BOVINE PARVOVIRUS AND BOVINE ENTEROVIRUS IN MIXED INFECTIONS

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. Introduction

In a mixed infection with viruses, two or more different viruses infect a single host at the same time. During such an infection, individual cells of that host may be infected with two or more different viruses at the same time.

Multiple infections are very similar to mixed infections, except that only one kind of virus is involved. In multiple infections, two or more virions of the same virus type infect a single host organism at the same time. Also, as often occurs in a mixed infection, an individual cell of a host is infected with two or more virions of the same virus type at the same time. Therefore, mixed and multiple infections often occur together.

The existence of mixed infections was first demonstrated by Syverton and Berry (1936). These investigators induced specific inclusions of two viruses (herpes simplex and vaccinia) in single cells of rabbit cornea. Similar experiments were performed by Anderson (1942). By using viruses that form recognizable intracellular inclusions, he found cytological evidence that individual cells may be infected by two different viruses at the same time. Such virus combinations as fowlpox and herpes simplex virus, laryngotracheitus virus and vaccinia virus, and herpes simplex and vaccinia or rabies virus were used by Anderson to demonstrate mixed infection.

It is apparent that the intestinal tract of man and other animals can be infected with a variety of different virus types. For example, the human intestinal tract at various times is infected with RNA viruses such as picornaviruses (enteroviruses) and reoviruses, and DNA viruses such as adenoviruses and parvovirus-like viruses (i.e. hepatitis virus and non-bacterial gastroenteritis virus). Similarly, the bovine intestinal tract can be infected with picornaviruses, reoviruses, coronaviruses, adenoviruses, and parvoviruses. The interactions of these viruses may ultimately affect any disease produced. It is difficult to assess the significance of mixed infections with these viruses since little is known about the interactions which occur in single cells infected with a DNA virus that replicates in the nucleus and an RNA virus that replicates in the cytoplasm.

In the present study, a model system was used to determine the nature of the interactions which occur in a mixed infection involving a DNA and an RNA virus. Cultures of bovine fetal spleen (BFS) cells were mixedly infected with bovine parvovirus type 1 and bovine enterovirus, strain 71-3-13W. These two viruses were originally isolated from the intestine of a calf in which they were occurring as a mixed infection.

The objectives of this investigation were:

1. To define the growth cycles of bovine parvovirus

and enterovirus during single infection.

A. To determine the effect of each virus on cellular macromolecular syntheses in single infections.

B. To determine the rate of bovine parvovirus DNA synthesis in cells singly infected with bovine parvovirus.

2. To investigate the effects of simultaneous mixed infection of cells with bovine parvovirus and enterovirus.

A. To determine the effect of this mixed infection on the rate of bovine parvovirus DNA synthesis.

B. To determine the effect of this mixed infection on the production kinetics of each virus.

3. To study the effect of enterovirus infection on bovine parvovirus replication in cell cultures preinfected with bovine parvovirus.

A. To investigate the effect of this mixed infection on total cellular RNA and protein synthesis.

B. To determine the effect of this mixed infection on the rate of bovine parvovirus DNA synthesis.

C. To determine the effect of this mixed infection on the production kinetics of each virus.

2. Literature Review

2.1. Properties of Bovine Parvovirus.

2.1.1. Biochemical and Biophysical Properties:

Bovine parvovirus contains single-stranded DNA, which weighs 1.5×10^6 daltons. The capsid of each virion is composed of two or possibly three different proteins. By using the electron microscope, it was determined that the virions were icosahedral in shape and had a diameter of 20 to 22 nm (Bates, 1972). Also the buoyant density of complete virions was found to be 1.42 g/cc. In addition, bovine parvovirus was found to be stable at 56 C and to be resistant to ether and extremes in pH.

2.1.2. Cellular Sites of Replication:

Through the use of immunofluorescence and electron microscopy it was determined that bovine parvovirus replicates and matures in the nucleus of the infected cell (Bates, 1972). Specific fluorescence was detected in the nuclei of cells as early as 12 hours after inoculation with bovine parvovirus. The site of synthesis of structural polypeptides of bovine parvovirus has not been determined, but for other DNA viruses replicating in the nucleus the viral protein synthesis occurs in the cytoplasm.

2.1.3. Cellular Requirements for Replication:

Some event which occurs during S phase of the cell cycle is required for the initiation of bovine parvovirus DNA synthesis. It was recently hypothesized that a factor (i.e. protein) produced during S phase, may be required for initiation of the replication of the bovine parvovirus DNA (Parris and Bates, 1975). Therefore, if synchronized cells are used, detectable rates of viral DNA synthesis are higher sooner than if nonsynchronized cells are used (Bates and Storz, 1973; Parris and Bates, 1974).

2.2. Properties of Bovine Enterovirus.

2.2.1. Biochemical and Biophysical Properties:

The virions of bovine enterovirus are icosahedral in shape, have a diameter of 25 to 27 nm and have a buoyant density of 1.34 g/cc in CsCl (Martin, Johnston, and Clements, 1970). Also, the sedimentation coefficient of the complete virion is approximately 165S. The capsid of the bovine enterovirus is composed of four polypeptides of molecular weight 34,000; 28,000; 26,000 and 9,000 (Johnston and Martin, 1971). These polypeptides were found to occur in molar ratios of 1:1:1:0.5, respectively. Capsids with both 32 and 42 capsomeres are compatible with the model postulated by Rueckert (1971). Within each capsid is found single-stranded RNA, which sediments as a major peak of 35S relative to 28S and 16S ribosomal RNA and has a base

composition characterized by high A (30%) and low U (21%) (Martin , Johnston and Clements, 1970).

2.2.2. Cellular Sites of Replication:

The replication and maturation of the bovine enterovirus occurs in the cytoplasm of the infected cell (Dales and Franklin, 1962; Baltimore et al., 1963; Penman et al., 1964; Schiff, 1970).

2.2.3. Cellular Requirements for Replication:

Recent studies indicate that the replication of bovine enteroviruses may be influenced by the stage of the cell cycle when infection occurs. Eremenko and coworkers (1972) studied poliovirus replication in the different phases of the life cycle of the HeLa cells. They found that viral replication does not take place at the same rate during the entire cell cycle, but that it occurs most readily in the S phase and to a lower extent in the G_1 phase. Further, there is very little viral replication at the end of G_1 and beginning of S, and in the M phase. If the same type of situation exists for the closely related bovine enterovirus, then it would be advantageous to use synchronized cells to study the replication process of this virus.

2.3. Types of Interactions Which May Occur in Mixed Infections.

2.3.1. Independent Replication of Both Viruses:

There are several different types of interactions which might occur when two different viruses infect the same cell. One possibility is that both viruses may replicate independently. Chicken cells support the normal replication of avian leukosis virus and several other viruses as mixed infections (Burmester et al., 1955; Rubin, 1960; and Rubin et al., 1961). Choppin and Holmes (1967) reported that monkey kidney cells preinfected with the paramyxovirus SV5 and superinfected with poliovirus showed no inhibition of either virus. Their results indicated that poliovirus inhibits cellular DNA-dependent RNA synthesis but not SV5 RNA-dependent RNA synthesis. Furthermore, poliovirus does not inhibit SV5 protein synthesis, but it does inhibit cellular protein synthesis, thus indicating that the poliovirus can distinguish between SV5 messenger and cellular messenger RNA. When rhesus monkey kidney cells were infected with SV40 for 3 days, SV5 for 7 days and measles virus for 14 days prior to superinfection with poliovirus, all four viruses replicated normally (Hsiung et al., 1966). If cells of lymphocytic choriomeningitis "carrier" mice are infected with lymphocytic choriomeningitis virus, ectromelia virus,

and Semliki Forest virus, all three viruses replicate independently (Mims and Subrahmanyan, 1966). Therefore, it seems quite possible that two different viruses can replicate independently in the same cell.

2.3.2. Complementation:

A second possible interaction is complementation which does not involve genetic recombination and which results in enhanced yields of one or both of the viruses. Newcastle disease virus, which normally does not produce cytopathic effects in swine testis cells until the seventh or eighth day after infection, produced cytopathic effects after only three days if the cells were also infected with hog cholera virus (Kumagai et al., 1961). Adenovirus-associated virus will not replicate unless adenovirus is replicating in the same cell (Parks et al., 1967; Ito et al., 1967). Without complementation by SV40, adenoviruses will not grow in rhesus monkey kidney cells (Lewis et al., 1966; Butel and Rapp, 1967). By viewing thin sections on the electron microscope, it was found that adenovirus would replicate in simian cells only if SV40 virus were infecting the same cells (O'Connor et al., 1963). Valle and Cantell (1965) demonstrated that the yield of infectious vesicular stomatitis virus in chicken or human cells was increased when the cells were preinfected with Sendai virus (parainfluenza type 1). In polyoma-transformed

BHK21 cells, Kisch and Gould (1973) observed a decrease in sensitivity to vesicular stomatitis virus. However, dual infection of these cells by vesicular stomatitis virus and Newcastle disease virus resulted in enhancement of plaque size and number.

2.3.3. Phenotypic Mixing:

Phenotypic mixing results when there is an association of phenotypes with non-homologous genotypes. Delbruck and Bailey (1946) first demonstrated this phenomenon when they observed yields of bacteriophages from bacteria which had been mixedly infected with T2 and T4 bacteriophages. In 1956, Streisinger showed that the yield from mixedly infected bacteria contained doubly neutralizable particles as well as the parental phenotypes. This type of occurrence was also observed with animal viruses. In mixed infections of two antigenically different strains of influenza virus, the production of doubly neutralizable particles was observed (Burnet and Lind, 1953; Hirst and Gotlieb, 1953; Gotlieb and Hirst, 1954). Phenotypic mixing occurs with influenza A and B and with influenza A and Newcastle disease virus (Granoff and Hirst, 1954). Also, it has been described with measles and Sendai viruses (Norrby, 1965). Doubly neutralizable particles were produced in single cells mixedly infected with ECHO7 and coxsackie A9 viruses (Itoh and Melnick, 1959). Holland and Cords (1964) showed the occurrence of phenotypic mixing in cells

mixedly infected with two different enteroviruses. If human epidermoid number 2 cells were preinfected with herpes simplex virus and superinfected with vesicular stomatitis virus, some of the progeny virions produced had a vesicular stomatitis virus genome enclosed within herpes simplex envelope antigens (Huang, Palma and Hewlett, 1974).

2.3.4. Genotypic Mixing:

In genotypic mixing, virions contain mixed but not recombined genetic material from more than one parent virus. Gotlieb and Hirst (1954) found that most phenotypically mixed influenza virions also had mixed genomes, and produced both parental types on further cloning. If a cell is mixedly infected with two viruses which both mature by budding through the cytoplasmic membrane, there often occurs one or more copies of the genome of each virus within a single envelope (Hirst, 1962).

2.3.5. Viral Attachment Interference:

Another possible type of interaction which can occur in a mixed infection is interference. Interference results in a depression of virus yield in one or both of the viruses. There are several different types of interference. Viral attachment interference involves the alteration or destruction of cellular receptors for virus. Crowell (1966) reported such interference in HeLa cells infected

with poliovirus. He believed the interference to be due to residual capsid material blocking the cellular receptors. In chicken cells infected with avian leukosis virus, there is viral attachment interference against Rous sarcoma virus (Rubin, 1960). Bovine fetal spleen cells, saturated with bovine enterovirus, adsorbed bovine viral dairrhea virus more slowly than untreated bovine fetal spleen cells (Schiff, Storz and Collier, 1973).

2.3.6. Homologous Intracellular Interference:

Intracellular interference involves the modification of viral replication within the infected cell. One type of intracellular interference is called homologous interference, which occurs only with homologous viruses. Henle and Rosenberg (1949) described a situation in which there was interference by UV-irradiated influenza virus on active influenza virus. Using the two serotypes (N.J. and IND.) of vesicular stomatitis virus, Cooper (1958) found an example of homologous interference that was heterotypic. UV-irradiated vesicular stomatitis virus-NJ interfered with active vesicular stomatitis virus-IND but not with active vesicular stomatitis virus-NJ, and vice versa.

Defective T particles partially purified from undiluted passage stocks of Indiana serotype vesicular stomatitis virus interfere with the replication of

homotypic infectious B particles in Krebs-2 mouse ascites tumor cells (Huang and Wagner, 1966). The T particle is shorter than the bullet-shaped B plaque-forming virion. The interference appears to take place during an early stage of viral protein and RNA synthesis in the replication cycle of B. These investigators hypothesized that the T particle interferes with the synthesis or function of virus-specific enzymes or RNA of B.

Roizman (1965) reported that in dog kidney cells mixedly infected with MPdk$^-$, a conditional lethal strain of herpes simplex virus, and with MPdk$^+$sp, a mutant capable of multiplying in dog kidney cells, the yield of infectious MPdk$^+$sp is greatly reduced. He suggested that this interference was brought about because MPdk$^-$ specifies nonfunctional peptides or proteins which form nonfunctional aggregates on polymerization with potentially functional subunits specified by MPdk$^+$sp.

In homologous interference with polioviruses, it seems to be a prerequisite that the interfering virus be able to multiply first, before the infection by the second virus occurs. Cords and Holland (1964) found that the interfering virus had to replicate for one hour before it was challenged if interference was to occur. The most likely explanation for this interference is that there is

competition between the two viruses for sites of replication or for some substrate needed for viral synthesis.

2.3.7. Heterologous Intracellular Interference:

Heterologous interference, a second type of intracellular interference, is active against a virus belonging to a different taxonomic group from the virus causing the interference.

Several unrelated types of viruses have been shown to induce interference against superinfection by Newcastle disease virus (Marcus and Carver, 1965). This particular type of interference was called intrinsic interference. The capacity to inhibit Newcastle disease virus was demonstrated with rubella virus, Sindbis virus, West Nile virus, poliovirus and lactic dehydrogenase virus. A protein or proteins coded for by the interfering viral genome presumably causes the actual interference.

Vaccinia virus and frog virus 3 are both DNA viruses that replicate in the cytoplasm of the infected cell. They are not related biologically, but some structural component of frog virus 3 can inhibit the replication of vaccinia virus in cells co-infected with both viruses (Aubertin and Kirn, 1969; Vilagines and McAuslan, 1970). In some manner, the structural component of the frog virus 3 associates with and blocks the transcription of uncoated poxvirus DNA.

Doyle and Holland (1972) reported that in doubly infected HeLa cells, poliovirus type 1 rapidly and completely dominated the production of infectious vesicular stomatitis virus. The poliovirus inhibited incorporation of amino acids into vesicular stomatitis-specific proteins within two hours after superinfection. Vesicular stomatitis virus-directed RNA synthesis was not affected by the poliovirus. Apparently the poliovirus interfered with vesicular stomatitis virion production only at the level of translation of viral mRNA.

In HeLa cells mixedly infected with poliovirus and Sindbis virus, only the poliovirus replicated (Sreevalsan and Rosemond-Hornback, 1974). Translation, but not replication of the poliovirus appeared to be needed for the interference of Sindbis virus. The polyribosomes present in Sindbis virus-infected cells became disaggregated when the poliovirus was added. In the presence of the poliovirus, Sindbis virus messenger RNA's did not attach to the host ribosomes. Their results indicated that the observed interference was due to a block in the protein synthesis of the Sindbis virus possibly at the level of initiation. The restriction of translation of mRNA's other than poliovirus RNA appears to occur quite often in mixed infections, since in cell cultures infected with herpes simplex virus, Newcastle disease virus, or vesicular stomatitis virus,

virus-specific RNA's were not translated subsequent to superinfection with poliovirus (Doyle and Holland, 1972; Ito, Okazaki and Ishida, 1968; Saxton and Stevens, 1972). Giorno and Kates (1971) found that vaccinia virus messenger RNA's did not associate with ribosomes in cells doubly infected with vaccinia and adenovirus.

When Schiff and Storz (1972) preinfected bovine embryo kidney cells or bovine embryo spleen cells with bovine viral diarrhea virus and then challenged with bovine enterovirus, they observed a reduction in the yield of bovine enterovirus produced after 20 hr of incubation. A short period of replication of the preinfecting virus was necessary before intracellular interference would take place. They believed that this interference resulted from either a competition between preinfecting virus and challenge virus for substrate or site of replication within the host cell.

Preinfection of fetal porcine kidney cell cultures with porcine parvovirus resulted in only a slight interference of the replication of porcine enterovirus, pseudorabies virus, vesicular stomatitis virus, or hemagglutinating encephalomyelitis virus (Mengeling, 1975). When porcine kidney cells were mixedly infected with porcine parvovirus and vesicular stomatitis virus, porcine parvovirus replication was severely inhibited.

Khoobyarian and Fischinger (1965) observed that the addition of heat inactivated (56 C, 1 hr) adenovirus 2 fluids to RHF-1 cell cultures prevented the formation of vaccinia plaques in those cells when they were superinfected with vaccinia virus. Replication of adenovirus was not necessary in order for interference to be initiated. That the inhibitory effect of the heated virus fluids was exerted at an intracellular site was verified by the fact that the percent of challenge vaccinia adsorbed was virtually the same in both treated and susceptible cultures.

An example of heterologous interference involving a DNA virus that replicates in the nucleus and an RNA virus that replicates in the cytoplasm was studied by Bablanian and Russell (1974). In their work they infected HeLa cells, pre-infected with adenovirus, with poliovirus in the presence of quanidine. Guanidine selectively inhibits the replication of poliovirus but does not affect poliovirus inhibition of host protein synthesis. Also, guanidine selectively inhibits cellular polypeptide synthesis and prevents the formation of adenovirus particles. From their experiment they found that superinfection of adenovirus-infected HeLa cells with poliovirus in the presence of guanidine can dramatically alter the course of adenovirus infection. These results seem quite plausible since it has been found previously that in single infections of

poliovirus, there is a reduction in cellular polypeptide and cellular RNA synthesis. Zimmerman and coworkers (1963) found that poliovirus significantly reduces the host cell polypeptide synthesis. The mechanism of host cell protein synthesis inhibition by poliovirus appears to be an inactivation of the host cell messenger RNA. It is hypothesized that the host cell messenger RNA becomes incapable of associating with the host cell ribosomes (Willems and Penman, 1966). The inhibition appears to be due to a product of the viral genome which is stable for at least 1 hr in the absence of protein synthesis and seems to be specific for the host cell mRNA. Replication of poliovirus occurs normally in cells in which cellular protein synthesis has been stopped (McCormick and Penman, 1968; Doyle and Holland, 1972). Bablanian and coworkers (1965) reported that poliovirus significantly inhibits the RNA synthesis of infected cells within 1 to 2 hr after infection and the inhibition increases with time.

3. Materials and Methods

3.1. Preparation of Cell Cultures.

Primary cultures of bovine fetal spleen (BFS) cells and bovine fetal kidney (BFK) cells were prepared from the organs of five to six month old bovine fetuses by the method of Youngner (1954). Both cell types were grown in Earles minimal essential medium (MEM) (See appendix I) supplemented with 10% lamb serum for growth and with 3% lamb serum for maintenance. Also contained in the medium were penicillin-G (100 units/ml) and streptomycin (100 μg/ml).

Secondary cell cultures were prepared from confluent monolayers of each cell type by trypsin-versene (See appendix I) dispersion of the cells from the glass surface. Tenth to twentieth passage cells were used in this study. The cells were seeded into appropriate experimental vessels at a concentration of 1.6×10^5 to 2.5×10^5 cells/ml of medium.

After the seeding procedure, actively dividing, but asynchronous, cell cultures were incubated in a CO_2 incubator at 37 C for 24 to 48 hr before they were used in virus propagation and plaque assay procedures.

Synchronized cell cultures were obtained by seeding cells in media containing hydroxyurea (HU) at a concentration of 2×10^{-3} M. HU, at this concentration, blocks the cells

at the G_1/S border after 32 hr of incubation in 5% CO_2 at the temperature of 37 C (Parris et al., 1975). After 32 hr incubation, the HU block was removed by washing the cells three times with Dulbecco's phosphate buffer (See appendix I).

3.2. Origin of the Viruses.

Bovine parvovirus strain 71-1-20W and bovine enterovirus strain 71-3-13W were isolated as a natural mixed infection from a calf with neonatal diarrhea (Bates et al., 1972). The two viruses were separated by the use of specific antiserum. Bovine parvovirus 71-1-20W was determined to be antigenically identical to bovine parvovirus type 1 (Bates et al., 1972). The serological subgrouping to which bovine enterovirus 71-3-13W belongs was not determined.

3.2.1. Preparation of Bovine Parvovirus:

Ten to twelve plastic flasks (Falcon, 250 ml) were seeded with 2.75×10^6 BFS cells and incubated at 37 C for 18 hr. After 18 hr, the medium was removed and one ml of bovine parvovirus stock was added to each flask and the virus adsorbed to the cells for 1 hr. Then 20 ml of growth medium containing 10% lamb serum was added to each flask and they were incubated at 37 C until 95% of the cell layer showed cytopathic changes. The cultures were then frozen at -20 C and thawed three times. After

this procedure, the contents of the flasks were centrifuged at 10,000 x g for 15 min. The pellets were discarded and the supernatant, containing the virus, was stored at -20 C.

The plaque assay method was used to titer the virus. BFS cells were seeded in plastic petri dishes (60 mm) at a concentration of 1 x 10^6 cells per plate. Twenty-four hr after seeding, the cell cultures were washed two times with 1 ml of MEM and 0.25 ml of serial 10-fold dilutions of the bovine parvovirus stock was adsorbed for 30 min at 37 C. After adsorption, 8 ml of overlay consisting of 1X MEM, 1% ionagar and 10% lamb serum was added to each plate. The plates were incubated for 5 to 7 days at 37 C in a CO_2 incubator. The plaques were visualized by staining with neutral red or crystal violet.

3.2.2. Preparation of Bovine Enterovirus:

BFS cells were grown to confluency in glass bottles. To each of the cell cultures was added 1 ml of high multiplicity bovine enterovirus. The cell cultures were incubated at 37 C until 95% of cells showed cytopathic changes. Then the cells were frozen at -20 C and thawed three times. Following this, the contents of the bottles were centrifuged at 10,000 x g for 15 min. The pellets were discarded and the supernatant, containing the virus was stored at -20 C.

Titration of the bovine enterovirus was also carried out using the plaque method. BFS cells were seeded into plastic petri dishes (60mm) at a concentration of 1 x 10^6 cells per plate. Forty-eight hr after seeding, the cell cultures were washed two times with 1 ml of MEM. Then 0.25 ml of serial 10-fold dilutions of bovine enterovirus was adsorbed to the cells for 30 min at 37 C. After adsorption, 8 ml of overlay consisting of 1X MEM, 1% ionagar and 3% lamb serum was added to each plate. The plates were incubated for 48 to 72 hr in a CO_2 incubator at 37 C. The plaques were visualized by staining with neutral red or crystal violet.

3.3. Purification of Viruses.

3.3.1. Purification of Bovine Parvovirus:

BFS cells were seeded at a density of 30 x 10^6 cells per roller bottle and infected with 1 ml of bovine parvovirus stock at 18 to 24 hr after seeding. After 30 min adsorption, 50 ml of MEM supplemented with 10% lamb serum was added. When 95% of the cell culture exhibited cytopathic effects (CPE), the culture fluids and detached cells were removed and centrifuged at 10,000 x g for 15 min. The cell pellets were resuspended in a total volume of 5 ml of 0.05 M Tris-0.1 M NaCl pH 7.5 buffer and frozen and thawed three times. This cell suspension was treated with a VirSonic Cell Disrupter (VirTis) at a setting of 50 (four

5 sec bursts). Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5% and the mixture incubated at room temperature for 30 min. The 5 ml sample was layered on a discontinuous 10-30% sucrose gradient (prepared in 0.05 M Tris-0.1 M NaCl-0.5% SDS, pH 7.5) and centrifuged at 90,000 x g for 3 hr. The upper layers of the gradient were re-centrifuged at 135,000 x g for 4 hr. The supernatant was discarded and the pellets were soaked in 0.05 M Tris-0.1 M NaCl pH 7.5 buffer and then resuspended by sonication. CsCl gradients with a mean density of 1.42 g/cc were prepared and 0.5 ml of virus suspension was layered on the top. The gradients were centrifuged at 100,000 x g for 24 hr. The bands were visualized by indirect lighting and collected from the bottom with a fractionator (Buchler Instruments). The density of each band was determined with a refractometer (Abbe-3L, Bausch & Lomb). The bands were dialyzed against 0.05 M Tris-0.1 NaCl pH 7.5 buffer for 24 hr.

Negative stains were prepared for electron microscopy by applying samples on parlodion and carbon coated grids (300 mesh) followed by staining with 1% uranyl acetate for 30 sec. The grids were examined with a JEM 100B electron microscope.

3.3.2. Purification of Bovine Enterovirus:

BFK cell monolayers were prepared in roller bottles

and infected with 1 ml of bovine enterovirus. After 30 min adsorption, 75 ml of MEM supplemented with 2% lamb serum was added. When 95% of a cell culture exhibited CPE, the culture was frozen and thawed 3 times. The culture fluids were centrifuged at 10,000 x g for 15 min. The pellets were discarded and the supernatants centrifuged at 135,000 x g for 6 hr. The supernatants were discarded and the pellets were soaked in 0.05 M Tris-0.1 M NaCl pH 7.5 buffer for 36 hr. The softened pellets were treated with a VirSonic Cell Disrupter at a setting of 50 (four 5 sec bursts). The virus suspension was layered on the top of a discontinuous 10-30% sucrose gradient. The gradient was centrifuged at 90,000 x g for 2 hr. The upper layers of the gradient were re-centrifuged at 135,000 x g for 4 hr. The pellet was resuspended as described above, the density adjusted to 1.34 g/cc with CsCl, and centrifuged at 100,000 x g for 16 hr. The bands were visualized by indirect lighting and collected with a fractionator. The density of each band was determined with a refractometer. The bands were dialyzed against 0.05 M Tris-0.1 M NaCl pH 7.5 buffer for 24 hr. Samples were prepared for electron microscopy using the procedure described in Section 3.3.1.

3.4. Serologic Procedures.

3.4.1. Preparation of Immune Sera:

Antiserum against bovine parvovirus was prepared in a rabbit. Purified virus prepared by the procedure described in section 3.3.1. was used as the antigen. The purified fraction having a buoyant density in CsCl of 1.42 g/cc was injected into the rabbit. One intravenous (I.V.) injection of 1 ml via the ear vein and two intramuscular (I.M.) injections, into each hind leg, of 2 ml of the purified virus suspended in Freund's adjuvant were given. The rabbit was bled by cardiac puncture at two week intervals for the next sixteen weeks. The antiserum was separated from the clotted blood and stored at -20 C.

Antiserum against bovine enterovirus was also prepared in a rabbit. Purified virus prepared by the procedure described in section 3.3.2. having a buoyant density in CsCl of 1.34 g/cc was used to inject the rabbit. One I.V. injection of 1.5 ml was given initially and this was followed by two I.M. injections of 4.0 ml of the virus suspended in Freund's adjuvant at two week intervals. Two weeks after the last injection, the rabbit was test bled. When another two weeks had elapsed, the rabbit was given an I.V. injection of 0.5 ml of the virus and an I.M. injection of 3.0 ml of the virus suspended in Freund's adjuvant. Then the rabbit was test bled at two week

intervals for the next sixteen weeks. Each blood sample was collected, prepared, and stored as described above.

3.4.2. Hemagglutination Inhibition Procedure:

Using a microtiter dropper, 0.25 µl of 0.85% saline was added to the wells of V-bottomed microtiter plates. Then all of the anti-parvovirus antisera to be tested were heated at 56 C for 30 min to inactivate the complement. Then, using microtiter diluters, 2-fold dilutions of each antiserum in the wells of the microtiter plates were made. Then 0.25 µl of bovine parvovirus having an hemagglutination titer of 64 was added to each well with a microtiter dropper. The contents of the plates were mixed and then incubated at room temperature for 30 min. After the incubation period, 0.25 µl of a 0.5% suspension of guinea pig red blood cells was added to each well. Upon completion of this step, the contents of the plates were thoroughly mixed and then incubated at room temperature for 2 hr. Two types of controls were included. A red blood cell control consisted of 0.25 µl of 0.85% saline plus 0.25 µl of guinea pig red blood cells. The virus control contained 0.25 µl of saline, 0.25 µl of giunea pig red blood cells, and 2-fold dilutions of the bovine parvovirus. These controls were also incubated for 2 hr at room temperature. After the incubation, the titers of each of the antisera were determined.

3.4.3. Serum Neutralization Procedure:

Tube cultures, each of which contained 1 x 10^5 BFS cells and 1 ml of MEM supplemented with 10% lamb serum, were prepared. These were incubated at 37 C until a cell monolayer was formed.

The anti-enterovirus serum was heat inactivated at 56 C for 30 min. After inactivation, two-fold dilutions of the antiserum were prepared in Dulbecco's phosphate buffer.

Ten-fold dilutions of the stock virus were made. Then 0.4 ml of the virus dilution containing 100 Tissue Culture Infective Dose (TCID) 50/0.1 ml was added to 0.4 ml of each serum dilution. The serum-virus mixtures were incubated at room temperature for 30 min. Following the 30 min incubation period, 3.2 ml of MEM containing 3% lamb serum was added to each serum-virus mixture.

Then the medium was removed from the tube cell cultures and each was washed one time with 1 ml of Dulbecco's phosphate buffer. After the washing procedure was completed, 1 ml of the serum-virus samples was added to the tube cell cultures. The cultures were examined daily until the cells of the virus control showed 100% cytopathic effect. The controls were prepared by adding virus and no antiserum to three of the tube cultures.

3.5. One-Step Growth Curves.

3.5.1. Bovine Parvovirus:

BFS cells were seeded in plastic petri dishes (60 mm) at a concentration of 8×10^5 cells per plate. To each plate was added 5 ml of MEM supplemented with 10% lamb serum and 2×10^{-3} M HU. After 32 hr of incubation at 37 C, the synchronized cells were washed three times with Dulbecco's phosphate buffer in order to remove the HU block. Then 2.0 ml of bovine parvovirus, having a multiplicity of 10 PFU/cell, was added to each cell culture. The virus was allowed to adsorb to the cells for 60 min at 37 C. After the 1 hr adsorption period, the virus was removed from the plates and each was washed two times with Dulbecco's phosphate buffer. Then 5 ml of MEM containing 10% lamb serum was added to each plate and they were placed at 37 C in a CO_2 incubator.

Using the procedure described below, samples were taken at 2, 4, 6, 8, 10, 12, 16, 20 and 24 hr postinfection (p.i.). At each sample time two plates were removed at random from the 37 C CO_2 incubator. The cell culture fluid was removed from each plate with a sterile pipette and placed in two sterile centrifuge tubes and centrifuged for 5 min at 200 x g. The supernatant was collected and labelled the extracellular fraction. The pellets were resuspended and added to the cell-associated

fraction. Five ml of trypsin-versene was added to each of the two plates. These were then incubated at 37 C for 10 min. After the incubation, a sterile pipette was used to transfer the cells to centrifuge tubes which were spun at 200 x g for 5 min. The supernatant was discarded and the pellets were resuspended in 10 ml of MEM. This volume was then transferred to a sterile tube and labelled the cell-associated fraction. Before analyzing the cell-associated samples with the plaque assay, they were sonified for 30 sec (three 10 sec bursts) at a setting of 50 with a VirSonic Cell Disrupter.

3.5.1.1. Hemagglutination:

Since bovine parvovirus hemagglutinates red blood cells, the hemagglutination method was used to determine the amount of hemagglutinating viral antigen in each of the samples. Twenty-five µl of 0.85% saline was added to the wells of a V-bottomed microtiter plate. Using microtiter diluters, two-fold dilutions of the samples were made. Then 25 µl of a 0.5% suspension of guinea pig red blood cells was added to each well. After an incubation period of 2 hr at room temperature, the results were read.

3.5.2. Bovine Enterovirus:

The procedure described in section 3.5.1. was also used when doing the one-step growth curve of bovine enterovirus. However, the multiplicity of the virus was

5 PFU/cell and plates were inoculated with a volume of 0.5 ml.

3.6. Determination of Cellular Macromolecular Syntheses in Cells Singly Infected with Bovine Parvovirus or Bovine Enterovirus.

3.6.1. Preparation and Inoculation of Cell Cultures:

BFS cells were seeded at a concentration of 8×10^5 cells per plastic petri dish (60 mm) in 5 ml of MEM supplemented with 10% lamb serum and 2×10^{-3} M HU. After 32 hr of incubation at 37 C, the HU block was removed as described in section 3.1.

When a single infection by bovine parvovirus was analyzed, 2 ml of MEM containing 10% lamb serum and 10 PFU/cell of bovine parvovirus was added to each plate. In single infection experiments with bovine enterovirus, 2 ml of MEM containing 10% lamb serum and 5 PFU/cell of bovine enterovirus was added to each plate. For each of these experiments, uninfected control plates were prepared. To each of these plates was added 2 ml of MEM supplemented with 10% lamb serum. These were referred to as mock-infected cultures. After the parvovirus was allowed to adsorb to the cells for 1 hr at 37 C and the enterovirus was adsorbed for 30 min, the inoculum was removed, and the plates were washed two times with Dulbecco's phosphate buffer. The mock cultures were processed similarly. Then

5 ml of MEM containing 10% lamb serum was added to all cultures and they were incubated at 37 C in a CO_2 incubator.

3.6.2. Radioactive Labelling Technique and Collection of Samples:

When the synthesis of total DNA was being analyzed, the procedure was different than when total RNA and total protein synthesis were being measured.

In DNA synthesis experiments, at each sample time two infected cultures and two mock-infected cultures were removed at random from the CO_2 incubator. To each culture was added 50 µl of ^{3}H-thymidine (Amersham/Searle, S.A. = 18.6 Ci/m mole) at a final concentration of 0.5 µCi/ml. After the cultures were incubated at 37 C in a CO_2 incubator for 1 hr, the label was removed. Then the cultures were washed one time with 1 ml of cold Dulbecco's phosphate buffer.

One ml of cold phosphate buffered saline (PBS) (See appendix I) was then added to each plate and the cells were scraped from the surface of the plates with rubber policemen. The cells, suspended in the PBS, were then poured into sterile plastic tubes and quick-frozen in liquid nitrogen. Each tube was then stored at -20 C.

With the RNA and protein determinations, the procedure was slightly different. At each sample time, two infected plates and two mock-infected plates were selected at random

from a 37 C CO_2 incubator. After the medium was removed from the plates, they were washed two times with Dulbecco's phosphate buffer. Then to each plate was added 5 ml of MEM containing no amino acids but supplemented with 3% lamb serum. To the four plates, 50 µl of ^{3}H-uridine (Amersham/Searle, S.A. = 28 Ci/m mole) at a final concentration of 0.5 µCi/ml and 50 µl of ^{14}C labelled protein hydrolysate (Amersham/Searle, S.A. = 55 Ci/m atom) at a final concentration of 0.1 µCi/ml were added. After the plates were incubated in a CO_2 incubator for 1 hr, the label was removed and discarded. When each plate had been washed one time with 1 ml of cold Dulbecco's phosphate buffer, 1 ml of cold PBS was added to each plate and the cells were scraped with rubber policemen into sterile plastic tubes. After being quick-frozen in liquid nitrogen the tubes were stored at -20 C.

3.6.3. Preparation of Samples for Liquid Scintillation:

The method of Regan and Chu (1965) was used and will be described briefly. After the samples were thawed, they were sonified for 30 sec at a setting of 55 with a VirSonic Cell Disrupter (two 15 sec bursts). One-hundred µl of each sample was then applied in duplication to Whatman No. 3 MM filter paper discs. When the discs had dried, they were rinsed three times for 10 min each, in 5% trichloracetic acid (TCA) at 4 C. Following this, they were rinsed three

times in 95% ethanol for 10 min at room temperature and then one time in ether. After the discs were thorougly dry, they were placed in glass vials with 5 ml of a liquid scintillation fluid which contained 1.98 g of 2,5-diphenyloxazole and 0.25 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per liter of toluene. Counting was done in a liquid scintillation spectrometer (Packard Tri Carb, Model 3310).

3.6.4. Determination of Protein Content:

The amount of protein in each sample was measured by using a slight modification of the method of Lowry and coworkers (1951). Before the protein determination was made, the samples were sonified as described in section 3.6.3. Two glass test tubes were set up for each sample to be analyzed. To the first tube of each pair was added 0.05 ml of the particular sample and to the second tube 0.1 ml of the sample. Bovine serum albumen (BSA) standards were set up in separate tubes by adding 0.1, 0.2, 0.3, 0.4, and 0.5 ml of BSA of a concentration of 250 µg/ml. Then 0.15 ml of PBS was added to the first tube of each sample pair and 0.1 ml to the second tube. To the five BSA standards 0.2 ml of PBS was added. At this point a blank tube was prepared to which 0.2 ml of PBS and 0.8 ml of sterile distilled water were added. Sterile distilled water was added to all other tubes to bring the final volume to 1.0 ml. Copper reagent was prepared immediately before use by

mixing one part 2% Na_2CO_3 in 0.1 N NaOH with 50 parts of 2% sodium-tartarate and with one part 1% $CuSO_4$. Following the addition of 5 ml of this reagent to each sample tube, BSA standard and blank, the contents were mixed. The tubes were then incubated at room temperature for 10 min. During the 10 min incubation period, the phenol reagent was prepared by mixing two parts 2N phenol reagent (Folin & Ciocalteu, Fisher) with one part 1.0 N NaOH. Phenol reagent was added to all of the tubes at a volume of 0.5 ml per tube. Each tube was mixed immediately following the addition. Then the tubes were incubated at room temperature for 30 min. After the incubation period, the absorbance of each tube was read on a Bausch and Lomb Spectrophotometer 20 at a wavelength of 650 nm. The determination was made in 16 mm colorimeter tubes.

3.7. Determination of Bovine Parvovirus DNA Synthesis in Cells Singly Infected with Bovine Parvovirus and in Cells Mixedly Infected with Bovine Parvovirus and Bovine Enterovirus.

3.7.1. Preparation of Cell Cultures:

BFS cells seeded in plastic petri plates (60mm) were synchronized by the HU method. In the single infection study, immediately after removal of the HU block an appropriate number of cultures were infected with bovine parvovirus at multiplicity of 10 PFU/cell and the cultures

were processed as described in section 3.6.1. Mock-infected cultures were also prepared as described in section 3.6.1.

In the mixed infection studies, two different schedules of infection were followed. In both cases the cells were prepared in the same manner as described in section 3.6.1. However, in the initial mixed infection study, immediately upon release from the HU block, the cells were simultaneously infected with bovine parvovirus having a multiplicity of 10 PFU/cell and with bovine enterovirus having a multiplicity of 5 PFU/cell. The two viruses were allowed to adsorb to the cells for 1 hr at 37 C. In the later mixed infection study, the synchronized cells were pre-infected with bovine parvovirus immediately upon release from the HU block and superinfected with bovine enterovirus at 8 hr post release (p.r.). The same titers of the two viruses were used in this mixed infection study. Bovine parvovirus adsorbed to the cells for 1 hr and bovine enterovirus adsorbed to the cells for 30 min. In both mixed infection studies, plates singly infected with bovine parvovirus were run as controls.

3.7.2. Extraction of Bovine Parvovirus DNA:

A modification of the procedure of Hirt (1967) was used to extract the bovine parvovirus DNA and will be described briefly. At each sample time, three of each

type of cell culture were removed at random from the 37 C CO_2 incubator. To each plate was added 50 µl of ^{3}H-thymidine (Amersham/Searle, S.A. = 18.6 Ci/m mole) having a final concentration of 0.5 µCi/ml. Then the plates were incubated 1 hr in a CO_2 incubator at 37 C. After the incubation, the media was removed from each plate and they were washed two times with Dulbecco's phosphate buffer. When the washing procedure was complete, 5 ml of MEM supplemented with 10% lamb serum was added to each plate and they were incubated at 37 C in a CO_2 incubator for 1 hr. At the end of this incubation, the media was removed and 0.5 ml of 0.6% SDS plus 0.1 M EDTA and 50 µl of pronase were added to each plate. The pronase was at a concentration of 20 mg/ml and had been self digested by heating at 80 C for 10 min and 37 C for 6 hr. After this addition, the plates were incubated at 37 C for 1 hr. At the end of this incubation, the contents of three plates, all of the same type, were scraped with a sterile rubber policeman into one sterile centrifuge tube and to each tube 0.375 ml of 5 M NaCl was added to make the final concentration of each tube 1 M. Then the tubes were placed at 4 C for 8 hr, after which they were centrifuged at 17,300 x g for 30 min. The supernatant from each tube was carefully removed and placed in a sterile centrifuge tube. These were again centrifuged at 17,300 x g for 30 min. Again the supernatant was saved and the pellet was discarded. Each of these samples was

then prepared and counted as described in section 3.6.3.

3.8. Mixed Infection Studies.

Two different types of mixed infections were studied as described in section 3.7.1.

3.8.1. Preparation of Cell Cultures:

The cell cultures were prepared as described in section 3.7.1. Controls, singly infected with enterovirus and singly infected with bovine parvovirus were prepared.

3.8.2. Collection of Samples:

For each sample, two plates, similarly treated were removed from the incubator. They were first checked for CPE. Then, using a sterile rubber policeman, the cells were removed from the plastic surface and suspended in the 5 ml of medium. After the two plates had been prepared in this manner, the contents of both, which consisted of a 10 ml volume, were transferred into one sterile plastic tube, using a sterile 10 ml pipette. Then the tube was frozen at -20 C. Each sample was then thawed and sonified with a VirSonic Cell Disrupter (two 15 sec bursts at a setting of 55).

3.8.3. Determination of Rates of Total Cellular RNA Synthesis and Total Cellular Protein Synthesis:

These two determinations were performed only on the mixed infection in which the synchronized cells were preinfected with bovine parvovirus immediately upon removal of the hydroxyurea block and superinfected with bovine enterovirus at 8 hr post release. The procedure described in section 3.6. was used to analyze this type of mixed infection, as to these two parameters.

3.8.4. Plaque Assay of Samples:

The level of infectivity in each sample was measured by using the plaque assay technique described in sections 3.2.1. and 3.2.2. In the mixed samples, 0.5 ml of neutralizing antiserum was added to 0.5 ml of each sample. For example, if the infectivity of bovine parvovirus was being determined, anti-enterovirus serum was added and vice versa. Then the procedure described in the above two sections was followed. In later experiments, each dilution blank contained a 10-fold dilution of the specific neutralizing antiserum.

3.8.5. Hemagglutination Assay:

The hemagglutination assay described in section 3.5.1.1. was used to measure the level of bovine parvovirus hemagglutinating antigen in the mixedly infected samples and in the controls singly infected with bovine parvovirus.

4. Results

4.1. Purification of Bovine Parvovirus.

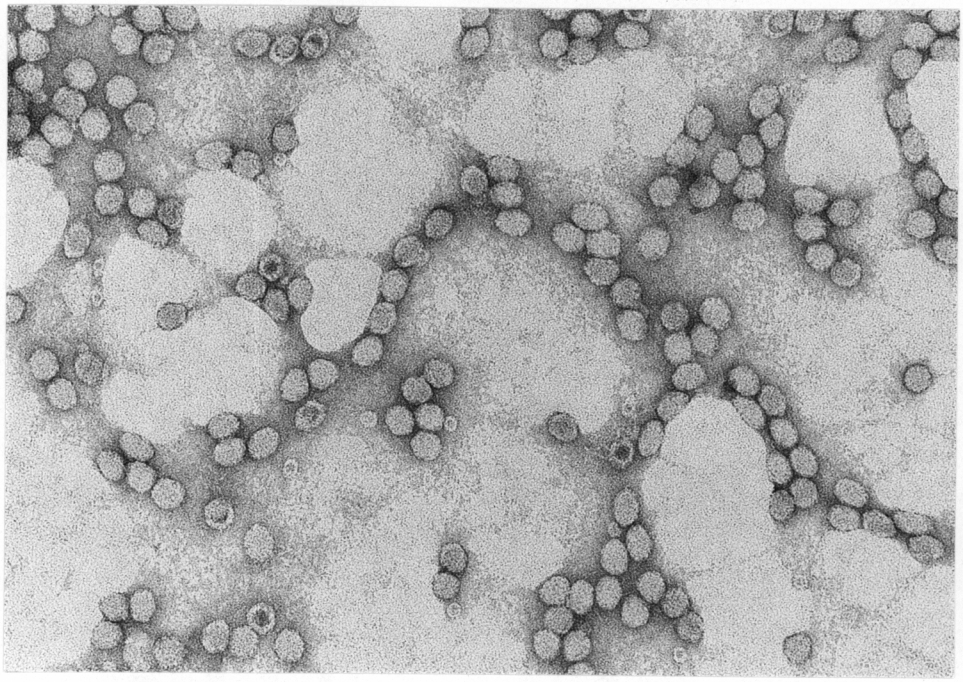
After the CsCl gradient had been centrifuged at 100,000 x g for 24 hr, there were three distinct bands. These bands had densities of 1.42 g/cc, 1.36 g/cc, and 1.32 g/cc. Observation of each band with the electron microscope indicated that the most dense band contained mostly complete virions (Fig. 1). In the band with a density of 1.36 g/cc there was a mixture of complete virions and empty capsids, whereas, only empty capsids and other debris were in the least dense band.

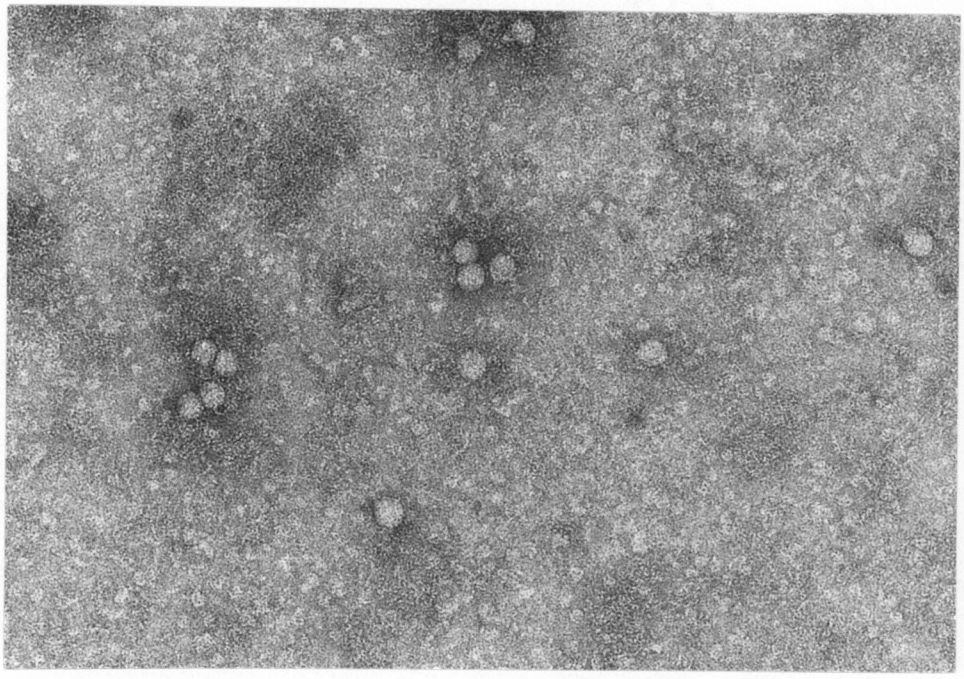
4.2. Purification of Bovine Enterovirus.

When the centrifugation of the CsCl gradient was complete, there were four distinct bands. Two of the bands were very close to each other and were collected into the same tube. The combined bands had a density of 1.348 g/cc and contained mostly complete virions with an occasional empty capsid present (Fig. 2). The densities of the two separate bands were 1.304 g/cc and 1.300 g/cc. These bands were not examined by electron microscopy.

Fig. 1. Electron photomicrograph of negatively stained bovine parvovirus from a CsCl gradient at a density of 1.42 g/cc. Magnification: x133,000.

Fig. 2. Electron photomicrograph of negatively stained bovine enterovirus from a CsCl gradient at a density of 1.348 g/cc. Magnification: x 107,000.





4.3. Serologic Studies.

4.3.1. Hemagglutination Inhibition of Anti-Bovine Parvovirus Serum:

Hemagglutination inhibition (HI) titers for anti-bovine parvovirus serum ranged from 16 to 5120 (table 1).

4.3.2. Serum Neutralization of Anti-Bovine Enterovirus Serum:

Titers of the different sera collected ranged from 64 to 8192 (table 2).

4.4. One-Step Growth Curve of Bovine Parvovirus.

Cell-associated and extracellular or free virus titers were determined after infecting synchronized bovine fetal spleen (BFS) cells with a high multiplicity of bovine parvovirus immediately after release from hydroxyurea (HU) block. The titer of the unadsorbed inoculum was 4.46×10^6 PFU/ml and that of the adsorbed inoculum was 5.5×10^6 PFU/ml. Therefore, the adsorbed multiplicity of virus was 5.5 PFU per cell. The data on the growth cycle of bovine parvovirus in BFS cells is presented in Figure 3. No increase in cell-associated virus titers was detected until 10 hr after inoculation. After 10 hr postinfection (p.i.), cell-associated progeny virus titers increased exponentially and reached 1.4×10^6 PFU/ml at 24 hr. However, no measurable extracellular virus appeared until after 20 hr p.i., thus

Table 1

Titer of Anti-Bovine Parvovirus Serum

Days after Injection	Titer[1]
0	16
10	512
24	2560
30	2560
42	5120
60	2560

[1]determined by hemagglutination inhibition.

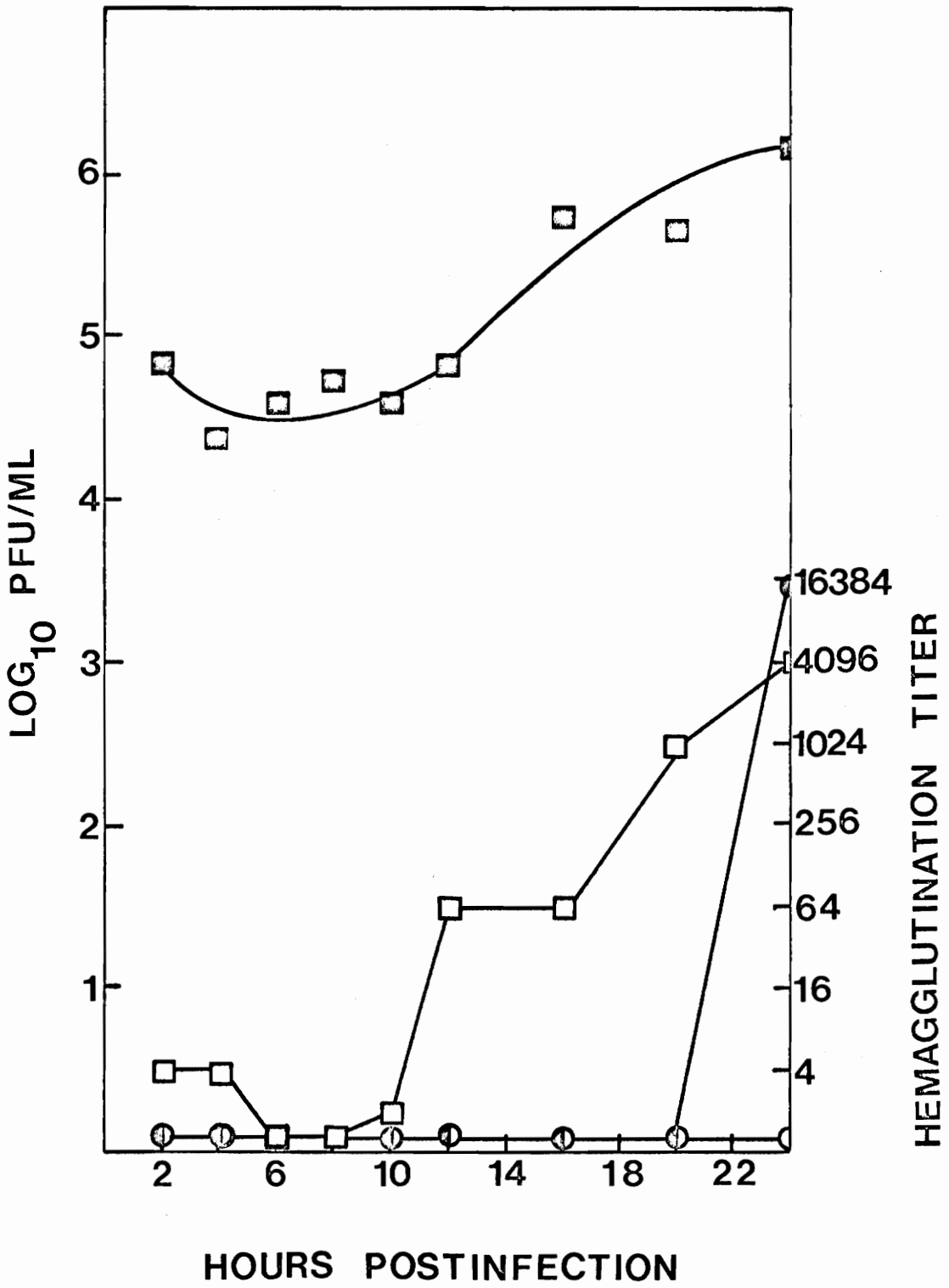
Table 2

Titer of Anti-Bovine Enterovirus Serum

Days after Injection	Titer[1]
0	0
25	512
45	2048
50	4096
64	8192
72	8192

[1]determined by serum neutralization.

Fig. 3. One-step growth curve of bovine parvovirus replication in bovine fetal spleen cells. Cell-associated virus (■——■); extracellular virus (●——●); cell-associated hemagglutinins (□——□); extracellular hemagglutinins(○——○).


6
5
4
3
2
1
LOG10 PFU/ML
16384
4096
1024
256
64
16
4
HEMAGGLUTINATION TITER
2
6
10
14
18
22
HOURS POSTINFECTION

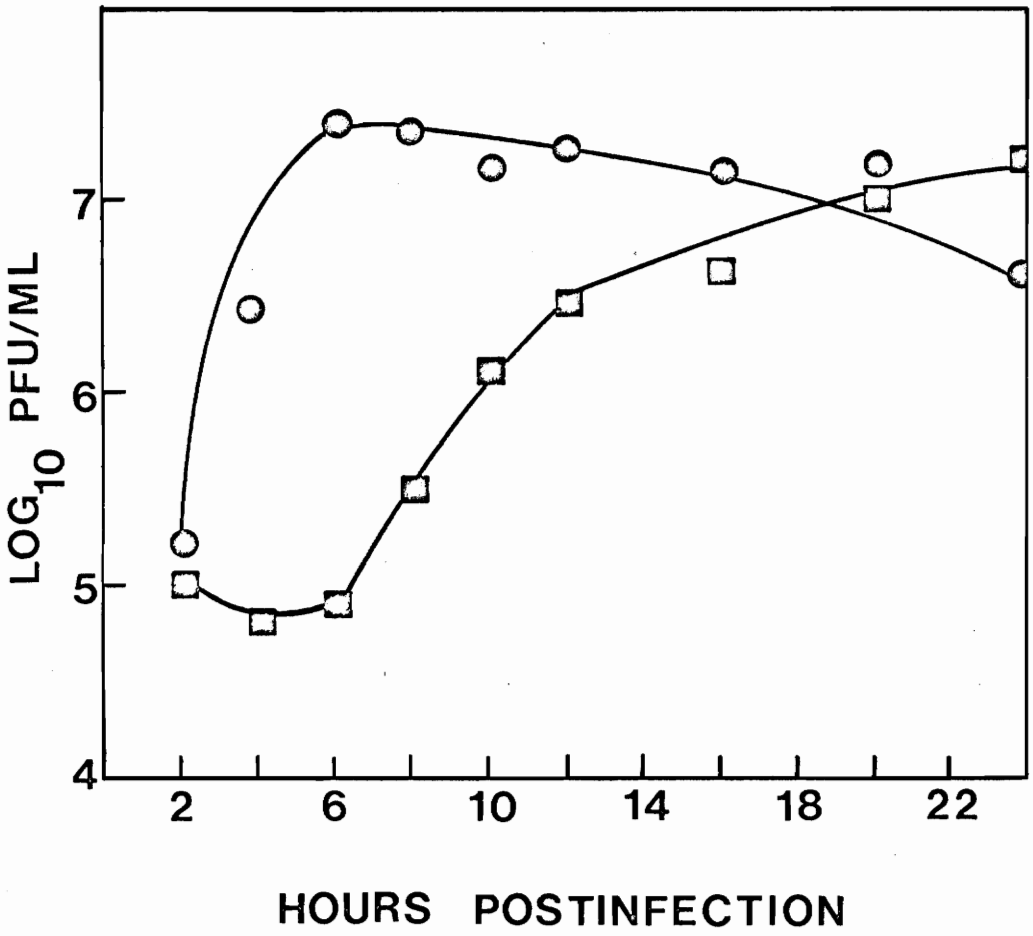
indicating that the bovine parvovirus remains closely associated with the infected cells during this period. The cell-associated virus titer was considerably higher than the extracellular virus titer at 24 hr p.i. In this experiment, cytopathic changes were first observed at 8 hr p.i. when approximately 15% of the cells were rounded. At 24 hr p.i., 95% of the cells were showing cytopathic changes, when peak cell-associated virus titers were detected.

The production of cell-associated hemagglutinins (Fig. 3) paralleled infectivity. Viral hemagglutinating antigens began to increase 8 hr after inoculation and reached a titer of 4096 at 24 hr. No measureable level of viral hemagglutinins was detected in any of the extracellular virus samples during the 24 hr period.

4.5. One-Step Growth Curve of Bovine Enterovirus.

The growth cycle of bovine enterovirus in synchronized BFS cells is summarized in Figure 4. Samples were taken at intervals until 24 hr p.i. The cell-associated and extracellular fractions were assayed separately for virus by the plaque method. The cells, immediately after release from the HU block, were adsorbed with 0.5 ml of virus stock which had a titer of 7.8×10^7 PFU/ml. Cell-associated virus increased exponentially between 2 and 6 hr p.i., reaching a maximum titer of 2.48×10^7 PFU/ml at 6 hr.

Fig. 4. One-step growth curve of bovine enterovirus replication in bovine fetal spleen cells. Cell-associated virus (o——o); extracellular virus (□——□).


LOG10 PFU/ML
7
6
5
4
2
6
10
14
18
22
HOURS POSTINFECTION

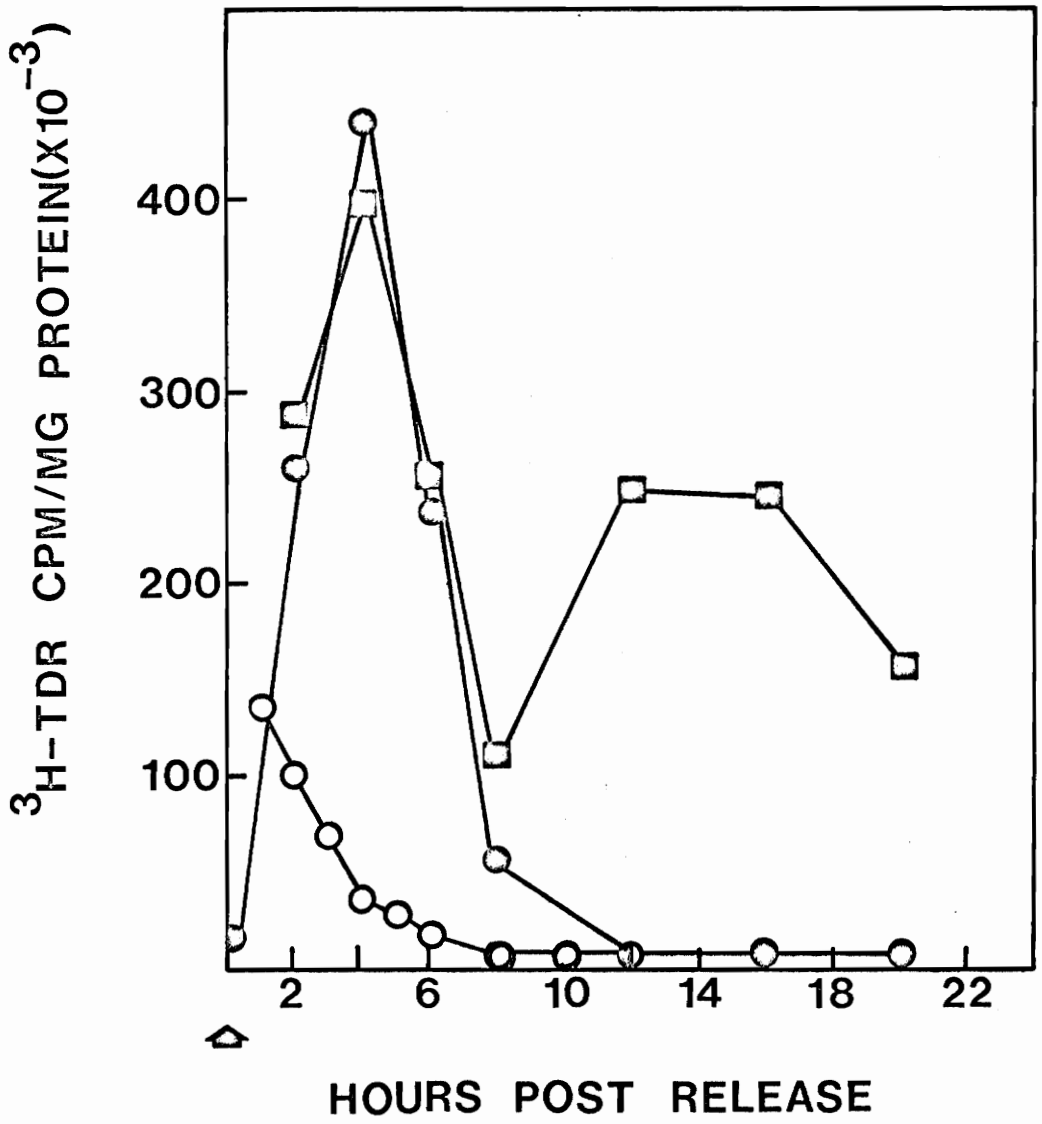
After 6 hr, cell-associated virus titers leveled off before gradually decreasing. In contrast to above, an increase in extracellular virus titers did not begin until 6 hr p.i. Extracellular virus titers were consistently lower than cell-associated virus titers until 20 to 24 hr p.i. As early as 6 hr p.i., 80% of the cells showed cytopathic changes, but few cells were released into the medium. At 24 hr p.i., 100% of the cells were rounded and only a few cells were still attached.

4.6. Cellular Macromolecular Syntheses in Singly Infected Cells.

4.6.1. Rate of DNA Synthesis in Bovine Parvovirus and Bovine Enterovirus Infected Cells:

A comparison of the rates of DNA synthesis in mock-infected cells and cells singly infected immediately upon release from the HU block with bovine parvovirus and bovine enterovirus is presented in Figure 5. Maximum levels of DNA synthesis in mock-infected and bovine parvovirus-infected cells were observed at 4 hr post release (p.r.). The rates of total DNA synthesis in these cells were very similar between 2 and 8 hr p.r. In contrast to parvovirus-infected cells, the rate of DNA synthesis in enterovirus-infected cells was rapidly inhibited to background levels by 4 hr p.r., a time when maximal rates were detected in mock-infected and parvovirus-infected cells.

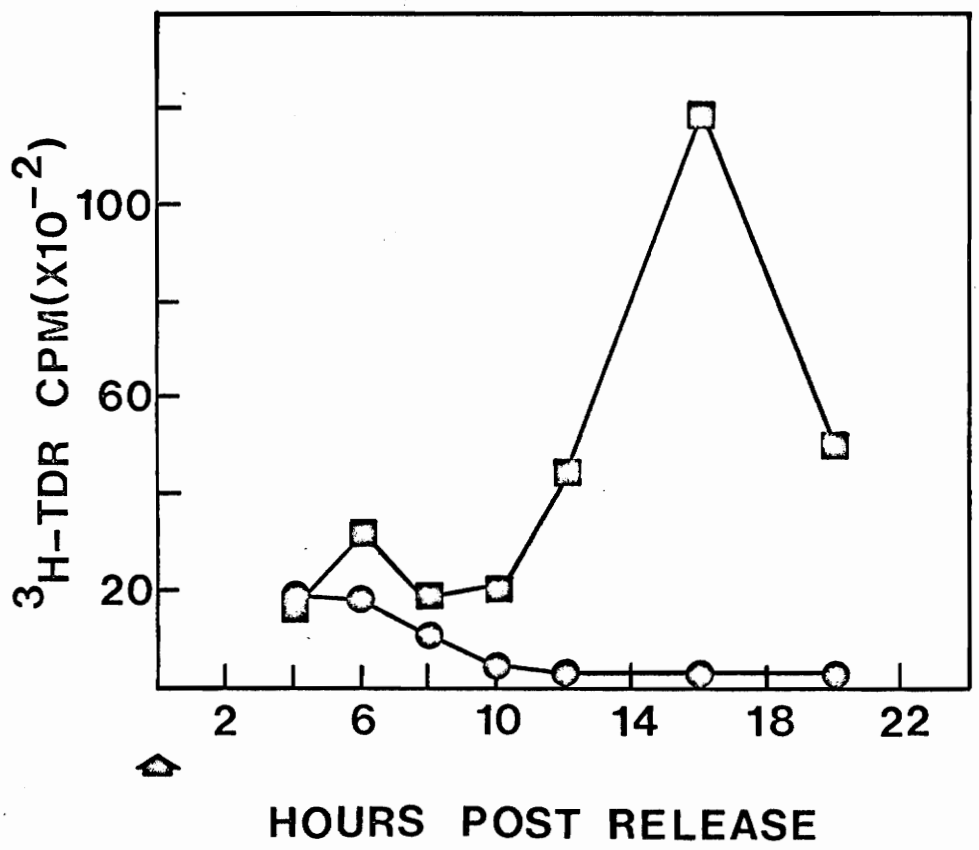
Fig. 5. Rate of total DNA synthesis in singly infected cells. Mock-infected (●——●); bovine parvovirus-infected (■——■); bovine enterovirus-infected (○——○); ⌂ indicates time of infection of cells.


^{3}H-TDR CPM/MG PROTEIN(X10^{-3})
400
300
200
100
2
6
10
14
18
22
HOURS POST RELEASE

Between 4 and 20 hr p.r., the rate of DNA synthesis in enterovirus-infected cells remained at background levels. The rate of DNA synthesis in mock-infected and parvovirus-infected cells decreased in parallel between 4 and 8 hr p.r. However, after 8 hr, the rate of DNA synthesis increased in parvovirus-infected cells, reaching a plateau at 12 to 16 hr, while the rate of DNA synthesis in mock-infected cells continued to decline to background levels. The increased rate of DNA synthesis in the bovine parvovirus-infected cells may result from the induction of cellular DNA synthesis or represent the synthesis of viral DNA.

To test this hypothesis, it was necessary to differentiate between cellular and viral DNA synthesis in parvovirus-infected cells. This was accomplished by using the Hirt extraction procedure (1967) in which the cellular DNA is precipitated and the low molecular weight viral DNA remains in the supernatant. Cells, mock-infected and singly infected with parvovirus, were sampled at intervals for 20 hr (Fig. 6). Between 8 to 10 hr p.r., the rate of DNA synthesis in parvovirus-infected cells began to increase and reached a maximum level at 16 hr. This increase is not due to cellular DNA synthesis since only background levels were observed in the supernatants of mock-infected samples, which were collected in a similar manner. Therefore, the increase in the rate of DNA

Fig. 6. Rate of bovine parvovirus DNA synthesis in singly infected cells. Mock-infected (●——●); bovine parvovirus-infected (■——■); ⌓ indicates time of infection of cells.


^{3}H-TDR CPM(X10^{-2})
100
60
20
2
6
10
14
18
22
HOURS POST RELEASE

synthesis after 8 hr in parvovirus-infected cells represents the synthesis of parvovirus DNA (Fig. 5, Fig. 6).

4.6.2. Rate of Total RNA Synthesis in Bovine Parvovirus and Bovine Enterovirus Infected Cells:

In order to determine the effect of infection of each virus on total RNA synthesis in synchronized cells, the following experiment was done. Immediately upon release from the HU block, cells were singly infected with bovine parvovirus and bovine enterovirus or mock-infected. At intervals up to 20 hr, the rate of total RNA synthesis was determined in virus-infected cells and compared to the levels in mock-infected cells, resulting in a per cent of mock measurement (Fig. 7, Table 3). The rate of RNA synthesis in cells singly infected with parvovirus did not decrease below that of the mock-infected cells until after 8 hr p.r. Between 8 and 12 hr, there was a rapid decrease in the rate of RNA synthesis, but after 12 hr the rate of synthesis was maintained at a level of approximately 70% of that observed in mock-infected cells. However, in cells singly infected with enterovirus, a rapid inhibition of RNA synthesis was observed starting at 1 hr p.r. and by 3 hr the rate of RNA synthesis was only 27.5% of that in mock-infected cells. However, at 3 hr p.r. the rate of RNA synthesis sharply increased, reaching a level of 74.1% of the mock at 4 hr. Following this

Fig. 7. Rate of total RNA synthesis in singly infected cells. Bovine parvovirus-infected (■——■); bovine enterovirus-infected (●——●); ⌂ indicates time of infection of cells.

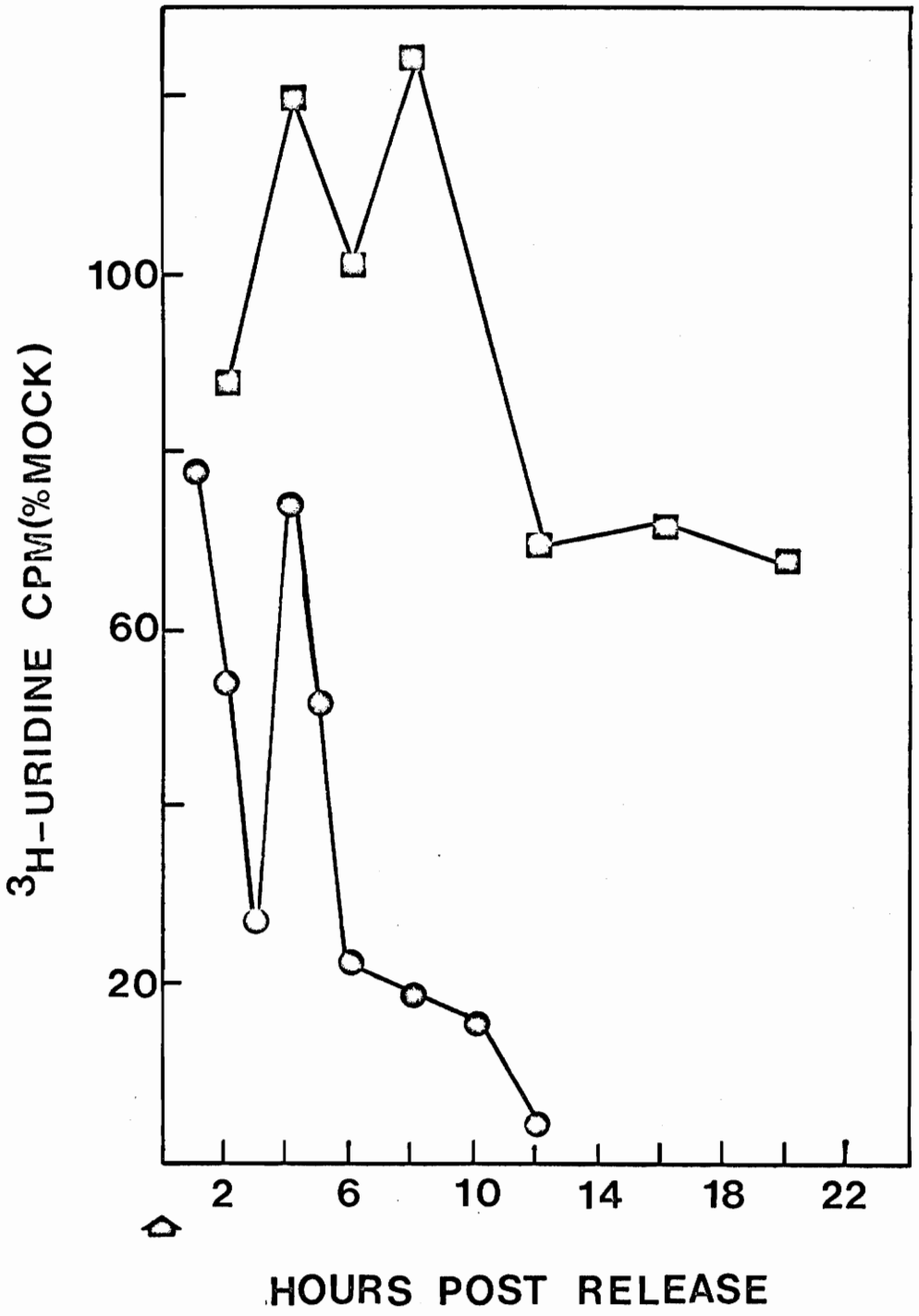

^{3}H-URIDINE CPM(%MOCK)
100
60
20
2
6
10
14
18
22
HOURS POST RELEASE

Table 3

Rate of Total RNA Synthesis in Bovine Parvovirus and Bovine Enterovirus Infected Cells

	Bovine Parvovirus			Bovine Enterovirus		
Hours Post Release	Mock	Infected	% Control[1]	Mock	Infected	% Control
1	n.d.[2]	n.d.		42.9[3]	33.5[3]	78.1
2	58.1[3]	51.3[3]	88.2	54.5	29.7	54.4
3	n.d.	n.d.		49.9	13.8	27.5
4	60.2	72.5	120.3	54.2	40.2	74.1
5	n.d.	n.d.		62.4	32.5	52.1
6	72.2	73.6	102.0	62.5	14.6	23.3
8	61.1	76.0	124.3	41.5	7.7	18.6
10	n.d.	n.d.		49.1	8.0	16.2
12	73.7	51.6	70.0	49.2	2.2	4.5
16	63.2	45.4	71.8	n.d.	n.d.	
20	61.7	41.3	67.8	n.d.	n.d.	

[1]per cent of control is infected cpm/µg protein ÷ mock cpm µg/protein x 100.

[2]not determined.

[3]cpm/µg protein

exponential increase in the rate of RNA synthesis (which probably was due to enterovirus RNA synthesis), the rate rapidly declined in infected cells and by 12 hr was at a level of only 4.5% of that in mock-infected cells. From these results, it is apparent that bovine enterovirus more rapidly and severely alters the synthesis of total RNA in singly infected cells that does bovine parvovirus.

4.6.3. Rate of Total Protein Synthesis in Bovine Parvovirus and Bovine Enterovirus Infected Cells:

In addition to examining the effect of the two viruses on total RNA synthesis, a parallel experiment was designed to determine whether single infections with bovine parvovirus and bovine enterovirus had corresponding effects on total protein synthesis. Similar to the effect on RNA synthesis, single infection by parvovirus did not cause an inhibition of protein synthesis until after 8 hr p.r. (Fig. 8, Table 4). Further, a decrease in the rate of protein synthesis was detected between 8 and 12 hr, followed by a leveling off after this time. The pattern of protein synthesis in cells singly infected with enterovirus was similar to that observed for RNA synthesis in these cells (Fig. 8, Table 4). A peak in the rate of protein synthesis was seen at 4 hr p.r. and corresponds to the peak in RNA synthesis which also occurs at this time.

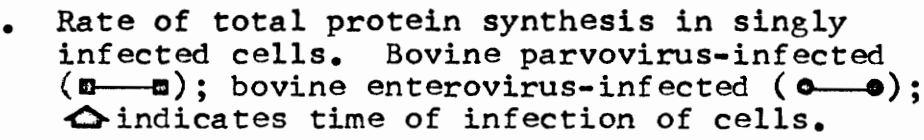


Fig. 8. Rate of total protein synthesis in singly infected cells. Bovine parvovirus-infected (■——■); bovine enterovirus-infected (○——●); ⌂ indicates time of infection of cells.

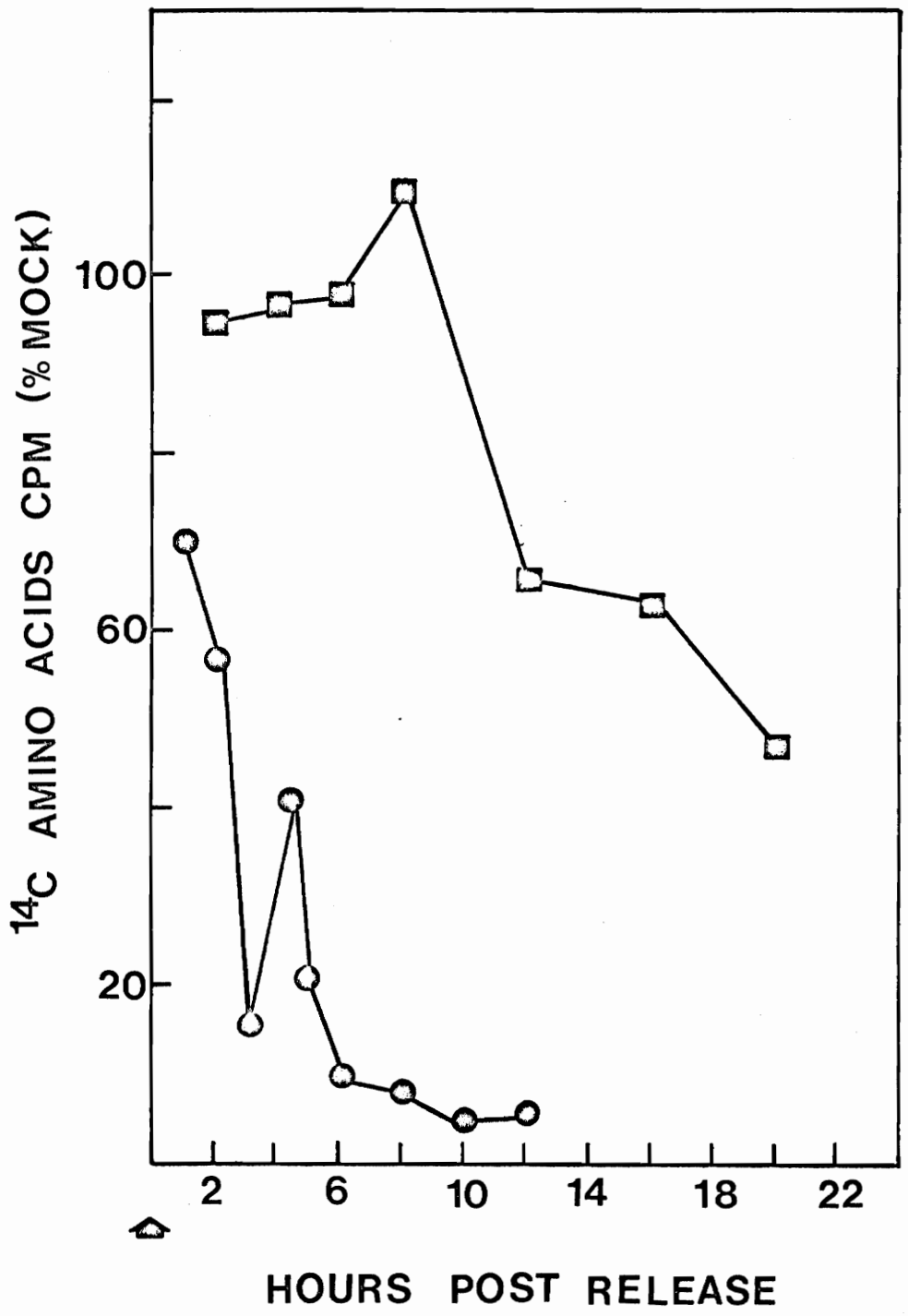

^{14}C AMINO ACIDS CPM (% MOCK)
100
60
20
2
6
10
14
18
22
HOURS POST RELEASE

Table 4

Rate of Total Protein Synthesis in Bovine Parvovirus and Bovine Enterovirus Infected Cells

	Bovine Parvovirus			Bovine Enterovirus		
Hours Post Release	Mock	Infected	% Control[1]	Mock	Infected	% Control
1	n.d.[2]	n.d.		80.0[3]	60.2[3]	75.2
2	108.6[3]	103.3[3]	95.1	68.0	38.9	57.2
3	n.d.	n.d.		65.2	10.1	15.6
4	102.1	98.8	96.7	64.2	26.4	41.2
5	n.d.	n.d.		82.3	17.1	20.8
6	109.2	106.6	97.7	70.3	7.2	10.2
8	100.4	110.6	110.2	74.7	6.3	8.5
10	n.d.	n.d.		63.0	2.9	4.7
12	119.0	78.9	66.3	54.0	3.2	6.0
16	89.7	56.2	62.7	n.d.	n.d.	
20	91.2	42.9	47.0	n.d.	n.d.	

[1]per cent of control is infected cpm/µg protein ÷ mock cpm/µg protein x 100.

[2]not determined

[3]cpm/µg protein.

4.7. Mixed Infection Studies.

4.7.1. Simultaneous Infection with Bovine Parvovirus and Enterovirus at 0 hr Post Release:

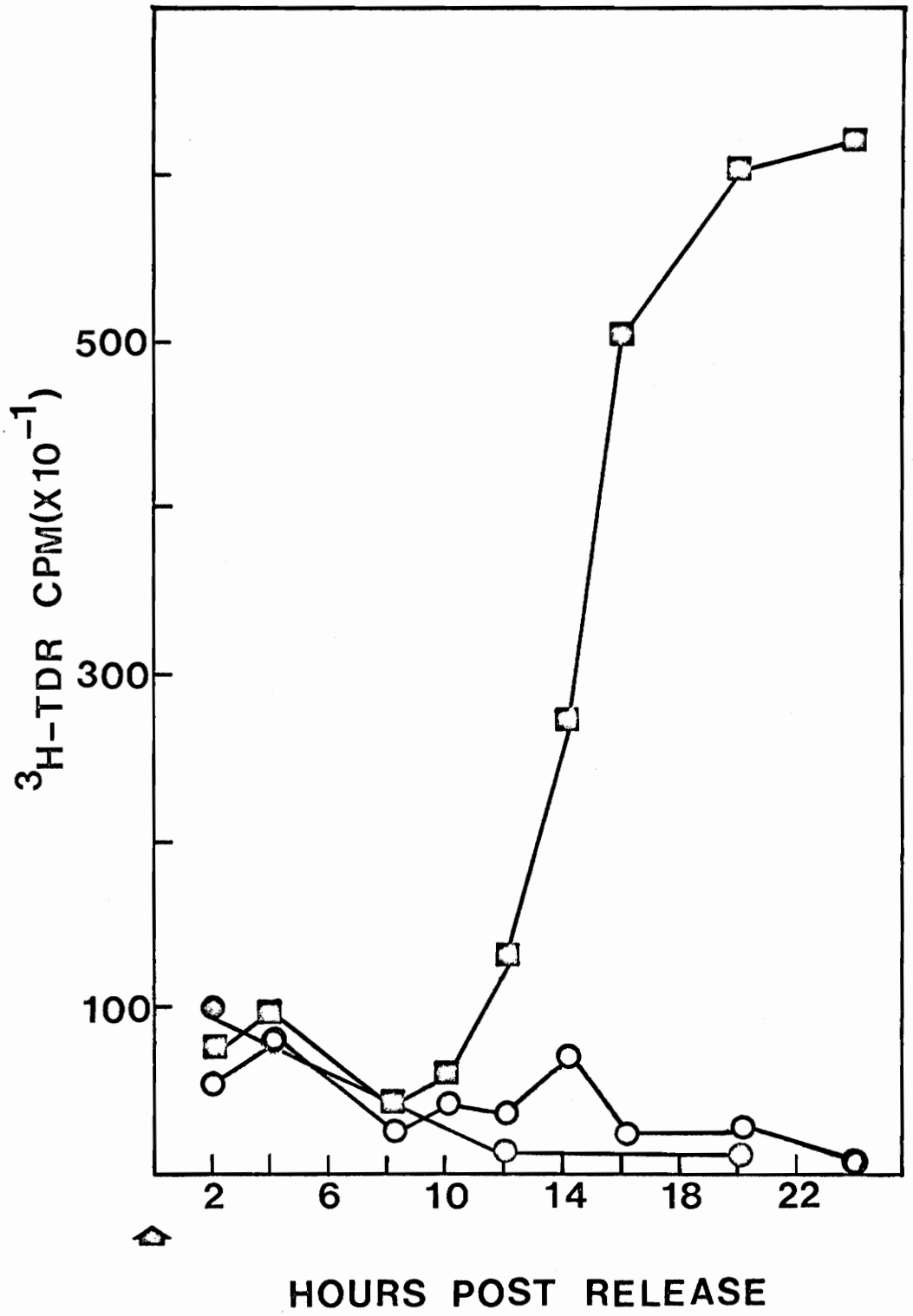
4.7.1.1. Rate of Bovine Parvovirus DNA Synthesis:

In order to assess the effect of simultaneous mixed infection by bovine parvovirus and bovine enterovirus on the subsequent synthesis of parvovirus DNA the following experiment was done. BFS cells were infected with both viruses immediately after release from the HU block and the rate of bovine parvovirus DNA synthesis was determined at intervals for 24 hr (Fig. 9). An increase in the rate of parvovirus DNA synthesis in both single and mixed infections is first observed between 8 and 10 hr p.r. However, the rate continues to increase exponentially in singly infected cells and is 3.7-fold higher at 14 hr p.r., when maximal levels are seen in mixedly infected cells. At 20 hr p.r., the rate of DNA synthesis in singly infected cells is 25-fold higher than that in cells mixedly infected with parvovirus.

4.7.1.2. Titers of Infectious Bovine Parvovirus and Hemagglutinating Antigen:

The titers of infectious virus were determined in cultures singly infected with bovine parvovirus and in mixedly infected cultures after neutralization of bovine

Fig. 9. Rate of bovine parvovirus DNA synthesis in cells simultaneously infected with bovine parvovirus and bovine enterovirus. Mock-infected (●——●); bovine parvovirus-single infection (■——■); bovine parvovirus-mixed infection (○——○); ⏢ indicates time of infection of cells.


500
300
100
^{3}H-TDR CPM(X10^{-1})
2
6
10
14
18
22
HOURS POST RELEASE

enterovirus with specific antiserum (Fig. 10). In addition, the amount of hemagglutinating antigen in these samples was determined (Fig. 10). A gradual increase in the titer of parvovirus was observed in singly infected cells between 8 and 14 hr p.r. followed by an exponential increase in titer. At 24 hr the titer of parvovirus in singly infected cells had reached a level that was 1000-fold greater than that at 14 hr. In contrast, in mixedly infected cultures, only residual infectivity was detected during the 14 hr period after release, and no infectivity was detected between 16 and 24 hr, a period corresponding to rapid production of virus in singly infected cells. Hemagglutinating antigen was not detected at any time during the mixed infection, however, hemagglutinating antigen was detected beginning at 14 hr p.r. in singly infected cells and increased to a titer of 64 by 24 hr. These results demonstrate that production of infectious bovine parvovirus and hemagglutinating antigen, is severely reduced or inhibited during simultaneous infection with bovine enterovirus.

4.7.1.3. Titers of Infectious Bovine Enterovirus:

The titers of infectious virus were determined in cultures singly infected with bovine enterovirus and in mixedly infected cultures after neutralization of bovine parvovirus with specific antiserum (Fig. 11). The data

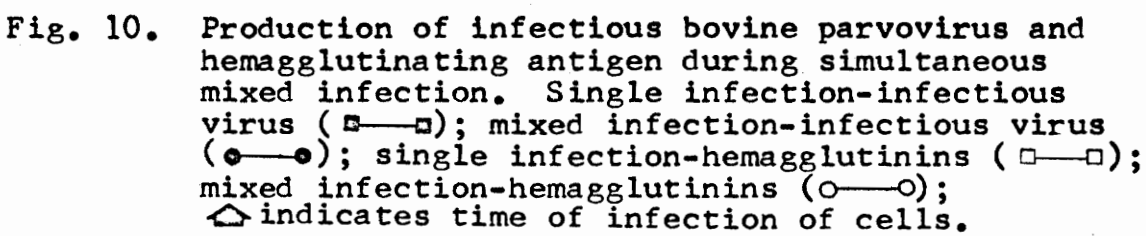


Fig. 10. Production of infectious bovine parvovirus and hemagglutinating antigen during simultaneous mixed infection. Single infection-infectious virus (▫——▫); mixed infection-infectious virus (●——●); single infection-hemagglutinins (□——□); mixed infection-hemagglutinins (○——○); ⌂ indicates time of infection of cells.

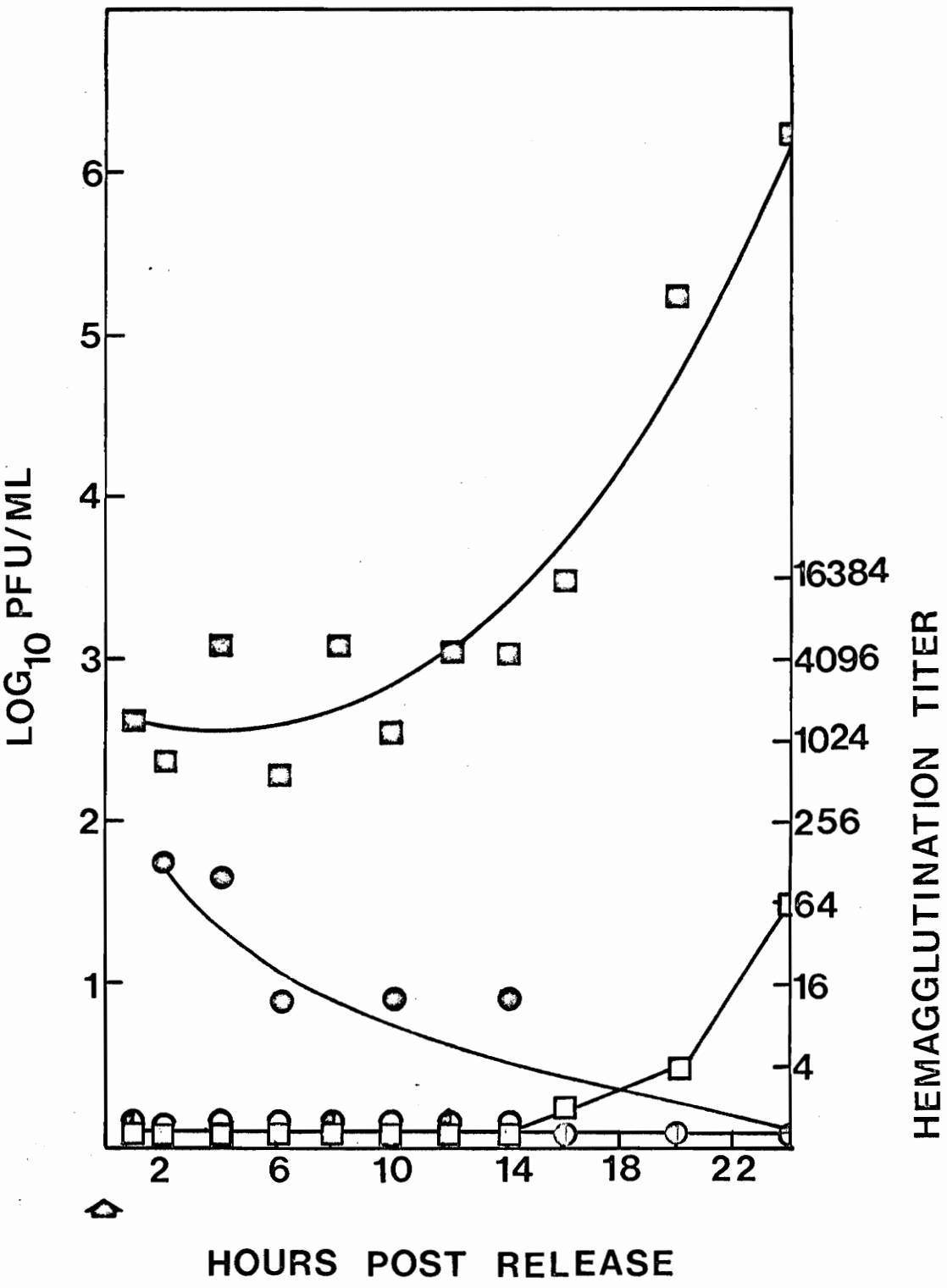
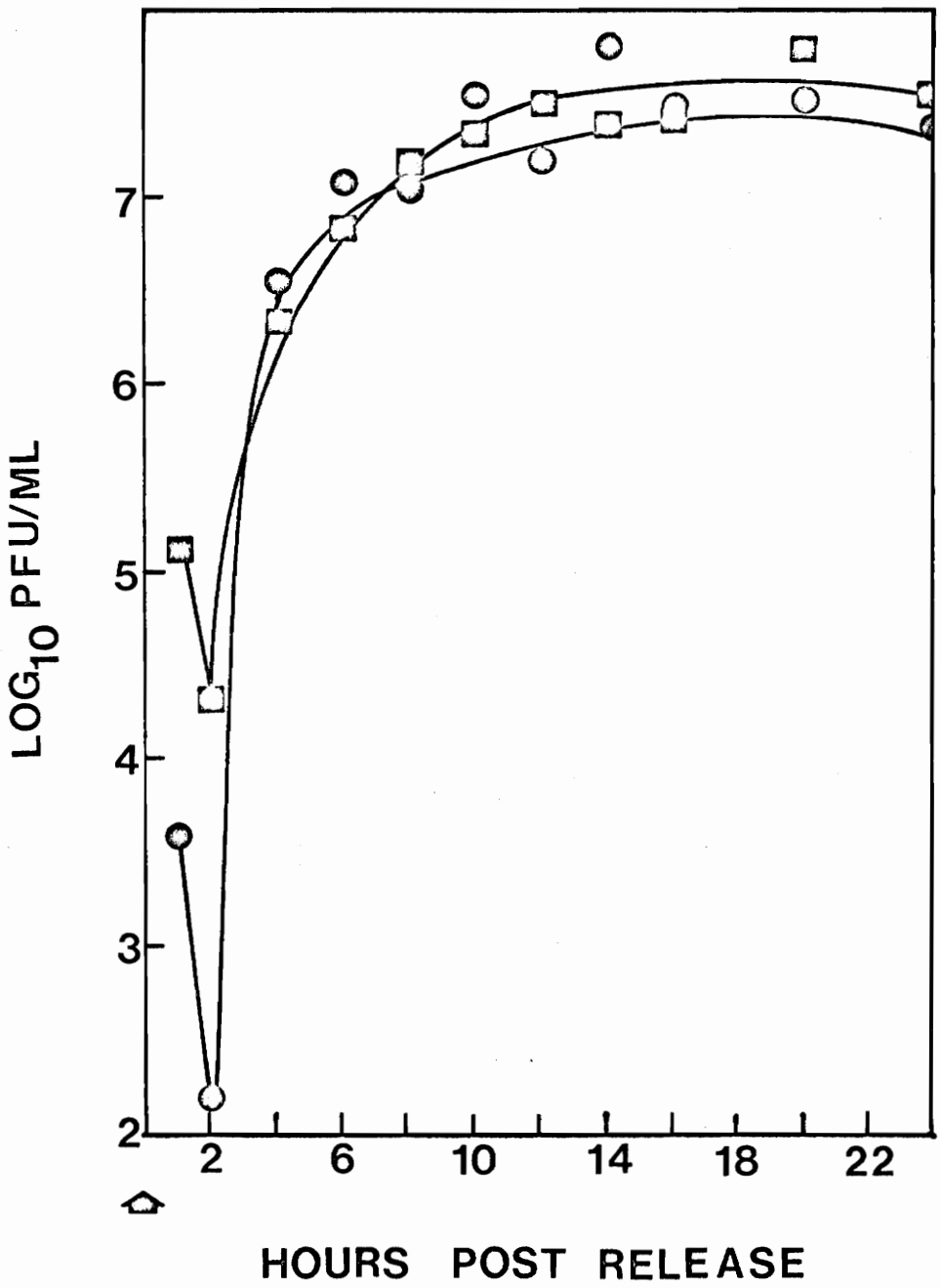

LOG10 PFU/ML
6
5
4
3
2
1
16384
4096
1024
256
64
16
4
HEMAGGLUTINATION TITER
2
6
10
14
18
22
HOURS POST RELEASE

Fig. 11. Production of infectious bovine enterovirus during simultaneous mixed infection. Single infection-infectious virus (■——□); mixed infection-infectious virus (●——○); ⌂ indicates time of infection of cells.


LOG10 PFU/ML
7
6
5
4
3
2
2
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14
18
22
HOURS POST RELEASE

indicate that there was no significant difference in the production of bovine enterovirus in cells singly infected with enterovirus and cells simultaneously infected with enterovirus and parvovirus. Therefore, simultaneous infection with bovine parvovirus does not inhibit the production of bovine enterovirus.

4.7.2. Pre-infection with Bovine Parvovirus at 0 hr Post Release and Superinfection with Bovine Enterovirus at 8 hr Post Release:

It was necessary to use a modified schedule of infection in order to study a mixed infection in which both viruses could replicate, since it was shown that simultaneous infection with bovine enterovirus led to essentially complete inhibition of bovine parvovirus replication. Cells were preinfected with parvovirus immediately upon release from the HU block and superinfected with enterovirus at 8 hr p.r. Superinfection with enterovirus was made at this time when parvovirus DNA synthesis was being initiated to determine the effect of enterovirus replication on the synthesis of parvovirus DNA and subsequent progeny virus production.

4.7.2.1. Rate of Bovine Parvovirus DNA Synthesis:

The effect of superinfection by bovine enterovirus on the rate of bovine parvovirus DNA synthesis in mixed

infections is presented in Figure 12. A parallel increase in the rate of bovine parvovirus DNA synthesis was observed beginning at 12 hr p.r. in cells singly infected with parvovirus and in cells mixedly infected with parvovirus and enterovirus. However at 16 hr, the level of viral DNA synthesis in the singly infected cells was 4.4-fold higher than that observed in mixedly infected cells. At 20 hr, a time when maximal levels of viral DNA synthesis were detected in singly infected cells, the rate in singly infected cells was 6-fold higher than that in mixedly infected cells.

4.7.2.2. Rate of Total Cellular RNA and Protein Synthesis:

As a result of the observed inhibition of bovine parvovirus DNA synthesis by superinfection with bovine enterovirus, it was necessary to examine the effect of superinfection with enterovirus on the rates of total RNA and protein synthesis in this mixed infection to determine whether a similar inhibition would occur. In cells singly infected with parvovirus, the rate of RNA synthesis increased between 2 and 6 hr p.r. (2 and 6 hr p.i.), reaching a maximum level of 136% of mock-infected cells (Fig. 13). After 6 hr, the rate of RNA synthesis gradually decreased and was 56% of the level in mock-infected cells at 24 hr. Following single infection with bovine enterovirus at 8 hr p.r., the rate of RNA synthesis was 150% of the

Fig. 12. Rate of bovine parvovirus DNA synthesis in cells preinfected with bovine parvovirus at 0 hr post release and superinfected with bovine enterovirus at 8 hr post release. Mock-infected (●——●); bovine parvovirus-single infection (■——■); bovine parvovirus-mixed infection (○——○); ⌂ indicates time of infection of cells at 0 hr with bovine parvovirus and at 8 hr with bovine enterovirus.

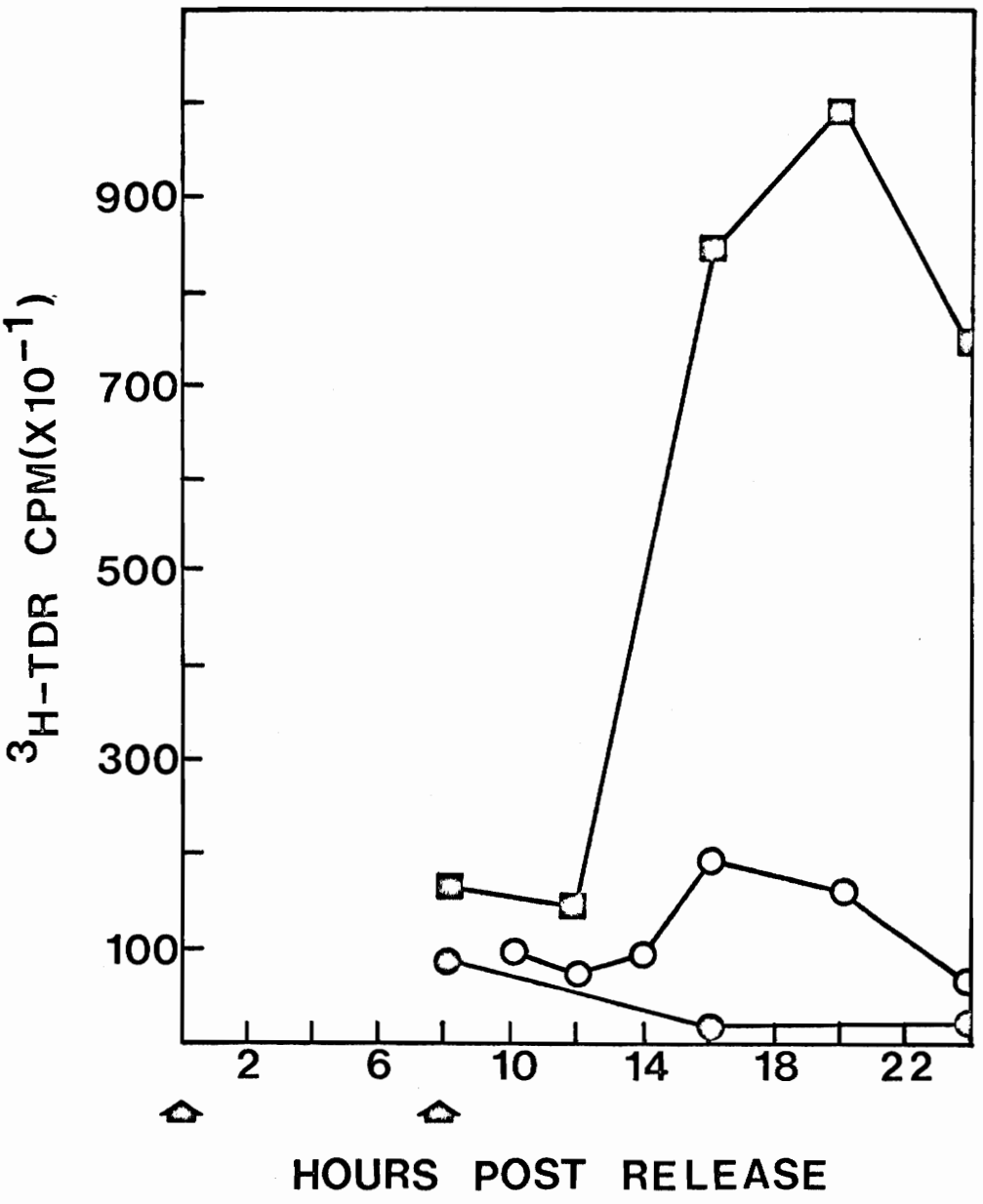
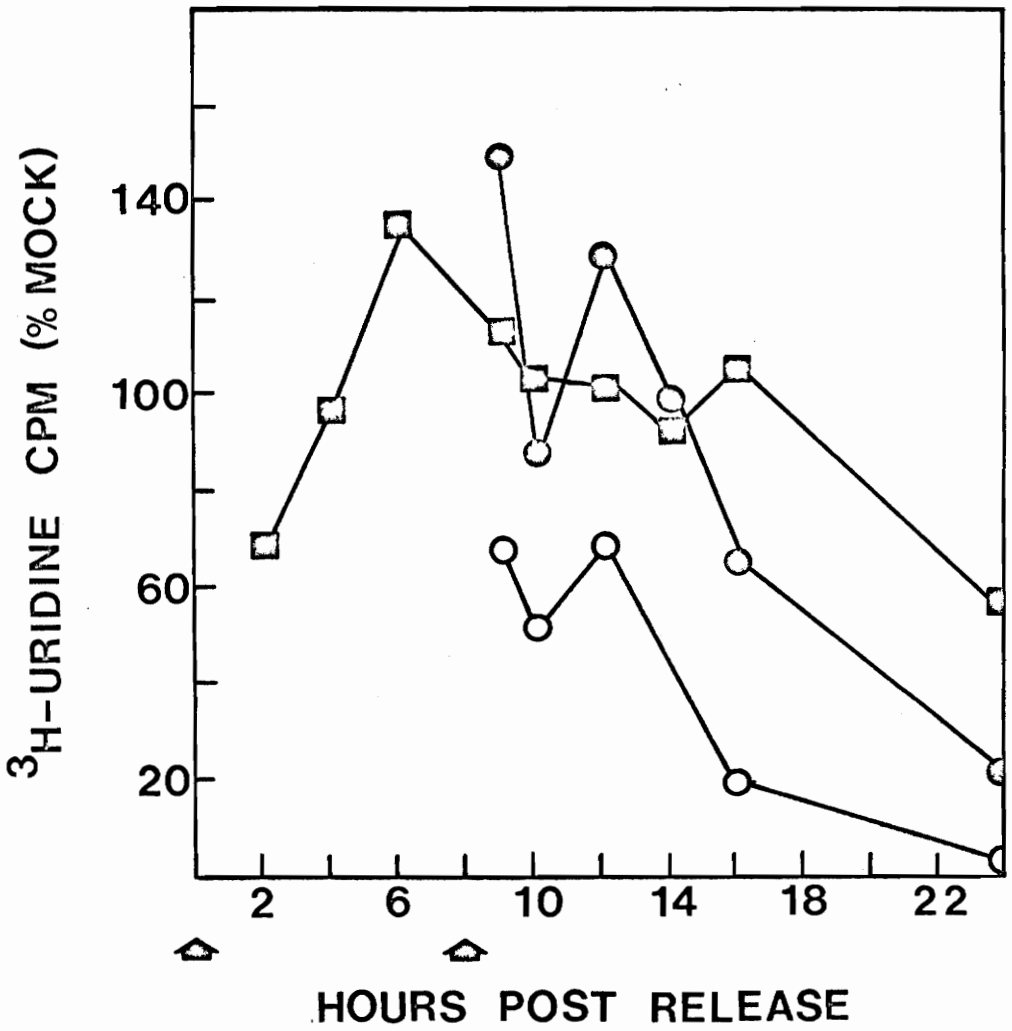

900
700
500
300
100
^{3}H-TDR CPM(X10^{-1})
2
6
10
14
18
22
HOURS POST RELEASE

Fig. 13. Rate of total cellular RNA synthesis in cells preinfected with bovine parvovirus at 0 hr post release and superinfected with bovine enterovirus at 8 hr post release. Bovine parvovirus-single infection (▫——▫); bovine enterovirus-single infection (●——●); bovine parvovirus and enterovirus-mixed infection (○——○); ⌂ indicates time of infection of cells at 0 hr with bovine parvovirus and at 8 hr with bovine enterovirus.

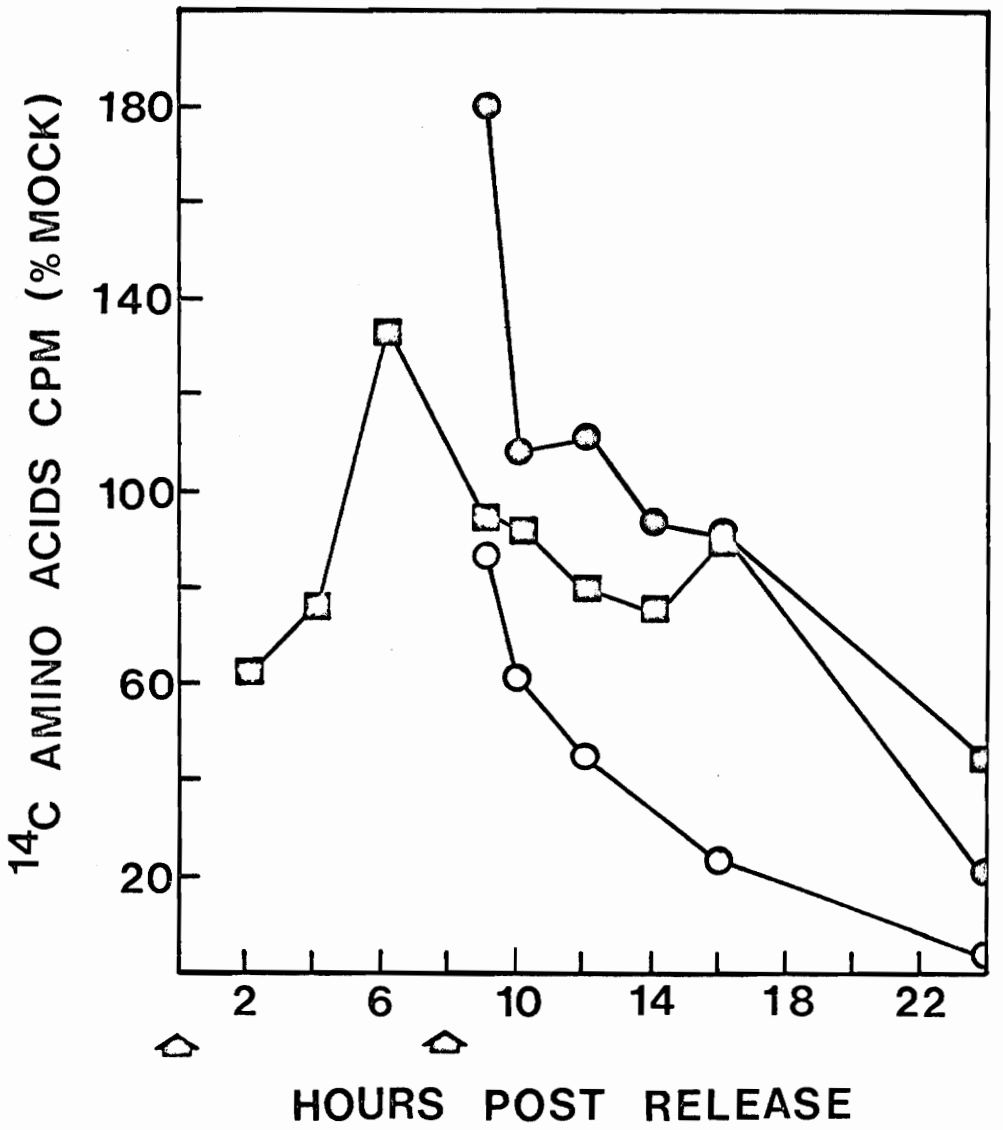

^{3}H-URIDINE CPM (% MOCK)
140
100
60
20
2
6
10
14
18
22
HOURS POST RELEASE

level in mock-infected cells at 1 hr p.i. (9 hr p.r.) but rapidly decreased to 88% at 2 hr p.i. (10 hr p.r.). At 4 hr p.i. (12 hr p.r.), the rate of RNA synthesis reached a peak of 129% of the level in mock-infected cells, whereas, the rate of RNA synthesis in cells singly infected with parvovirus was similar to mock-infected cells at this time. After 4 hr p.i. (12 hr p.r.) the rate of RNA synthesis decreased much more rapidly in cells singly infected with enterovirus than in cells singly infected with parvovirus. The rate of RNA synthesis in cells preinfected with parvovirus and superinfected with enterovirus at 8 hr p.r. followed the pattern seen during single infection with enterovirus, but was at levels considerably below mock-infected cells throughout the course of the experiment. Although the pattern of RNA synthesis in this mixed infection was similar to that seen in single infection with enterovirus, the reduced levels of RNA synthesis were apparently due to a combined effect of infection by both viruses. A similar depression of the rate of protein synthesis is also seen during mixed infection with these viruses (Fig. 14).

4.7.2.3. Titers of Bovine Parvovirus and Hemagglutinating Antigen:

The titers of infectious bovine parvovirus were examined in cells singly infected with parvovirus and in

Fig. 14. Rate of total cellular protein synthesis in cells preinfected with bovine parvovirus at 0 hr post release and superinfected with bovine enterovirus at 8 hr post release. Bovine parvovirus-single infection (□——□); bovine enterovirus-single infection (●——●); bovine parvovirus and bovine enterovirus-mixed infection (○——○); ⌂ indicates time of infection of cells at 0 hr with bovine parvovirus and at 8 hr with bovine enterovirus.


^{14}C AMINO ACIDS CPM (% MOCK)
180
140
100
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2
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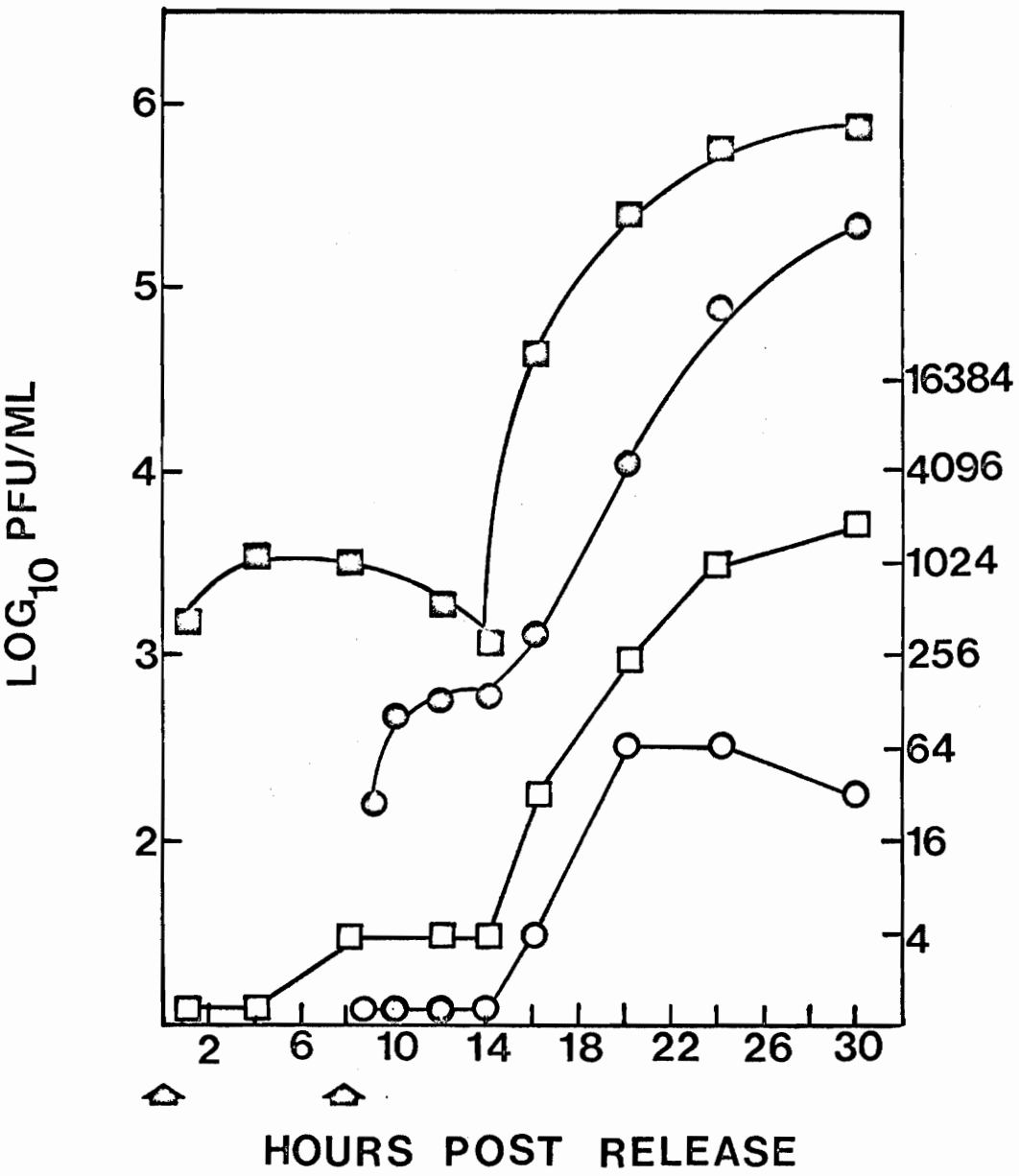
HOURS POST RELEASE

mixedly infected cell cultures, after infectious bovine enterovirus had been neutralized with specific antiserum (Fig. 15). In addition, the amount of hemagglutinating antigen in these samples was determined (Fig. 15). Beginning at 14 hr p.r., a parallel exponential increase was observed in the titer of infectious virus and hemagglutinins in both singly and mixedly infected cells. However, the amount of infectious virus and hemagglutinating antigen in the mixedly infected cells was lower than in the singly infected cells at each sampling time. At 30 hr, the titer of infectious virus in the single infection was approximately 4-fold more than in the mixed infection. The level of hemagglutinating antigen in the single infection at 30 hr, was 64-fold higher than in the mixed infection. Although the amounts of infectious virus and hemagglutinating antigen were reduced, significant levels of bovine parvovirus and hemagglutinating antigen were produced in this mixed infection.

4.7.2.4. Titers of Infectious Bovine Enterovirus:

The titers of infectious virus were determined in cultures singly infected with bovine enterovirus and in mixedly infected cultures after neutralization of bovine parvovirus with specific antiserum (Fig. 16). The titer of infectious enterovirus in both singly and mixedly infected cells increased exponentially beginning at

Fig. 15. Production of infectious bovine parvovirus and hemagglutinating antigen in cells preinfected with bovine parvovirus at 0 hr post release and superinfected with bovine enterovirus at 8 hr post release. Single infection-infectious virus (■——■); mixed infection-infectious virus (●——●); single infection-hemagglutinins (□——□); mixed infection-hemagglutinins (○——○); ⏢ indicates time of infection of cells at 0 hr with bovine parvovirus and at 8 hr with bovine enterovirus.


LOG10 PFU/ML
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HEMAGGLUTINATION TITER
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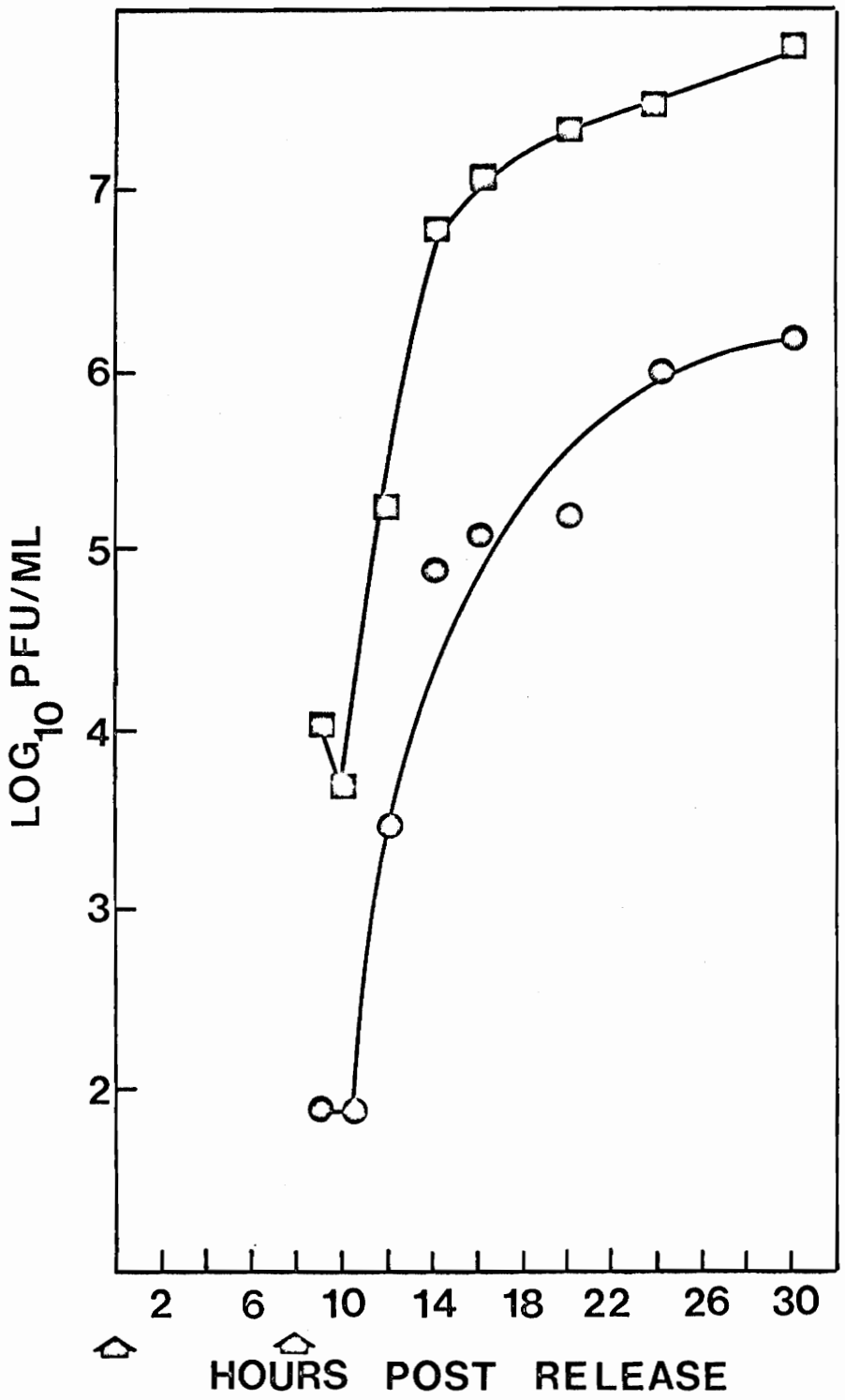
HOURS POST RELEASE

Fig. 16. Production of infectious bovine enterovirus in cells preinfected with bovine parvovirus at 0 hr post release and superinfected with bovine enterovirus at 8 hr post release. Single infection-infectious virus (▫——▫); mixed infection-infectious virus (○——○); ⌂ indicates time of infection of cells at 0 hr with bovine parvovirus and at 8 hr with bovine enterovirus.


LOG10 PFU/ML
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HOURS POST RELEASE

2 hr p.i. (10 hr p.r.) and continued to rise through 22 hr p.i. (30 hr p.r.). At this time, the titer of infectious virus in the singly infected cells was 43-fold higher than in mixedly infected cells. Therefore, in this type of mixed infection, the production of infectious bovine enterovirus was more severely inhibited than the production of infectious bovine parvovirus.

5. DISCUSSION

5.1. Growth Cycles of Bovine Parvovirus and Bovine Enterovirus in Single Infections.

Single infection of synchronized cells with bovine enterovirus and bovine parvovirus resulted in latent periods of 2 and 10 hr, respectively (Fig. 3, Fig. 4). Although the latent period for enterovirus replication in synchronized cell cultures has not been reported, the latent period of poliovirus, a virus in the same taxonomic group as bovine enterovirus, was determined in synchronized HeLa cells (Eremenko et al., 1972). A minimum latent period of 2 hr occurred when synchronized cells were infected with poliovirus during S phase of the cell cycle. Therefore, the latent periods for these two members of the picornavirus group are identical when synchronized cells are infected. Evidence from several laboratories suggests that parvoviruses are closely dependent on the cell cycle for initiation of their replication (Rose, 1974). It is not surprising that the minimum latent period for parvovirus replication results when synchronized cells are infected. Recent studies in our laboratory have shown that the optimum time of infection for bovine parvovirus is early S phase (Parris and Bates, 1975).

Maturation of enterovirus occurs very rapidly beginning at 2 hr p.i. and reaching maximum levels in the cell-associated fraction 4 hr later. Coincident with the

production of progeny virus, cytopathic changes appear in the cell cultures. By 6 hr, the cells begin to lyse releasing virus into the extracellular fluids. On the contrary, maturation of bovine parvovirus was detected for 10 hr in the cell-associated fraction before any progeny were released into the extracellular fluids. These results demonstrate that parvovirus remains more closely cell-associated than does enterovirus during its growth cycle. In part, the difference in the time of release of the two viruses is related to their intracellular sites of replication. Cytoplasmic membranes are more easily lysed than are nuclear membranes. Therefore, a virus which replicates in the cytoplasm (i.e. enterovirus) is more quickly released from the infected cells than is one which replicates in the nucleus (i.e. parvovirus).

5.2. Macromolecular Syntheses in Cells Singly Infected with Bovine Parvovirus and Bovine Enterovirus.

5.2.1. DNA Synthesis:

When comparing the rate of total DNA synthesis in cells infected with bovine parvovirus and in mock-infected cells, it is apparent that infection by parvovirus does not cause inhibition of cellular DNA synthesis during S phase since there is essentially no difference in the rates observed between 0 and 8 hr p.i. (Fig. 5). However, infection by enterovirus results in a severe reduction of cellular DNA

synthesis, beginning as early as 1 hr after infection. A similar rapid inhibition of cellular DNA synthesis is observed in cells infected with other picornaviruses (Levintow, 1974). Levintow postulated that the inhibition of DNA synthesis in poliovirus-infected cells is a secondary effect of virus-induced inhibition of synthesis of regulatory proteins. From my results I predicted that the rapid inhibition of cellular DNA synthesis in enterovirus-infected cells would severely affect the replication of a virus (i.e. parvovirus) which is dependent on cellular DNA synthesis during S phase.

The peak of DNA synthesis observed at 16 hr p.r. in cells singly infected with parvovirus occurs at a time when cellular DNA synthesis is at background levels (Fig. 5, Fig. 6). Although it is apparent that parvovirus DNA is being synthesized at this time, it is not known whether this DNA is double-stranded replicative forms or single-stranded progeny DNA. Initiation of parvovirus DNA synthesis does not begin until 8 hr p.r. (8 hr p.i.) which corresponds to late S phase. The reason for this delay has not been determined. However, it has been hypothesized by Parris and Bates (1975) that some factor (i.e. protein) produced during the S phase of the cell cycle may be required for initiation of viral DNA synthesis.

5.2.2. RNA and Protein Synthesis:

The rate of total RNA synthesis in cells singly infected with bovine parvovirus is increased over the levels observed in mock-infected cells during the first 8 hr after infection (Fig. 7). This increase may be the result of the synthesis of viral specific RNA species as well as those of cellular origin. The nature of this increased RNA synthesis will require additional studies in order to determine whether parvovirus mRNA is synthesized at this time after infection. The rate of total protein synthesis in parvovirus-infected cells is similar to that in mock-infected cells until 8 hr p.r., when a peak of activity is seen (Fig. 8). This may represent the synthesis of viral specific proteins. The temporal appearance of mRNA and protein in the replication cycle has not been determined yet for any virus in the parvovirus group. Although the rates of total RNA and protein synthesis decreased after 8 hr in parvovirus-infected cells, the level is maintained at 60 to 70% of that observed in mock-infected cells. This corresponds to the time when cytopathic effects are beginning to appear in the cell cultures and synthesis of viral DNA is initiated. It is apparent from the data presented that infection with parvovirus does not result in rapid inhibition of RNA and protein synthesis in infected cells.

In contrast to parvovirus-infected cells, the rates of total RNA and protein synthesis in bovine enterovirus-infected cells are at levels of 80% or less of mock-infected cells at all intervals after infection (Fig. 7, Fig. 8). At 4 hr p.r. there is a sharp increase in the rate of RNA and protein synthesis in the enterovirus-infected cells. These activities most likely represent the synthesis of enterovirus RNA and proteins, since the kinetics are similar to those reported for the replication of poliovirus in synchronized HeLa cells (Eremenko et al., 1972 and Levintow, 1974). In contrast to the effect of parvovirus infection on total RNA and protein synthesis, infection with enterovirus results in a very rapid inhibition of these syntheses. Similar inhibition of cellular RNA and protein synthesis has been described for poliovirus-infected cells (Levintow, 1974). Levintow suggested that virus-specified proteins produced very early in the infection cause reduction of these host syntheses. These early proteins inhibit cellular mRNA at the level of transcription and translation. The mechanism of inhibition of transcription is not well understood, but inhibition of translation is a result of the dissociation of host mRNA from the polyribosomes.

5.3. Mixed Infections.

5.3.1. Simultaneous Infection with Bovine Parvovirus and Bovine Enterovirus:

As could be predicted by the results obtained from the single infection experiments, in simultaneous infection with these viruses the replication of the enterovirus is favored as a result of the shorter replication cycle and rapid inhibition of cellular macromolecular syntheses. In fact, replication of the enterovirus followed kinetics similar to those seen in cells singly infected with enterovirus. However, the rate of parvovirus DNA synthesis and production of infectious parvovirus and hemagglutinating antigens was severely reduced or inhibited. Similar results were obtained when porcine kidney cells were simultaneously infected with porcine parvovirus and vesicular stomatitis virus (Mengeling, 1975). The porcine parvovirus replication was severely inhibited.

There are two possible reasons why the bovine parvovirus production was so drastically reduced in this simultaneous infection. The main cause was probably heterologous interference by the bovine enterovirus, since in the single infection studies it was found that enterovirus severely inhibited total cellular RNA,protein, and DNA synthesis, resulting in the appearance of cytopathic changes early in the infection. With the almost complete inhibition of

cellular DNA synthesis during S phase by the enterovirus, it would be predicted that the replication of parvovirus DNA would be sharply reduced as was observed in the Hirt extraction of the simultaneous mixed infection. In addition to heterologous interference, another possible reason for the inhibition of parvovirus production in the simultaneous infection might have been viral attachment interference by bovine enterovirus. Poliovirus, which is closely related to enterovirus, caused viral attachment interference in HeLa cells (Crowell, 1966).

5.3.2. Preinfection with Bovine Parvovirus at 0 hr p.r. and Superinfection with Bovine Enterovirus at 8 hr p.r.:

In an attempt to optimize conditions for the replication of both viruses in mixed infection, it was necessary to preinfect with parvovirus at 0 hr p.r. and then superinfect with enterovirus at 8 hr p.r. The superinfection with enterovirus, at a time when initiation of parvovirus DNA synthesis occurs, results in greater than 80% inhibition of viral DNA synthesis. It is expected that this viral DNA synthesis is required in the maturation of progeny virus, since the appearance of infectious virus coincides with synthesis of viral DNA. Although a significant level of infectious virus was detected, there was approximately a 75% decrease in comparison to singly infected cells. This

inhibition of the production of infectious parvovirus was probably due to heterologous interference by bovine enterovirus. Bablanian and Russell (1974) found similar results when they preinfected HeLa cells with adenovirus, a DNA virus which replicates in the nucleus, and superinfected these cells with poliovirus in the presence of guanidine. Even though the guanidine selectively inhibited the replication of poliovirus, the infection of adenovirus was dramatically altered. In this mixed infection protocol, the replication of enterovirus was more adversely affected than was that of parvovirus, since the level of infectious enterovirus in the mixed infection was approximately 98% lower than that in singly infected cells. Although this represents a much greater decrease in virus titer as compared to cells singly infected with enterovirus, more enterovirus plaque forming units are produced by 30 hr (1.52×10^6 PFU/ml) than parvovirus plaque forming units by 30 hr (2.18×10^5 PFU/ml) in this mixed infection. Heterologous interference by parvovirus is probably what caused the drastic decrease in the levels of bovine enterovirus in the mixed infection. By being preinfected 8 hr earlier than the enterovirus, the parvovirus was at an advantage in the competition with the enterovirus for use of the host cells' ribosomes and for substrates needed for the replication.

The rates of synthesis of total RNA and of total protein were both dramatically reduced in the mixedly infected cells in comparison to cells singly infected with either virus. For both RNA and protein the patterns of synthesis in the mixed infection closely resembled those in cells singly infected with enterovirus. However, a combined effect by both viruses most likely was required in order to cause such a severe decrease of both syntheses. The small peak in the rate of total RNA synthesis at 12 hr p.r. in the mixedly infected cells is probably due to the synthesis of enterovirus specified RNA, since a similar peak is seen at the same time in cells singly infected with enterovirus.

5.4. Concluding Remarks.

The results of this research provided documentation for the following statements:

1. Single infection of synchronized BFS cells with bovine parvovirus resulted in no inhibition of cellular DNA synthesis within 20 hr p.r. and only slight inhibition of total RNA and protein synthesis during the same time period.
2. If synchronized BFS cells were singly infected with bovine enterovirus, there resulted a rapid reduction in total DNA, RNA, and protein synthesis within 2 hr after infection.

3. When bovine parvovirus and bovine enterovirus simultaneously infected synchronized BFS cells, heterologous interference occurred as the parvovirus was dramatically inhibited and the enterovirus replicated normally.
4. In a mixed infection, in which BFS cells were preinfected with bovine parvovirus at 0 hr p.r. and superinfected with bovine enterovirus at 8 hr p.r., the levels of both viruses were reduced as a result of heterologous interference.

From the results obtained when studying these two different protocols of mixed infection, it can be easily seen that different interactions can occur in a mixed infection. The times of infection of the two viruses determine what interactions result in a particular mixed infection. Since these two viruses are known to occur in natural mixed infections in calves, it is quite obvious that the interactions between the two viruses could very well determine what symptoms might occur in the infected animal. Two viruses very similar to bovine parvovirus and bovine enterovirus are known to mixedly infect the human intestinal tract. These viruses are the hepatitis or non-bacterial gastroenteritis virus and human enterovirus. Therefore, by studying the various interactions of bovine parvovirus and bovine enterovirus in mixed infections, one could become more knowledgeable of what happens during a

mixed infection involving hepatitis virus and human enterovirus in man. In this research, only preliminary studies were performed on this particular mixed infection. Further experimentation in this area would involve the testing of other protocols of infection as to what virus-host interactions and virus-virus interactions occur. Immunofluorescence and electron microscopy would be very useful methods with which to determine the actual location and time of appearance of the different viral specific antigens and to follow the morphogenesis of the two viruses in infected cells.

6. LITERATURE CITED

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7. APPENDIX I

Minimum Essential Medium (MEM), Earle Base

Ingredients per liter:

l-Arginine HCl	126.98	mg
l-Cystine	24	mg
l-Glutamine	292	mg
l-Histidine HCl·H_2O	41.88	mg
l-Leucine	52	mg
l-Isoleucine	52	mg
l-Lysine HCl	58	mg
l-Methionine	15	mg
l-Phenylalanine	32	mg
l-Threonine	48	mg
l-Trytophan	10	mg
l-Tyrosine	36	mg
l-Valine	46	mg
Choline Chloride	1	mg
Biotin	1	mg
Folic Acid	1	mg
Inositol	2	mg
Ca-D-Pantothenate	1	mg
Pyridoxal HCl	1	mg
Thiamine HCl	1	mg
Nicotinamide	1	mg
Riboflavin	0.1	mg
Sodium Chloride	8	g
Potassium Chloride	0.4	g
Calcium Chloride	0.2	g
Magnesium Sulfate	0.0977	g
Monosodium Phosphate·H_2O	0.14	g
Dextrose	1	g
Phenol Red	0.02	g
Sodium Bicarbonate	2.2	g
Streptomycin	0.1	g
Penicillin	100,000	units

Dulbeccos Phosphate Buffer

Ingredients per liter:

Sodium Chloride	8	g
Disodium Phosphate·H_2O	1.12	g
Potassium Chloride	0.2	g
Magnesium Chloride·H_2O	0.1	g
Monopotassium Phosphate	0.2	g
Calcium Chloride	0.1	g
Phenol Red 1%	0.005	g
Streptomycin	0.1	g
Penicillin	100,000	units

Trypsin-Versene

Ingredients per liter:

Sodium Chloride	8	g
Sodium Bicarbonate	0.35	g
Phenol Red	0.02	g
Streptomycin	0.1	g
Potassium Chloride	0.4	g
Glucose	1	
Trypsin (1:250)	0.5	g
Penicillin	100,000	units

Phosphate Buffered Saline (PBS), pH 7.5

0.01 M Potassium Phosphate
0.15 M Sodium Chloride

8. VITA

The author was born in Mount Hope, West Virginia on April 7, 1951. He graduated from Mount Hope High School in June of 1969 and entered West Virginia Wesleyan College in August of the same year. In May of 1973, he graduated with a Bachelor of Science degree, having a major in biology and a minor in chemistry.

As a result of his interest in microbiology, the author entered the graduate school of Virginia Polytechnic Institute and State University in September of 1973 and began working toward a Master of Science degree.

Following completion of his Master's work, the author will enter the School of Veterinary Medicine at Ohio State University.

Ralph Benjamin Dorsey

BOVINE PARVOVIRUS AND BOVINE ENTEROVIRUS IN MIXED INFECTIONS

by

Ralph Benjamin Dorsey

(Abstract)

Bovine fetal spleen cells synchronized with 2mM hydroxyurea were infected with bovine parvovirus and bovine enterovirus in order to study the events occurring when DNA and RNA viruses mixedly infect single cells.

The objectives of this research were threefold. First, to determine the effects of single infection of synchronized bovine fetal spleen cells by bovine parvovirus and bovine enterovirus on cellular macromolecular syntheses. Second, to study the effect of simultaneous infection of synchronized cells by bovine parvovirus and bovine enterovirus. Third, to investigate the interactions which occur when synchronized cells are preinfected with bovine parvovirus and superinfected with bovine enterovirus.

Single infection of cells with bovine parvovirus upon release from hydroxyurea does not affect cellular macromolecular syntheses until 8 hr after infection; whereas, single infection with bovine enterovirus results in a rapid decrease in the rates of total DNA, RNA, and protein

synthesis by 2 hr after infection. In simultaneously infected cells, the enterovirus replication is not inhibited while the level of parvovirus is severely reduced. However, in cells preinfected with bovine parvovirus and superinfected with bovine enterovirus, the replication of both viruses is dramatically decreased.

It can be seen from the results obtained in the study of two protocols of mixed infection, that many different virus-host interactions and virus-virus interactions can occur in a mixed infection. The time sequence of infection of the two viruses determines what interactions take place.