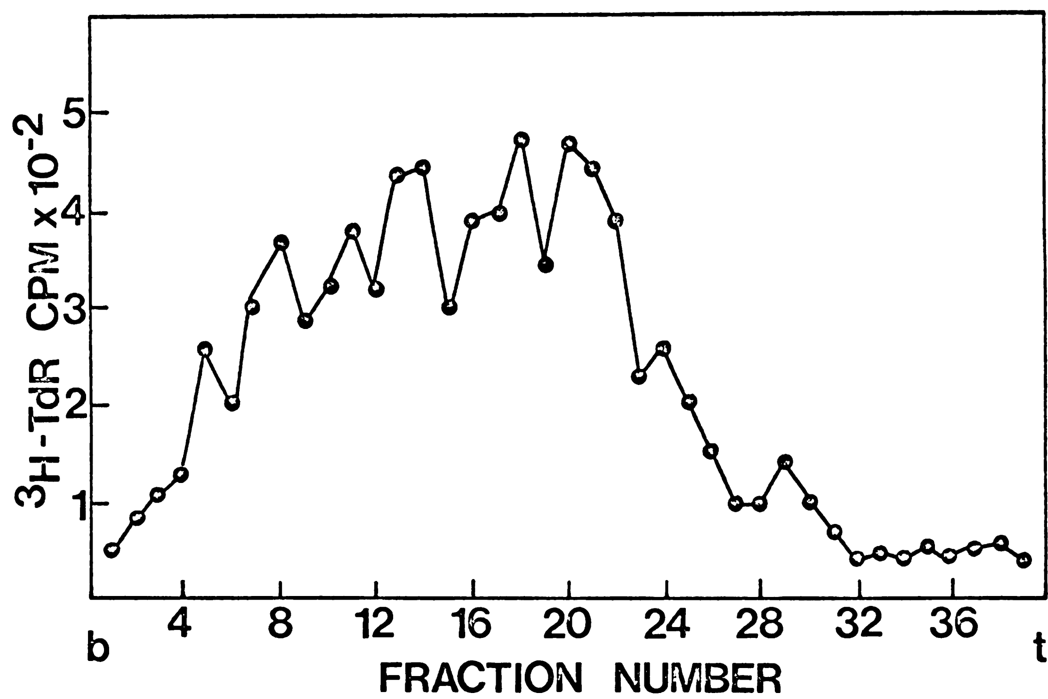
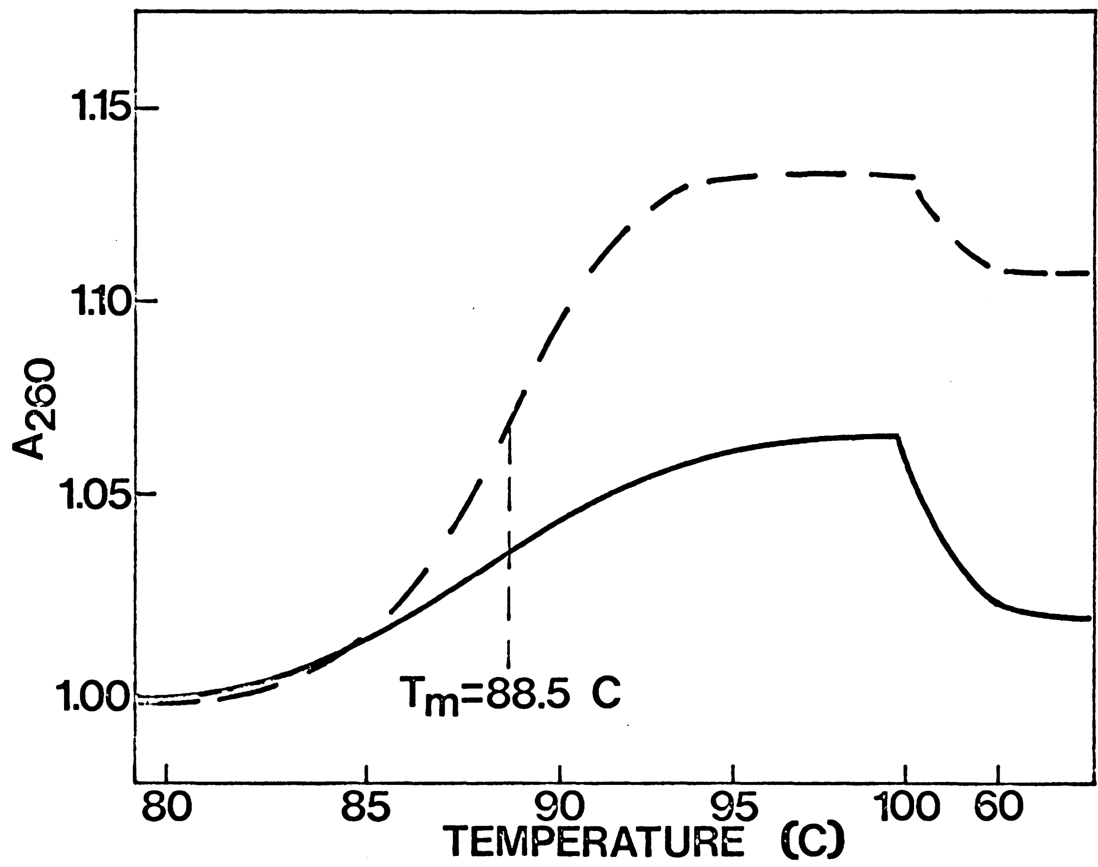

3H-TdR CPM x 10-2
5
4
3
2
1
b
4
8
12
16
20
24
28
32
36
t
FRACTION NUMBER

Fig. 18. Kinetics of thermal denaturation and renaturation of double stranded BPV and *Bacteroides fragilis* DNA. (———) BPV DNA; (– – –) *B. fragilis* DNA.


1.15
1.10
1.05
1.00
A_{260}
T_m=88.5 C
80
85
90
95
100
60
TEMPERATURE (C)

increased by 0.135 representing a 34% hyperchromic shift. BPV DNA at 99 C had an A_{260} of 0.0675 units above that observed at 65 C representing a 22% hyperchromic shift. The temperature at which 50% of the hyperchromic shift had occurred (the T_m) was 88.5 C for both DNA preparations.

The G+C content of BPV DNA was determined from the T_m using the equation of Marmur and Doty (1962). According to this equation, the T_m for *B. fragilis* DNA, corresponding to its known G+C content of 42%, is 86.6 C although that obtained in this experiment was 1.9 C higher due to the higher ionic strength buffer used. Therefore, 1.9 C was substracted from the T_m of 88.5 C observed for BPV DNA before its introduction into the Marmur and Doty formula. These calculations demonstrated the G+C content of BPV double stranded DNA to be 42%. Due to the complementary pairing of guanine and cytosine residues, the G+C content of single stranded virion DNA is also 42%.

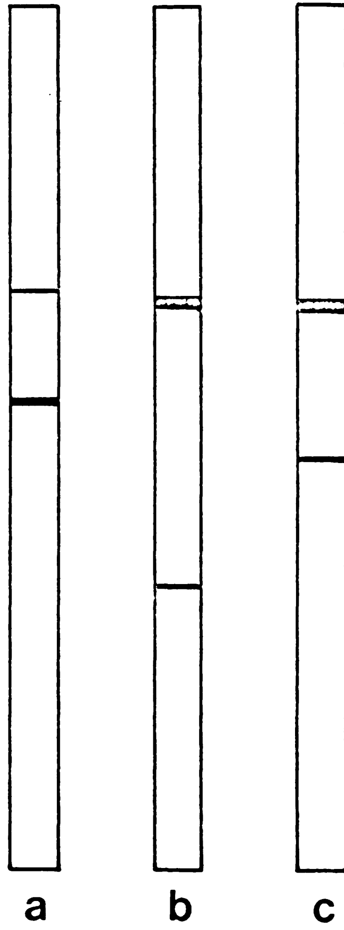
After BPV and *B. fragilis* DNA were maximally denatured at 99 C, the temperature was rapidly decreased to determine the extent and rate of reannealing. Upon cooling, BPV DNA lost 79% of the hyperchromicity acquired during melting, while only 16% of the hyperchromicity of linear *B. fragilis* DNA was lost. Therefore, most of the BPV DNA present in this preparation demonstrated renaturation kinetics not seen by linear double stranded DNA molecules. A rapidly reannealing rate is consistent with a covalently

closed circular conformation. Further evidence for this configuration was obtained by observing the renaturation kinetics of BPV DNA isolated by the more vigorous batch HAP procedure. Although DNA isolated in this way showed identical melting characteristics to that shown for BPV DNA in Figure 18, the rate of renaturation was similar to that of *B. fragilis* DNA. Therefore, the harsher treatment probably resulted in slight breakage of the closed circular forms preventing rapid reannealing.

4.5.4. Migration of Double Stranded Bovine Parvovirus DNA Subjected to Agarose Gel Electrophoresis

The migration of double stranded BPV DNA, prepared by HAP column chromatography, was observed in 1% agarose gels to determine if more than one species of DNA was present. As a control, migration of ØX 174 replicative form (RF) DNA was also observed to determine the pattern of migration of covalently closed circular DNA and open circular (nicked) DNA. Since covalently closed circular DNA has a superhelical configuration, it migrates in 1% gels more rapidly than open circular DNA (M. H. Edgell, personal communication). Figure 19 shows that ØX 174 RF DNA migrates as 2 bands in agarose. Although BPV DNA also migrates as 2 bands, only the uppermost band is in a position analogous to a ØX 174 RF band. *E. coli* plasmid (PML 21) DNA also migrated as 2 bands in 1% agarose.

Fig. 19. Electrophoretic pattern of double stranded BPV DNA, ØX 174 RF DNA, and *E. coli* plasmid DNA in 1% agarose gels. a. Plasmid DNA b. ØX 174 DNA; c. BPV DNA.


a
b
c

However, the faster moving covalently closed circular form was separated from the open circular form to a lesser degree than ØX 174 RF forms are separated.

5. Discussion

5.1. Effectiveness of Hydroxyurea as a Synchronizing Agent for Bovine Fetal Spleen Cells

Although HU has been shown to be an effective synchronizing agent for established cell lines (Sinclair, 1965; Pfeiffer and Tolmach, 1967; Kim *et al*., 1967; Adams and Lindsay, 1967; Plagemann *et al*., 1974), its effectiveness in synchronizing fetal cells has not been previously reported. Although no DNA synthesis was occurring after BFS cells were exposed to 2 mM HU for 32 hr, immediately after release cells began to incorporate ^{3}H-TdR into acid-insoluble material. The number of cells entering S phase increased rapidly and maximum rates of DNA synthesis occurred only 2 to 4 hr after removal of HU.

This high degree of synchronous progression of cells into S phase is not evident in BFS cells synchronized with 10% serum. In serum stimulated cells, the length of time required for maximum rates of DNA synthesis to be achieved following initiation of S phase was three times that observed for HU-synchronized cells. In fact, S phase was twice as long in serum stimulated cells as in HU-synchronized cells. Antimetabolites have been reported to decrease the time required for S phase (Mitchison, 1971). However, the results show that not only do HU-synchronized

BFS cells enter S phase more synchronously than serum stimulated cells, they also exit S more synchronously. Therefore, the longer time required for cells to complete DNA synthesis following serum stimulation probably reflects asynchrony of cells traversing S rather than a longer S period per cell.

HeLa, CHO, and BHK-21 cells, exposed to concentrations of HU completely inhibitory for DNA synthesis, continued to synthesize RNA and protein at near normal rates (Pfeiffer and Tolmach, 1967; Young and Hodas, 1964; Pollak and Rosenkranz, 1967). The synthesis of RNA and protein in the absence of DNA synthesis often resulted in a state of irreversible unbalanced growth leading to death of the cells. In fact, a rapid loss in viability occurred when CHO cells were exposed to HU for greater than 12 hr (Tobey and Crissman, 1972). In contrast, HeLa cells remained viable through at least 16 hr of HU exposure, and there was a high rate of survival of mouse L cells exposed to 2 mM HU for 18 hr (Pfeiffer and Tolmach, 1967; Kim _et al._, 1967; Adams and Lindsay, 1967). However, there was a rapid decrease in the percent survival of the population following exposure of these cells to HU for periods in excess of 18 hr.

Exposure of BFS cells to 2 mM HU for 32 hr did not result in a state of irreversible unbalanced growth since upon release, i) 85% of the cells were involved in DNA

replication; ii) RNA and protein synthesis continued; and iii) cells were able to support the replication of BPV. The ability of BFS cells to tolerate this long exposure to HU may reflect the reduced rate of RNA and protein synthesis observed in cells following exposure to HU for greater than 20 hr. This reduced rate of synthesis was accompanied by complete inhibition of DNA synthesis at 24 hr. Therefore, although RNA and proteins were synthesized in the absence of DNA synthesis, their levels were sufficient to maintain cell metabolism to allow cell cycle traverse to the G_1/S border, but not sufficient to cause severe, unbalanced growth.

Although HU has been reported to kill selectively S phase CHO cells, it has no lethal effect on S phase HeLa cells (Sinclair, 1967; Pfeiffer and Tolmach, 1967). Cells in S phase not killed by HU may become blocked in S upon exposure to HU while those cells at other stages of the cell cycle proceed to the G_1/S border. Therefore, upon release from HU, the cells previously blocked in S resume DNA synthesis out of phase with those cells moving into S from the G_1/S border. This phenomenon has been noted for cells synchronized with excess TdR. Application of a second TdR block following progression of all cells through S phase increased the degree of synchrony achieved following removal of the second TdR block (Galavazi *et al.*, 1966). A double HU block has also been reported to be

more effective in synchronizing Novikoff rat hepatoma cells than a single application (Plagemann et al., 1974). However, application of a second HU block did not increase the degree of synchrony obtained following a single exposure of BFS cells to HU. A possible explanation for these results is that no S phase cells were initially present because monolayered, stationary cells were always seeded in medium containing HU. Moreover, any differences observed in BFS cells exposed to HU may simply reflect inherent differences between primary and line cells.

5.2. Kinetics of Replication of Bovine Parvovirus

Asynchronous, actively dividing cells inoculated with BPV 18 hr after seeding in fresh medium containing 10% serum did not produce detectable infectious progeny virus until 16 hr p.i. (Bates and Storz, 1973). However, inoculation of HU-synchronized cells with BPV at the beginning of S phase resulted in a latent period of only 8 hr. Furthermore, the rate of increase of infectious progeny virus was greater in the synchronized cells, and maximum titers were detected 12 hr later. In contrast, maximum titers of infectious virus were not achieved in asynchronous cells until 32 hr after the latent period (Bates and Storz, 1973).

The latent period of 8 hr observed for BPV replication in HU-synchronized cells is comparable to that reported

for RV inoculated at a high m.o.i. (Salzman et al., 1972) and for Lu III virus (Siegl and Gautschi, 1973a). Furthermore, the number of cells involved in viral replication, as determined by immunofluorescence, was 85% for BFS cells inoculated with BPV but only 30% for rat embryo cells infected with RV (Salzman et al., 1972) and 55% for HeLa cells infected with Lu III virus (Siegl and Gautschi, 1973a). The high degree of synchrony achieved in BFS cells following treatment with HU may have enabled larger percentages of cells to initiate BPV replication rapidly than reported for cells infected with other parvoviruses.

The appearance of infectious BPV only 2 hr after viral DNA synthesis was first detected by the Hirt procedure (6 hr p.i.) may indicate that the rate limiting step in BPV maturation is the time required for production of BPV DNA. Further evidence that the rate of viral DNA synthesis governs the rate of maturation of BPV resides in the fact that as the rate of viral DNA synthesis declines after 16 hr p.i., so does the rate of production of new, infectious virions. In cell cultures synchronized with 40% serum and infected during early S with H-1 virus, Hirt-extractable DNA was also first detected at 6 hr p.i. (Rhode, 1974). In addition, a relationship was found to exist between the synthesis of H-1 viral antigens and viral DNA synthesis since antigen formation occurred at least 2 hr after the onset of H-1 DNA synthesis.

Initiation of BPV DNA synthesis occurs after the initiation of cellular DNA synthesis when BFS cells are inoculated upon HU removal. Furthermore, BPV DNA is not synthesized at a maximum rate at the same time as the maximum rate of cellular DNA synthesis, and BPV DNA synthesis continues after the completion of cellular S phase. Therefore, some of the requirements for initiation and continued replication of cellular DNA synthesis may be distinct from those required for the synthesis of BPV DNA.

Rhode (1973) concluded that since H-1 viral DNA synthesis required for subsequent HA production (HA-DNA synthesis) did not occur until late S phase, a particular event which occurs during S phase was required. BPV DNA synthesis is also initiated during late S phase of the cell cycle and continues during G_2 phase when BFS cells are infected immediately upon release from the HU block. Furthermore, BPV replicates in BFS cells continuously exposed to HU and unable to initiate synthesis of cellular DNA. Thus, initiation of BPV replication is not dependent upon cellular DNA synthesis *per se*. The fact that the latent period for progeny BPV production and the onset of viral DNA synthesis is extended to 16 hr in HU blocked cells may reflect the lack of accumulation of critical levels of a factor required for initiation of BPV replication until this time.

It is possible that the factor(s) required for BPV replication may be synthesized continuously throughout the cell cycle but produced at the highest level during mid to late S phase. Therefore, this factor would be synthesized slowly or not at all in noncycling, stationary cells which synthesize RNA and protein at very low rates. Cells synthesizing the required factor at similar levels except during S phase would be expected to show similar kinetics of replication of parvovirus when infected at any stage other than early to mid S. This would explain why stationary rat embryo cells support very little replication of RV, while cycling cells infected after the completion of S phase initiate viral replication prior to the subsequent period of cellular DNA synthesis (Tennant et al., 1969). The asynchronous increase in Lu III progeny virus when cells were infected during late S or G_2 phase similar to the increase in virus titers observed when asynchronous cells were inoculated (Siegl and Gautschi, 1973a), supports the hypothesis of continued synthesis of this factor throughout the cell cycle.

Although BFS cells blocked prior to S phase with HU are not cycling, they are distinctly different from stationary cells because RNA and protein are synthesized at significant rates and because DNA synthesis begins immediately after removal of HU. Therefore, these cells would be expected to synthesize the factor required for

initiation of BPV DNA synthesis and progeny virus production at a rate more comparable to that in cycling cells than in stationary cells. The fact that the latent period of 16 hr for BPV replication in HU blocked cells is the same as that in asynchronous cells (Bates and Storz, 1973) further suggests that HU blocked cells synthesize the required factor at levels similar to those in asynchronous cells.

Since parvovirus DNA synthesis always precedes production of viral progeny and appears to govern the rate of synthesis of viral antigens, the factor provided by cycling or HU blocked cells which is required for initiation of parvovirus replication is most likely related to viral DNA synthesis. Therefore, enzymes or nucleotide precursors required for DNA replication are plausible candidates for this factor. The enzymes shown to achieve maximum levels in S phase HeLa cells are thymidine kinase, deoxycytidine deaminase (Stein and Baserga, 1972), DNA polymerase α, and DNA polymerase γ (Spadari and Weissbach, 1974). The effect of these enzymes on parvovirus replication needs to be studied to determine if the absence of one or more is responsible for the lack of parvovirus replication. If temperature sensitive mutants of cells for synthesis of these enzymes were available, the extent of replication of parvoviruses at nonpermissive temperatures could be determined.

Enzyme levels in BFS cells continuously exposed to HU could also be determined. It is feasible that since cellular DNA synthesis is completely inhibited in these cells while BPV DNA synthesis is not, those enzymes whose synthesis is strictly dependent on cellular synthesis may occur at extremely low levels. However, those enzymes that are required for BPV DNA replication may occur at levels comparable to those found in cycling cells.

The maximum level of infectious BPV produced and the level of DNA synthesis in infected cells in the presence of HU are lower than those in the absence of HU. Furthermore, increased levels of HU decreased the production of BPV hemagglutinating antigen. Therefore, although BPV DNA synthesis is less sensitive to HU than is cellular DNA synthesis, it is inhibited to some extent. Although the ultimate mode of action of HU in BFS cells is unknown, HU may cause the reduction of pool sizes of nucleotide precursors of DNA as reported for CHO cells by Walters et al. (1973). Since the genome of BPV is probably smaller than the average unit of replication of the host genome, the critical level of nucleotides required for initiation may also be smaller. However, the reduced level of precursors would reduce the rate of BPV DNA synthesis resulting in fewer molecules of DNA and thus, fewer infectious virions in a given period of time.

5.3. Effect of Bovine Parvovirus Replication on Cellular Macromolecular Synthesis

BFS cells inoculated with BPV at high or low m.o.i. at the beginning of S phase exhibited neither a reduced rate of incorporation of TdR into DNA nor a change in the rate of progression of cells through S phase. In contrast, rat embryo cells inoculated with RV showed a marked decrease in total DNA synthesis by 5 hr p.i. with respect to controls (Tennant, 1971). In the mock-infected controls, cellular DNA synthesis did not begin until 10 hr, while the rate of total DNA synthesis remained low in infected cells at this time. Viral inhibition of S phase in this instance probably resulted from a commitment by the infected cells to synthesize viral precursors by the time S phase would have been initiated. In order to determine if cellular DNA synthesis proceeds unobstructed in cells inoculated with BPV prior to S phase, it will be necessary to infect synchronous populations of G_1 BFS cells. Such cells may best be obtained by mitotic selection of asynchronous cells with the use of colcemid, or by selection of mitotic cells following HU-synchronization.

After 6 hr p.i. the rate of DNA synthesis in BPV-infected cells began to increase over levels observed in mock-infected cells. The increase in DNA synthesis was most likely due to the synthesis of viral DNA since BPV DNA was first detected in the Hirt supernatant at the

same time. Furthermore, DNA extracted by the Hirt procedure contains only viral species since no significant radioactivity was detected in the crude Hirt supernatant from mock-infected cells nor at any density representative of DNA following isopycnic centrifugation of the mock-infected Hirt supernatant. However, simultaneous induction of host DNA synthesis cannot be ruled out based on these experiments alone. In order to ascertain definitively if host species of DNA are synthesized during the second period of DNA synthesis in infected cells, it is necessary to determine if the DNA produced in infected cells following S phase hybridizes to cellular DNA to a greater extent than does purified BPV DNA.

Both uridine and amino acid incorporation were reduced in cells infected with BPV relative to controls. The levels of RNA and protein synthesis in cells infected with an m.o.i. of 40 PFU/cell decreased below control rates 4 hr earlier than in cells infected with an m.o.i. of 2. Therefore, the level of production of viral precursors may influence the decreases observed. Cells inoculated with a high m.o.i. would be expected to accumulate greater amounts of viral precursors earlier than cells infected with a low m.o.i. because of the larger number of virions involved in the replication process. These precursors may either be toxic to cells directly or their synthesis may compete with the host cell for substrates required for

RNA and protein synthesis. Interestingly, CPE became evident at the same time as the decrease in RNA and protein synthesis was observed. Therefore, the reduced levels of RNA and protein synthesis in infected cells probably reflects the total commitment of cells to BPV replication.

Only H-1 virus has previously been reported to cause a decrease in the total rate of uridine incorporation in infected cells (Fong et al., 1970). However, the effect was evident only after 20 hr, when DNA synthesis was severely inhibited. It was argued that the reduced rate of RNA synthesis was an indirect result of the inhibition of DNA synthesis in H-1 virus-infected cells (Tennant, 1971). Indeed, this is not the case in BFS cells infected with BPV, since S phase was completed without inhibition by the time when reduced rates of RNA and protein synthesis were evident.

Inhibition of RNA and protein synthesis in BPV-infected cells is immediately preceded at 6 hr p.i. by a peak in the rate of RNA and protein synthesis in excess of rates in control cells. This increased synthesis may reflect the synthesis of viral specific species. However, inhibition of host specific RNA and protein synthesis may occur well before 6 hr p.i. masking any small increase in the synthesis of viral species early after infection. This effect was noted for cells infected with RV. Although total RNA synthesis was virtually unaffected by

RV replication (Tennant, 1971; Salzman _et al._, 1972), hybridization studies revealed that there was a progressive reduction in host specific RNA species (Salzman and Redler, 1974). Therefore, the control rates of RNA and protein synthesis observed in RV-infected cells was due to high levels of synthesis of viral species.

To determine when viral specific RNA is first synthesized in BPV-infected cells, RNA isolated at different times postinfection should be monitored for its ability to hybridize BPV DNA. Synthesis of viral specific proteins may be monitored by comparing the electrophoretic pattern of proteins isolated from infected cells to those in mock-infected cells and in purified virus capsids. Similar techniques may also be used to determine when the decrease in host specific RNA and protein synthesis begins and to ascertain if all host species are reduced to the same extent by BPV replication.

The strand specificity for transcription of RNA from BPV DNA may also be determined using hybridization techniques. Heavy and light single stranded DNA separated by centrifugation of double stranded BPV DNA in alkaline CsCl, may be used to determine which strand of DNA is complementary to the RNA isolated from infected cells. If one assumes that this RNA contains predominantly functional mRNA species, the strand of DNA hybridizing most (if not all) of the RNA is the template for transcription.

5.4. Physical Properties of Bovine Parvovirus DNA

Isolation of single stranded BPV DNA from purified virions is difficult due to the compact structure of the viral capsid. DNA was not released from the capsids as a result of treatment of virions with SDS followed by phenol extraction. Extensive enzymatic treatment was successful in removing the single stranded DNA from purified BPV. However, the DNA obtained by this procedure was heterogeneous in its sedimentation pattern in sucrose gradients as well as in buoyant density, probably as a result of extensive hydrolysis of the genomic DNA. DNase may have been a contaminant in any of the enzymes used, particularly in the pronase. Even in minute quantities, the presence of DNase would be sufficient to catalyze extensive hydrolysis of DNA due to the long periods of exposure of the DNA to the enzymes. Enzymatic treatment of the capsids may be required for DNA isolation, but shorter periods of treatment may be optimum for extraction of the intact genome.

DNA isolated by the Hirt procedure contained double stranded species synthesized *in vivo* in infected cells. The double stranded species were successfully separated from protein, RNA, and single stranded DNA, also present in the Hirt supernatant, by HAP chromatography. The DNA was eluted from the HAP at high ionic strengths of PO_4 (0.48 M), but not at ionic strengths sufficient to elute

single stranded species (0.14 M). Furthermore, DNA eluted at the high ionic strength exhibited thermal denaturation kinetics of double stranded DNA. The 22% hyperchromic shift observed for BPV DNA is significantly less than that for *B. fragilis* DNA. However, it is greater than would be observed for single stranded DNA. Further, the G+C content of 42% calculated from the T_m agrees well with that of 40.9, 43.5, and 42.2% reported for the nondefective parvoviruses MVM, RV, and H-1 (Rose, 1974).

Covalently closed circular RF's are produced during the replication of ØX 174 (Fiers and Sinsheimer, 1962), but RF's in this configuration have not been reported in DNA isolated from cells infected with animal parvoviruses. However, Rhode (1974) reported that a minute amount of H-1 double stranded DNA sedimented in the presence of ethidium bromide at the high buoyant density characteristic of covalently closed circles. Rhode dismissed this observation as not significant. His results could indicate the synthesis of circular forms occurring *in vivo* although most of the molecules were nicked during the isolation procedure. Tattersall *et al.* (1973) have shown that 20 to 40% of the double stranded species of MVM isolated was resistant to exonuclease. Thus, there is precedence for the existence of anomalous forms of double stranded DNA isolated from parvovirus-infected cells.

Rapid renaturation kinetics of double stranded BPV DNA is consistent with the presence of covalently closed circular forms. However, the resolution of two distinct bands in the double stranded preparation subjected to electrophoresis in 1% agarose gels confirms the existence of at least two forms of double stranded DNA. Since the slower moving band of BPV DNA migrates at a rate comparable to open circular ØX 174 RF DNA, it is feasible that this configuration exists in BPV DNA preparations. The faster moving BPV DNA band migrates slower than the covalently closed circular ØX 174 RF band. However, the difference in migration rates may reflect different degrees of supercoiling. In fact, supercoiled *E. coli* plasmid DNA migrates only slightly faster than its open circular counterpart. Thus, the faster moving BPV band could be covalently closed circular DNA with less supercoiling than ØX 174 closed circular RF's.

Covalently closed circular forms of BPV DNA could be conclusively demonstrated if endonucleolytic attack of the DNA resulted in the disappearance of the faster moving band, but not the slower moving one. In addition, DNA extracted from uninfected cells by the Hirt procedure and subsequent HAP chromatography should be analyzed for its electrophoretic pattern to control for possible contaminating cellular species in the BPV DNA preparation.

5.5. Concluding Remarks

BPV replicates more readily in synchronized cell cultures inoculated at the beginning of S phase than in asynchronous cell cultures. By employing synchronized cells, the kinetics of viral replication were reproducible. Because the rates of synthesis of DNA, RNA, and protein were characterized in the synchronized cells, the effects of bovine parvovirus replication on cellular macromolecular synthesis were accurately and reproducibly accessed.

Replication of BPV has little effect on cellular macromolecular synthesis until after significant replication of BPV DNA has been initiated. However, corresponding to the increase in viral DNA synthesis, there is a rapid decrease in RNA and protein synthesis in infected cells. This effect represents the commitment of cells to the production of viral precursors at this time.

The efficient and synchronous replication of BPV in HU-synchronized BFS cells enabled the isolation of large quantities of double stranded DNA from infected cells. The replicative forms isolated existed in two species. Preliminary results indicate these species to be analogous to those reported for ØX 174 replicative forms. However, more extensive characterizations will be necessary to ascertain if one of these is in a covalently closed configuration.

BPV replication, like that of other nondefective parvoviruses, was shown to depend upon the physiological state of the host cell. Although the factor required for parvovirus replication is not efficiently provided by noncycling stationary cells, it is provided by HU blocked cells unable to initiate cellular DNA synthesis. Therefore, replication of BPV is not dependent on cellular DNA synthesis *per se*, but may be dependent on a factor produced at the highest levels in S phase. However, the nature of this factor has not been elucidated. More extensive characterization of enzyme levels, such as those of thymidine kinase or DNA polymerases α or γ, and of nucleotide pools in HU blocked cells may help to determine the means of initiation of parvovirus replication.

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7. Appendix

List of Abbreviations

List of Abbreviations

A_{260}	Absorbance at 260 nm
AAV	Adeno-associated virus
BFS	Bovine fetal spleen
BPV	Bovine parvovirus
CHO	Chinese hamster ovary
CPE	Cytopathic effects
ara C	Cytosine arabinoside
DNV	Densonucleosis virus
FPV	Feline panleukopenia
FA	Fluorescent antibody
FUdR	Fluorodeoxyuridine
HA	Hemagglutination
HSV	Herpes simplex virus
HAP	Hydroxylapatite
HU	Hydroxyurea
MEM	Minimum essential medium
MEV	Mink enteritis virus
MVC	Minute virus of canines
MVM	Minute virus of mice
m.o.i.	Multiplicity of infection
PBS	Phosphate buffered saline
PFU	Plague forming units
PPV	Porcine parvovirus
p.i.	Postinfection
RV	Rat virus
RF	Replicative form
Sarkosyl	Sodium dodecyl sarcosinate
SDS	Sodium dodecyl sulfate
ts	Temperature sensitive
TdR	Thymidine
$TCID_{50}$	Tissue culture infective dose
TCA	Trichloroacetic acid
TBS	Tris buffered saline
u.v.	Ultraviolet

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REPLICATION OF BOVINE PARVOVIRUS

by

Deborah Sue Parris

(ABSTRACT)

Bovine parvovirus (BPV) is a small icosahedral virus containing single stranded DNA and belongs to the group of nondefective parvoviruses. Research on this virus group has revealed that viral replication occurs in cycling cells only, and evidence exists that the required factor provided by cycling cells is produced during early or mid S phase. Due to this S phase dependence, parvoviruses replicate synchronously only in synchronized cells. This study was initiated in order to determine the kinetics of replication of BPV in highly synchronized cells, the effects of viral replication on host macromolecular synthesis, and the properties of the double stranded DNA produced in infected cells.

Bovine fetal spleen (BFS) cells, in which BPV replicates optimally, were synchronized at the G_1/S border by exposure of cells to 2 mM hydroxyurea (HU) for 32 hr. Immediately after release of cells from the HU block by washing, DNA synthesis began. Autoradiographic analysis

revealed that within 2 hr, 80 to 85% of the cells were synthesizing DNA.

The latent period for progeny BPV production was 8 hr in HU-synchronized cells infected at the beginning of S phase compared to a 16 hr latent period in asynchronous cells. In addition virus titers and the percentage of cells containing viral specific antigens increased more synchronously in HU-synchronized cells than in asynchronous cultures. Synthesis of BPV DNA always preceded the initial increase in virus titers and appeared to govern the rate of virus maturation. BPV DNA synthesis was always initiated during late S phase in cells infected at the beginning of S. Cellular S phase was not affected by BPV replication, but RNA and protein synthesis declined rapidly after the onset of BPV DNA synthesis in infected cells.

The double stranded BPV DNA produced during infection was isolated in the supernatant after selective precipitation of cellular DNA with NaCl and sodium dodecyl sulfate (Hirt procedure) followed by hydroxylapatite chromatography. Some of the molecules of DNA isolated in this way existed in a covalently closed circular configuration as demonstrated by rapid reannealing rates following thermal denaturation. In addition, agarose gel electrophoresis revealed bands of DNA in this preparation comparable to ØX 174 closed and open

circular replicative forms. No double stranded DNA isolated from cells infected with other animal parvoviruses has been shown to contain covalently closed circular forms.

Although BPV is replicated to only a minor extent in stationary BFS cells, BPV was shown to replicate in cells infected 32 hr after the cells were exposed to 2 mM HU but not released. Throughout replication, cells remained in HU and no cellular DNA synthesis was detected. However, BPV DNA and progeny BPV were produced beginning at 16 hr postinfection. This synthesis was not due to viral production in a few cells only, since approximately 75% of the cells were involved in BPV replication as demonstrated by immunofluorescent staining. Therefore, it appears that a factor associated with S phase of the cell cycle and required for optimum BPV replication is produced even in the absence of cellular DNA synthesis *per se*.