COMPARISON OF SELECTED CHARACTERISTICS OF FOUR STRAINS OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

by

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INTRODUCTION

The virus of infectious bovine rhinotracheitis (IBR) has been incriminated as the cause of a number of different disease syndromes in cattle. These include abortion (22, 32), conjunctivitis (1), encephalitis (9), infectious pustular balanoposthitis (27, 52), infectious pustular vulvovaginitis (IPV) (11), fatal disease of newborn calves (5), mastitis (15), and a mild to severe upper respiratory disease (30). The viruses which were associated with these syndromes were found to be serologically related or identical by cross serum neutralization tests. It has been suggested by Manktelow and Hansen (24), and Lukas et al. (22) that undetected antigenic differences may exist between isolates.

The objective of this study was to compare selected characteristics of four strains of IBR in a semi-standardized virus-cell culture system. Characteristics compared included plaque sizes, thermal stability, growth curves, and cross reciprocal serum neutralization kinetics among strains.

Part II of this research consists of experimental animal inoculation with the ISU-IBR 2 strain of IBR virus. This strain is a recent field isolate.

REVIEW OF LITERATURE

In 1954 Schroeder and Moys (47) reported that an acute upper respiratory infection had suddenly appeared in dairy cattle in the vicinity of Los Angeles, California. The main signs observed were a sudden drop in lactation, pyrexia, and loss of appetite. The nasal mucosa was inflamed, and a stringy mucus discharge was observed which later became mucopurulent. Signs referable to the respiratory system included increased respiration rate and coughing. Most of the affected cattle returned to normal in approximately 7 days.

The most characteristic post-mortem lesion was a severe hemorrhagic tracheobronchitis. Necrotic lesions approximately 4 mm in diameter were scattered throughout the larynx and pharynx. McIntyre (26) was able to reproduce the disease in experimental calves by inoculating combinations of blood, nasal discharge and feces by various routes. The signs and post-mortem lesions resembled those observed in natural outbreaks. McKercher et al. (28) in 1954 described an influenza-like disease in California, but was unable to reproduce the disease experimentally at that time. The name of "infectious necrotic rhinotracheitis" was given to a disease entity in Colorado in 1955 by Miller (37). Signs of the disease condition were similar to those described in California.

McKercher et al. (29) in 1955 were able to reproduce the influenza-like disease condition using bacteria-free nasal washings. This finding strongly incriminated a virus as the etiological agent. It was demonstrated by cross protection tests in cattle that the syndromes observed in California and Colorado were identical. The recommendation was made that the disease be referred to as "infectious bovine rhinotracheitis."

The virus of infectious bovine rhinotracheitis was first isolated in cell culture by Madin et al. (23) from nasal exudate of affected cattle. They reproduced the disease in experimental animals using various passages of cell culture fluid. In these experiments both a California and a Colorado isolate were utilized. Both strains produced similar cytopathic changes in cell cultures of bovine embryonic kidney, testicle, and lung. Both strains also produced similar clinical syndromes when inoculated intranasally into experimental cattle. Results of cross serum neutralization studies indicated that the strains were serologically identical. York et al. (58) in 1957 compared three strains of IBR (Colorado I, Blythe and Los Angeles) using reciprocal cross neutralization tests. They concluded that the viruses studied were closely related.

With the development of cell culture systems for virus isolation, propagation, and serum neutralization tests, the

possibility of developing vaccines to IBR was realized. In 1957 Schwarz et al. (48) developed a modified live virus vaccine by rapid serial passage in cell culture. It was demonstrated that cattle inoculated intramuscularly with 10^4 50 per cent tissue infective doses ($TCID_{50}$) of the fortieth passage were resistent to challenge with a virulent IBR virus. The virus was also shown to be stable to lyophilization. In 1961 Zuschek and Chow (59) developed a modified live virus vaccine and compared it with a formalin-killed vaccine. Both of the vaccines induced immunity in cattle to virulent IBR challenge virus.

Gillespie et al. (11) in 1957 reported that IBR could be transferred from infected calves to others by contact. Clinical signs included pyrexia, salivation, lacrimation, anorexia, and depression. By reisolation studies, it was determined that high virus concentrations occurred in the nasal mucosa after intranasal and intratracheal inoculation.

McKercher et al. (30) in 1958 reported that IBR could be transmitted to goats. It was also reported that cattle exposed by the ocular route underwent a mild form of the disease. Cattle inoculated by the intranasal route developed a rapid rise in antibody by 12 days after inoculation and were refractory to challenge for at least a year.

Some of the biochemical properties of IBR virus were investigated by Griffin et al. (16) in 1958. The virus was

shown to be stable over a pH range of 6.0 to 9.3 at 4 C for 25 days. It could be stored for 9 months at -60 C with no loss of titer. At 4 C it was stable for 30 days and at 22 C dropped only 1 log in 5 days. At 37 C a ten-fold decrease was observed by the third day with complete inactivation taking place by day 10. The virus was completely inactivated in 21 minutes at 56 C. When the virus was subjected to lyophilization a ten-fold drop in titer was observed. Subjecting the virus preparation to equal parts of ether, alcohol, or acetone resulted in rapid inactivation.

Tousimis et al. (53) in 1958 reported on some of the biophysical characteristics of IBR virus. The size of IBR was estimated to be 145-156 mμ in diameter based on ultracentrifugation studies. Examination of infected cell culture fluids by electron microscopy revealed particles 136 mμ in diameter. Similar size particles were observed in ultrathin sections of infected cell cultures.

In 1958 Nadel (38) reported results of a growth curve study using IBR virus in a calf kidney cell system employing roller tubes. Two doses of inoculum were used, $10^{4.5}$ and $10^{1.5}$ $TCID_{50}$ per 2 ml. The virus was maximally adsorbed in 2 hours. With the low inoculum, virus release started at 48 hours and reached peak yields at 146 hours. However, the high inoculum virus was released at 18 hours reaching a peak yield at 146 hours.

Gillespie et al. (12) in 1958 reported that a relationship existed between IPV and IBR. Cytopathic changes, virus growth and production of intranuclear inclusion bodies in bovine kidney cell culture were similar. Both IPV and IBR were capable of producing similar clinical and pathological features in cattle; however, some differences in intensity were noted. The IPV strain produced a milder illness when inoculated intranasally. Cattle immunized with IBR virus were protected against challenge with IPV and vice-versa. Reciprocal cross serum neutralization tests indicated that these agents were serologically related. Wagner and Gillespie (54) in 1959 reported similar findings when they compared a Canadian IPV strain to Gillespie's IBR isolates. McKercher et al. (31) confirmed these findings in 1959 by comparing a New York IPV isolate to a California IBR isolate.

After the initial report of IBR isolation in the United States by Madin, reports of similar disease syndromes came from various parts of the world. Two New Zealand researchers, Webster and Manktelow (57), in 1959 reported the isolation of a virus from a cow with nasal catarrh. They were able to reproduce the disease in susceptible cattle. Two of the animals infected experimentally with the virus exhibited nervous symptoms, and two had adrenal lesions. The isolates were identified as IBR by the use of specific antiserum in neu-

tralization tests. In 1960 similar findings were reported from Germany by Liess et al. (21). The virus was isolated in cell culture and experimental inoculation reproduced both the IBR and IPV disease syndrome. Serological tests identified the virus as IBR virus.

The effect of IBR-IPV virus on young calves was studied by Baker et al. (5) in 1959. Calves were exposed intravenously, orally, or by contact shortly after birth. All of the calves became ill, and some died. By virus reisolation studies and post-mortem lesions it was concluded that the virus also has an affinity for the digestive and lymphatic tissues in young calves. Before this report it was generally assumed that only the respiratory or vulvovaginal tracts were involved.

In 1961 Manktelow and Hansen (24) compared an IBR strain isolated in New Zealand to a New Zealand IPV strain. Their results confirmed the findings of Gillespie et al. (12) and McKercher et al. (31) that these agents were serologically related. Manktelow and Hansen (24) also noted that IBR virus induced vulvovaginitis indistinguishable from the disease induced by IPV virus. Whereas, IPV virus inoculated intranasally produced a mild febrile response, but none of the characteristic respiratory signs usually associated with IBR. These authors suggested that undetected antigenic differences

among virus strains may account for the different clinical diseases observed.

Conjunctivitis, which is another manifestation of IBR infection, was reported by Abinanti and Plumer in 1961 (1). In the feeder cattle affected, no respiratory signs were observed. The infectious agent was isolated in bovine embryonic kidney cell cultures. Cytopathic effects of the isolate resembled those of IBR. The agent was identified by serum neutralization tests using specific antiserum. Conjunctivitis could be reproduced in cattle by intrapalpebral inoculation of this isolate and a California IBR isolate. However, the California strain did not produce as severe a conjunctivitis as the original conjunctival isolate. Serological results by cross serum neutralization indicated that the viruses were closely related.

Armstrong et al. (3) proposed that IBR be classified with the Herpesvirus group. They based this proposal on the type of cytopathic effect (CPE) produced in cell culture, ether sensitivity, particle size (150 mμ) and morphology as observed in the electron microscope. The mode of viral replication as observed in ultrathin sections of infected cell cultures resembled that of other members of the Herpesvirus group. Grinyer et al. (17) in 1962 confirmed these findings.

In 1962, French (9) reported the isolation of a virus from the brain of native Australian cattle dying of encephalitis. The disease condition was reproduced by intranasal inoculation of experimental animals. Transient ocular discharge as well as clinical signs referable to an encephalomyelitis were observed. However, no rhinotracheitis was recorded for any of the cases. The isolated virus was identified by serum neutralization tests with IBR antiserum. French suggested that the apparent neurotropism of this strain represented a unique genetic nature of the viral genome.

McKercher (27) in 1963 reported the results of studies comparing the Los Angeles IBR strain to the etiologic agents of Blächenausschlag (Coital Vesicular Exanthema). The latter disease had been described in central Europe, particularly in Germany for many years and was believed to be synonymous with the IPV syndrome in the United States. The Blächenausschlag viruses used were identified as the Riems (German) isolate, the Vienna (Austrian) isolate, and the Ghent (Belgian) isolate. Cross immunity tests in calves established that the four isolates were immunologically related. Reciprocal cross-neutralization and cross complement-fixation tests using post inoculation serum samples established the fact that all strains were serologically related.

In 1963, McKercher et al. (33) reported the results of a study on the distribution and persistence of IBR virus in cattle following intranasal inoculation. They concluded that the virus multiplied in the upper respiratory tract and spread via the lacrimal ducts to the ocular tissue where secondary multiplication may occur. The virus was also reisolated from some of the lymph nodes draining these areas. Virus was isolated occasionally from the aqueous humor, brain, kidney, mesenteric lymph nodes, peritoneal fluid and cecum from some animals. It was suggested that the virus was transported to these areas by migrating leukocytes.

The first report of the use of the plaque technique for the quantitative study of IBR virus was by Rouhandeh and Werder (43) in 1963. They described the procedure for plaque production of IBR virus on primary bovine embryonic kidney cells. It was stated that the production of plaques could be inhibited by IBR hyperimmune serum.

Later in 1963 Sabina and Parker (46) reported plaque production by IBR virus on monolayers of a bovine kidney cell line (MDBK). The multiplication of IBR virus on MDBK monolayers was studied by one-step growth curves. With a multiplicity of 1 plaque forming unit (PFU) per cell, the eclipse phase was complete at approximately 6 hours. Extracellular virus increased logarithmically until the twelfth to fourteenth hour. Maximum free virus was obtained 24 hours post

infection, and the infective virus yield per cell was 210 PFU. The kinetics of thermal inactivation and serum neutralization were also investigated. The kinetics of both of these reactions were found to follow first order rates over the period studied. Results indicated that the half life of IBR virus at 37 C and 42 C were 16 and 3.5 hours respectively. Experiments on the kinetics of neutralization showed that anti-IBR bovine and anti-IBR rabbit sera had the same virus neutralizing potential. The IBR strain used throughout this study was an isolate from Colorado cattle.

Stevens and Groman (49) in 1963 also reported on a quantitative virus-cell culture system. The MDBK tissue culture cells and the Cooper strain of IBR virus (Colorado) were used in this study. In a one-step growth curve using a high multiplicity of virus (6 PFU/cell) an eclipse phase of 4.5 hours was observed. During the next 7 hours intracellular virus increased to a peak and remained at that level for the next 10 hours. The extracellular virus did not begin to increase until approximately 8.5 hours and then gradually reached a peak at 25 hours. The yield of virus per cell was estimated to be between 115 and 270 PFU. Data on thermal stability indicated that this strain had a half life of 10 hours at 37 C.

Hahnefeld et al. (18) in 1963 reported results on the stability of the Bläschenausschlage virus. Most of their findings were similar to results reported for IBR virus.

Lukas et al. (22) in 1963 reported that IBR virus had been isolated from a large number of cases of abortion in California. The abortions had occurred in herds with different histories such as IBR vaccinated and unvaccinated, upper respiratory signs, ocular signs alone, rhinitis only, or no clinical signs of IBR. They also produced abortion experimentally by inoculating pregnant cattle with a virus isolate (Fresno No. 2294). It was shown by serum neutralization tests that the abortion isolate was identical to the IBR virus. However, the authors suggested that this strain may posses different infective and antigenic properties which would account for its apparent tropism for the fetal tissue. McKercher and Wada (32) confirmed the above results in 1964. They were able to induce abortion with two isolates of IBR from aborted fetuses. These isolates were found to be indistinguishable from a known IBR strain (Los Angeles) by cross immunity tests in cattle and reciprocal cross serum neutralization tests in cell culture.

In 1964, Studdert et al. (52) reported on the experimental reproduction of infectious pustular balanopostitis using a field isolate from a case of IPV. Clinical syndromes

of bulls inoculated intrapreputially were divided into three categories depending on the severity of the disease. In general, the early lesions consisted of pustules distributed over the preputial folds and penis. Later, after sloughing of the pustular material, the lesions healed. An elevated temperature response, varying degrees of inappetence and neutropenia, were usually associated with the clinical disease.

McKercher (34) in 1964 compared the viruses of IBR, IPV and rinderpest. By using various serological procedures, antigenic similiarity was demonstrated between IBR and IPV. No antigenic relationship was detected between these two agents and rinderpest. In a study (35) using a plaque assay system, it was shown that the plaques produced by IBR and IPV were indistinguishable. However, these plaques could be readily differentiated from plaques produced by rinderpest. It was also reported that the plaques produced by IBR and IPV virus could be inhibited by their homologous or heterologous antiserum, but not by rinderpest antiserum.

Straub et al. (51) in 1964 reported on a study comparing an IPV isolate with an IBR isolate. These isolates had identical serological characteristics, as well as identical buoyant densities. By using carrier-free virus zone electrophoresis in a glucose density gradient, the two viruses showed different mobilities. The authors, therefore, con-

cluded that the viruses were not identical but sub-types of the virus group.

Peter (41) in 1964 studied the pathogenesis of an infection produced by IBR virus (ISU-IBR 1) isolated from a calf dying of mucosal disease. Fluorescent antibody technique was utilized to detect the viral antigen at various locations in the tissue of experimentally inoculated calves. The viral antigen was primarily located in lymphoid tissue; however, the organs being involved depended upon the route of inoculation.

In 1965, Greig and Bannister (15) reported on the production of mastitis by inoculation of IBR virus into the teat canal of the bovine mammary gland. The virus could be reisolated from the second to the fifteenth day in high titers. Shortly after the disappearance of virus from the mammary gland, serum and milk antibodies were detected.

PART I. VIRUS STRAIN COMPARISON

MATERIALS AND METHODS

Tissue Culture System

Glassware washing

New glassware was rinsed in triple distilled water before sterilization. Used glassware was rinsed in tap water immediately after use, and any glassware contaminated with bacteria, viruses, or molds was autoclaved and then subjected to the washing procedure. Most of the glassware was washed in a commercial laboratory dishwasher[1], using washing cycles, tap water and distilled water rinses as specified by the manufacturer. In addition to the machine distilled water rinse, all glassware was rinsed 3 additional times in distilled water and once in triple distilled water. After the glassware had drained dry, the bottles were capped and the other glassware appropriately wrapped for autoclaving. All glassware was autoclaved for 30 minutes.

Triple distilled water

Tap water was first distilled by a large steam distiller. This water was then passed through an ion exchange resin into

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[1]Heinicke Instruments Co., Hollywood, Florida.

a glass laboratory distilling apparatus. The water collected from this apparatus was stored in plastic carboys until used.

Solutions

Hanks' balanced salts solution with lactalbumin hydrolysate and yeast extract. (Hanks' LY) This medium supplemented with 10 per cent inactivated lamb serum was used for cell propagation. The formula of Melnick (36) was modified by reducing the amount of lactalbumin hydrolysate to .1 per cent and adding .1 per cent yeastolate.

NaCl	8.00 gm.
KCl	.40 gm
$MgSO_4 \cdot 7H_2O$	.10 gm
$MgCl_2 \cdot 6H_2O$	.10 gm
$NaHCO_3$	.35 gm
$CaCl_2$	.14 gm
Na_2HPO_4	.06 gm
KH_2PO_4	.06 gm
Glucose	1.00 gm
Phenol red	.02 gm
Lactalbumin hydrolysate[1]	1.00 gm
Yeastolate[2]	1.00 gm

[1]Difco Laboratories, Incorporated, Detroit 1, Michigan.

[2]Difco Laboratories, Incorporated, Detroit 1, Michigan.

Triple distilled water q.s.	1000 ml

The medium was sterilized by pressure filtration through a .22 mμ Millipore filter. After filtration, the medium was incubated at 37 C for 48 hours as a sterility check. An antibiotic mixture containing 10,000 units of penicillin and 10,000 μgs of streptomycin was added per 100 ml of medium. This medium was then stored at 4 C. Lamb serum was added prior to seeding cells in culture plates and tubes.

Eagles' basal medium This medium was used in the maintenance of cell cultures after inoculation of virus. Horse serum in concentrations of 2 to 5 per cent was usually added prior to use.

Eagles' basal medium 10x[1] - with Earle's salts - with L-glutamine	100 ml
$NaHCO_3$	2.2 gm
Penicillin	10,000 u
Streptomycin	10,000 μg
Triple distilled water q.s.	900 ml

This medium was sterilized by pressure filtration through a .22 mμ Millipore filter and stored at 4 C.

Saline used for cell washing procedures and virus dilutions. (Saline G) The saline used was prepared according

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[1]Grand Island Biological Company, Grand Island, New York.

to the formula of Ham and Puck (19).

NaCl	8.00 gm
KCl	.40 gm
$MgSO_4 \cdot 7H_2O$	.15 gm
$Na_2HPO_4 \cdot 7H_2O$	.29 gm
KH_2PO_4	.15 gm
$CaCl_2 \cdot 2H_2O$	.016 gm
Glucose	1.10 gm
Phenol red	.0012 gm
Lactalbumin hydrolysate	2.00 gm
Triple distilled water q.s.	1000 ml

This saline solution was sterilized by autoclaving at 15 pounds pressure for 20 minutes. After cooling, an antibiotic solution was added to give a final concentration of 100 units of penicillin and 100 µg of streptomycin per ml. The solution was stored at 4 C until used. If the saline G was to be used for virus dilutions, 1 per cent inactivated horse serum was added prior to use.

Trypsin solution This solution was used for the dispersion of cells. It was prepared according to the following formulation:

NaCl	8.00 gm
KCl	.40 gm
$Na_2HPO_4 \cdot 7H_2O$	.045 gm

KH_2PO_4	.03 gm
$CaCl_2.2H_2O$	.016 gm
Glucose	1.00 gm
Phenol red	.0012 gm
Trypsin	2.00 gm
Triple distilled water q.s.	1000 gm

In order to dissolve the trypsin, the solution was incubated in a 37 C water bath for 1 hour. Penicillin and streptomycin were added, and the solution was sterilized by pressure filtration through a .22 mu Millipore filter. The trypsin solution was stored at -20 C until used.

Overlay medium for plaque assay Eagles' solution was prepared as a 2X concentrate from powder media as described below.

Powder Medium BME[1] - with Earles salts - with L-Glutamine	9.29 gm
Sodium bicarbonate	2.20 gm
Penicillin	100,000 units
Streptomycin	100,000 µg
Triple distilled water q.s.	500 ml

The medium was sterilized by pressure filtration through a Millipore .22 mu filter and stored at -20 C. When used for

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[1]General Biochemicals, Chargin Falls, Ohio.

overlay, this medium was supplemented with horse serum to give a final concentration of 5 per cent. This mixture was warmed to 48 C and mixed with an equal volume of aqueous 2.2 per cent of Special Noble Agar.[1]

Serum

The lamb and horse serum were supplied by Grand Island Biological Company, Grand Island, New York.

Cell culture procedure

Bovine testicles were supplied by Rath Packing Company, Waterloo, Iowa. The testicles were removed from slaughtered calves, packed in plastic bags, and shipped in a container with ice. A time lapse of about six hours occurred between removal and processing the tissue.

The bovine testicles were processed as described by Gratzek (13). After the testicular tissue was trypsinized the cells were strained through 4 layers of sterile gauze. The cells were sedimented by centrifugation and washed twice with saline G. The packed cells were resuspended in saline G and 50 per cent serum before counts were made. Cells were added to Hanks' LY containing 10 per cent lamb serum to make a final concentration of 1 million cells per ml. This mixture

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[1]Difco Laboratories, Incorporated, Detroit 1, Michigan.

was dispensed into disposable petri dishes[1] or disposable glass tubes.[2] After 24 hours of incubation at 37 C, the cell culture medium was changed. Confluent monolayers usually formed in 3 to 4 days.

Virus Strains

Four isolates of IBR virus were compared. The Colorado vaccine strain and the Los Angeles strain were used as prototypes.[3] The passage level of these two strains was unknown.

The ISU-IBR 1 strain was isolated by Gratzek et al. (14) in bovine testicle cell culture from deep scrapings of a Peyer's patch from a calf that had died of mucosal disease. This virus was identified as IBR by neutralization with specific IBR antiserum. The pathogenesis of this isolate for calves was reported by Peter in 1964 (41). This strain had been passed 10 times at the onset of this study.

The ISU-IBR 2 strain was isolated in the fall of 1964 by Buening in cell cultures of bovine testicle from nasal swabs of beef cattle having a mild upper respiratory disease.

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[1]Falcon Plastics, Los Angeles, California.

[2]Scientific Products, Chicago, Illinois.

[3]These strains were obtained through the courtesy of Phillips, C. H., National Animal Disease Laboratory, Ames, Iowa.

This strain was identified as IBR by neutralization of cytopathic effects in cell cultures by specific IBR antiserum. Results of intranasal inoculation of experimental calves will be reported in Part II of this thesis. This strain had been passed 10 times at the onset of this study.

All of the strains were cloned by plaque selection. This process was repeated at least twice at the beginning of this study. The four strains were passed simultaneously in primary bovine testicle cell monolayers which were derived from the same trypsinized batch of cells.

Propagation of Stock Virus

For the propagation of virus stocks, primary bovine testicle cell monolayers were grown in 32 oz. prescription bottles. In order to insure uniformity the same trypsinized batch of cells was used for all strains.

Stock viruses used to hyperimmunize rabbits were propagated in monolayers maintained in Eagles' medium without serum. Cloned virus strains were inoculated onto the monolayers at concentrations of about 10^4 PFU per monolayer. When 75 per cent of the cells showed CPE the tissue culture fluids were harvested. The fluids were centrifuged at 2000 RPM for 20 minutes and the supernatant dispensed in screw cap vials. These vials were stored at -69 C until used. Virus prepared

in this manner was used in the plaque size study and serum neutralization kinetics study.

Stock viruses used in the one-step growth curve and thermal inactivation study were propagated in monolayers maintained in Eagles' medium with 2 per cent horse serum. In this case, the cloned virus strains were inoculated onto the monolayer at concentrations of about 10^5 PFU per monolayer. After 90 per cent of the cells showed CPE the bottles containing the tissue culture monolayers and fluids were frozen and thawed once. The fluids were centrifuged, and the supernatant fluid dispensed into screw cap vials and stored at -69 C.

Production of Specific Rabbit Antiserum

Rabbits were bled prior to inoculation and the serum tested for non-specific inhibitors of IBR virus. Hyperimmunization was carried out with cell culture fluids which contained approximately 10^6 PFU per ml. Two rabbits were inoculated with each of the virus strains. The inoculation schedule used is described in Table 1.

Hyperimmune serum was collected one week after the last inoculation. It was heat inactivated at 56 C for 30 minutes and stored at -20 C until used.

Table 1. Hyperimmunization schedule

Day	Amount of inoculum	Route	Freund's adjuvant[a]
1	1 ml	I/V[b]	none
	1 ml	I/D[b]	1 ml
7	1 ml	I/V	none
14	1 ml	I/V	none
21	1 ml	I/V	none

[a]Difco Laboratories, Incorporated, Detroit 1, Michigan.

[b]The intradermal (I/D) injection consisted of equal parts tissue culture virus and Freund's adjuvant. Injections were made over the back with about 0.1 ml injected per site.

Assay Procedure

Infectious virus was assayed throughout this study by a modification of the plaque technique as described by Dulbecco and Vogt (7). Monolayers of primary bovine testicle cells were grown in disposable petri dishes at 37 C in an incubator gassed with 5 per cent CO_2 in air. When complete monolayers had formed, the medium was decanted, and the monolayers were washed with 4 ml of saline G. A 1 ml aliquot of virus dilution was added to each 60 mm cell culture dish, and the virus was allowed to absorb for 2 hours at 37 C. During this period the plates were frequently rotated to insure even distribution of plaques. After the absorption period, the inoculum was

decanted and the monolayers washed twice with saline G. Five ml of overlay medium containing 95 per cent Eagles' medium and 5 per cent horse serum in a 1.1 per cent agar solution (48 C) was added to each petri dish. After the overlay had solidified, the petri dishes were returned to the CO_2 incubator.

Plaques could be observed unstained in 48 hours, but visualization was enhanced by the addition of a second overlay of 1:10,000 neutral red in a 1 per cent aqueous agar solution at the third or fourth day of incubation. After a staining period of 6 hours, the plaques were counted.

Plaque Size Study

Thirty monolayers of primary bovine testicle cells in 60 mm petri dishes were inoculated with 20 to 50 PFU per monolayer of each virus strain. Virus was allowed to absorb for 2 hours, after which the monolayers were washed twice with saline G. The monolayers were overlayed with 4.5 ml of overlay medium and after solidification were returned to the CO_2 incubator.

From the third day through the seventh day of incubation, six plates of each virus strain were randomly selected for plaque size analysis. These plates were stained, the agar overlays were removed, and five plaques per plate were selected

at random for measurement using a grid positioned below the plate. Plaque size was determined to the closest 0.1 mm by the use of a vernier caliper measuring the smallest diameter of the plaque. The daily mean plaque size and standard deviations were calculated.

Thermal Stability

Heat inactivation rates of the virus strains were measured at 37 C and 42 C. The diluent used in this study consisted of saline G containing 1 per cent horse serum. Screw cap tubes containing the diluent were prewarmed to the desired temperature in a water bath. At zero time a virus dilution was added to give a final virus concentration of approximately 1 x 10^6 PFU per ml. Samples were taken at 0, 6, 12, 18, 24 hours for the 37 C study and at 0, 3, 6, 9, 12 hours for the 42 C study. All samples were frozen and stored at -69 C until assayed by the plaque procedure. In experiments at a given temperature the same bottle of diluent was used. All virus strains were subjected to the treatment and assayed simultaneously in order to reduce experimental error. At least three monolayer plates were used per dilution.

Single-Step Growth Curves

In order to minimize experimental error due to the cell culture system, primary bovine testicle cell monolayers which had been derived from the same trypsinized batch were used for single-step growth curves for all IBR strains. The procedure was to inoculate monolayers with a specific IBR strain at half hour intervals, thus all strains were inoculated at least within 1.5 hours of each other. Such a method allowed all samples to be collected within a 26 hour period. Also monolayers which had been derived from the same trypsinized batch were used to assay all samples.

Single-step growth curves of each IBR virus strain were performed according to the method of Stevens and Groman (50). Monolayers containing approximately 1×10^6 cells were inoculated with 2×10^7 PFU of virus. This input multiplicity (20 PFU per cell) according to Poisson distribution statistics would insure infection of 99.99 per cent of the cells with at least 1 PFU. After virus absorption for 2 hours at 37 C, the plates were washed four times with 4 ml of saline G to remove unabsorbed virus. Pooled washings were sampled to determine the proportion of unadsorbed virus. Four ml of Eagles' medium containing 5 per cent horse serum was added to each plate prior to incubation.

At various time intervals over a 24 hour period, monolayers were removed from the incubator and the supernatant fluid sampled for extracellular virus. The monolayers were washed four times with 4.0 ml of saline G followed by the addition of 3.0 ml of saline G containing 1 per cent horse serum. The monolayers were sonicated for 1.5 to 2.0 minutes at 15 kilocycles with an ultra-sonic probe[1] in order to determine intracellular virus. Samples were quick frozen and stored at -69 C until assayed.

Neutralization Kinetics

Antigenic comparison of the IBR strains was determined by reciprocal neutralization kinetics. The method used was a modification of the procedure reported by Sabina (45, 46). By preliminary experiments the dilution of hyperimmune rabbit serum which would neutralize 1.5 to 2.0 logs of its homologous IBR strain in 30 minutes was determined. Diluted serum and approximately 2×10^5 PFU of virus were prewarmed to 37 C in a water bath. At 15 seconds prior to zero time, 1 ml of virus was added to 1 ml of serum dilution in a test tube covered with a metal cap and mixed. At 0 time plus 15 seconds, 0.1

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[1]Bronwill Scientific Division, Will Scientific Inc., Rochester 3, New York.

ml of the serum virus mixture was removed and immediately added to 100 ml of cold (4 C) saline G containing 1 per cent horse serum. This sample (10^{-3} dilution) and an additional 1:10 dilution (10^{-4}) were assayed for residual infective virus. This procedure was repeated at 5, 10, 15, and 20 minutes. The 30 minute sample was diluted 10^{-2} and 10^{-3} and assayed for residual virus.

Two monolayer plates were used for assay of each dilution. The plaque assay was carried out as previously described, and the average number of plaques per dilution was recorded.

Since neutralization of IBR virus has been shown to be a first order reaction by Sabina and Parker (46), the logarithm of the reciprocal of the per cent of surviving virus plotted against time on a linear plot will result in a straight line. The method of simple linear regression analysis was employed to determine a straight line which best fitted the experimental points. The procedure of regression analysis used was that described by Ostle (40). The slope of this line will equal the neutralization rate constant divided by 2.3 x the reciprocal dilution of the antiserum. Thus, the reciprocal neutralization slopes of a given antiserum can be compared statistically by using Student's t-test. In all cases the slope of the homologous system was compared to heterologous systems.

The neutralization rate constant (K) can also be calculated by using the following equation: $K=D/t\ 2.3\ \log_{10} V_o/V_t$ as described by McBride (25). In this case only two observations need be considered, the initial amount of virus (V_o) and the residual virus at time t (V_t). The term D or 1/C is equal to the dilution of antiserum used in the experiment. When this formula was used, an estimated value for $\log_{10} V_o/V_t$ at 30 minutes was calculated from the regression analysis data.

Letting C equal antibody concentration as described by Ashe and Scherp (4), K is then the expression of serum titer derived from the initial rate at which a concentration of virus is neutralized by a large excess of antiserum. The K values of different antiserums cannot be compared directly due to the fact that the absolute concentration of antibodies cannot be determined. However, this difficulty is surmounted by normalizing the K values (NK). In this calculation, the K value for the homologous antiserum-virus combination is assigned a value of 100. The heterologous systems are calculated as a percentage of the homologous system. Ashe and Scherp (4) used the criterion that combinations giving reciprocal NK values of 90 to 100 were serologically homologous.

Analysis of the data from reciprocal neutralization kinetics were subjected to both of the procedures described. By using both methods, conclusions relative to the serological relatedness of the strains could be drawn with more confidence.

RESULTS

Plaque Size Study

Plates inoculated with each IBR virus strain were removed from the incubator from the third through the seventh day. These plates were removed at approximately the same time each day and stained with a second overlay of 1: 10,000 neutral red agar solution. Approximately 6 hours after the second overlay, the entire agar overlay was removed by reaming the edge of the petri dish with a spatula and inverting the plate. Plaque size to the nearest 0.1 mm was determined by measuring the smallest diameter of the plaque with a vernier caliper.

A total of 30 plaques, picked at random, were measured per strain each day. This data was compiled and programed for computer analysis, in order to determine the mean plaque size, standard deviation, and coefficient of variation. Results of this analysis are presented in Tables 2 through 5. Coefficient of variation of plaque size tended to be higher, except for the ISU-IBR 1 strain, on the first three days of measurement. However, as the plaque diameter increased, the coefficient of variation decreased.

Table 2. ISU-IBR 1 plaque size variability pattern

Day	Mean plaque size mm	Standard deviation mm	Coefficient of variation %
3	1.0233	0.0177	9.0
4	1.1433	0.0297	14.3
5	1.6767	0.0409	13.4
6	2.0667	0.0399	10.6
7	2.4300	0.0609	13.7

Table 3. ISU-IBR 2 plaque size variability pattern

Day	Mean plaque size mm	Standard deviation mm	Coefficient of variation %
3	1.0000	0.0292	14.3
4	1.1233	0.0292	14.2
5	1.5667	0.0365	13.1
6	2.0467	0.0358	9.6
7	2.2400	0.0426	10.4

Table 4. Colorado plaque size variability pattern

Day	Mean plaque size mm	Standard deviation mm	Coefficient of variation %
3	.9467	0.0359	20.7
4	1.1433	0.0347	13.3
5	1.4700	0.0560	20.8
6	2.2267	0.0356	8.8
7	2.2933	0.0387	9.3

Table 5. Los Angeles plaque size variability pattern

Day	Mean plaque size mm	Standard deviation mm	Coefficient of variation %
3	.9433	0.0346	20.0
4	1.1067	0.0394	19.6
5	1.5033	0.0395	14.6
6	1.9767	0.0433	12.0
7	2.1700	0.0539	13.6

Histograms of plaque size distribution per day were constructed. These histograms are presented in Figures 1 through 5. Plaque size classes were assigned using a range of 0.3 mm. The variation in plaque size of each virus strain appeared to increase with time, however, the histograms tended to follow a normal distribution curve.

By plotting the mean daily plaque size of the various strains against time, a curve was obtained which is illustrated in Figure 6. A sigmoid curve can best be fitted to the experimental points. Mean plaque size of the different virus strains are very similar on day 3 and 4. Subsequently increased variation in mean plaque size existed between strains, but these variations fell within the confidence intervals of the entire experiment and were judged as insignificant.

Representative monolayers containing plaques of each of the IBR virus strains were photographed on day 4. The photographs were taken after the monolayers were stained and before removal of the agar overlay and are presented in Figures 7 through 10.

Thermal Stability

The rates of thermal inactivation of the four IBR virus strains were compared at 37 C and 42 C. Experimental points

Figure 1. Histogram of plaque sizes of four IBR strains on the third day after inoculation

PLAQUE SIZE

DAY 3

GROUPS

A .5-.7mm
B .8-1.0mm
C 1.1-1.3mm
D 1.4-1.6mm

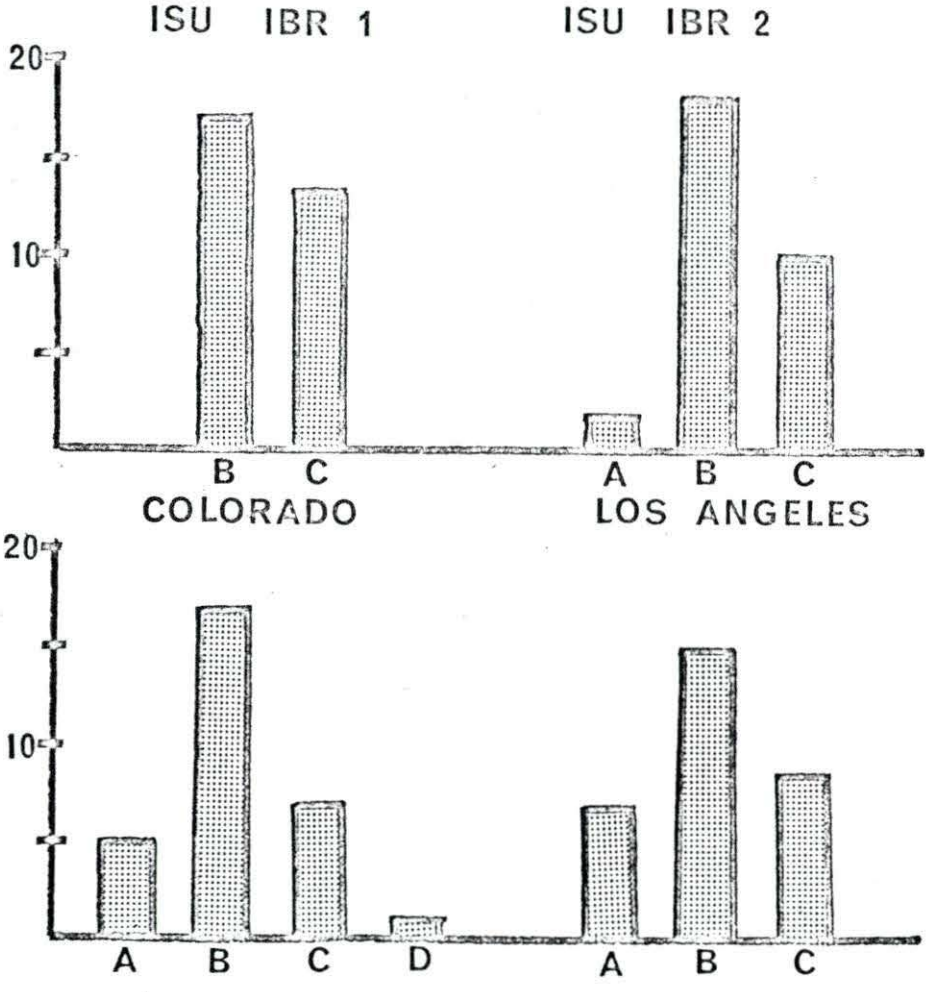


Figure 2. Histogram of plaque sizes of four IBR strains on the fourth day after inoculation

PLAQUE SIZE

DAY 4

GROUPS

A .5 - .7mm
B .8-1.0mm
C 1.1-1.3mm
D 1.4-1.6mm
E 1.7-1.9mm

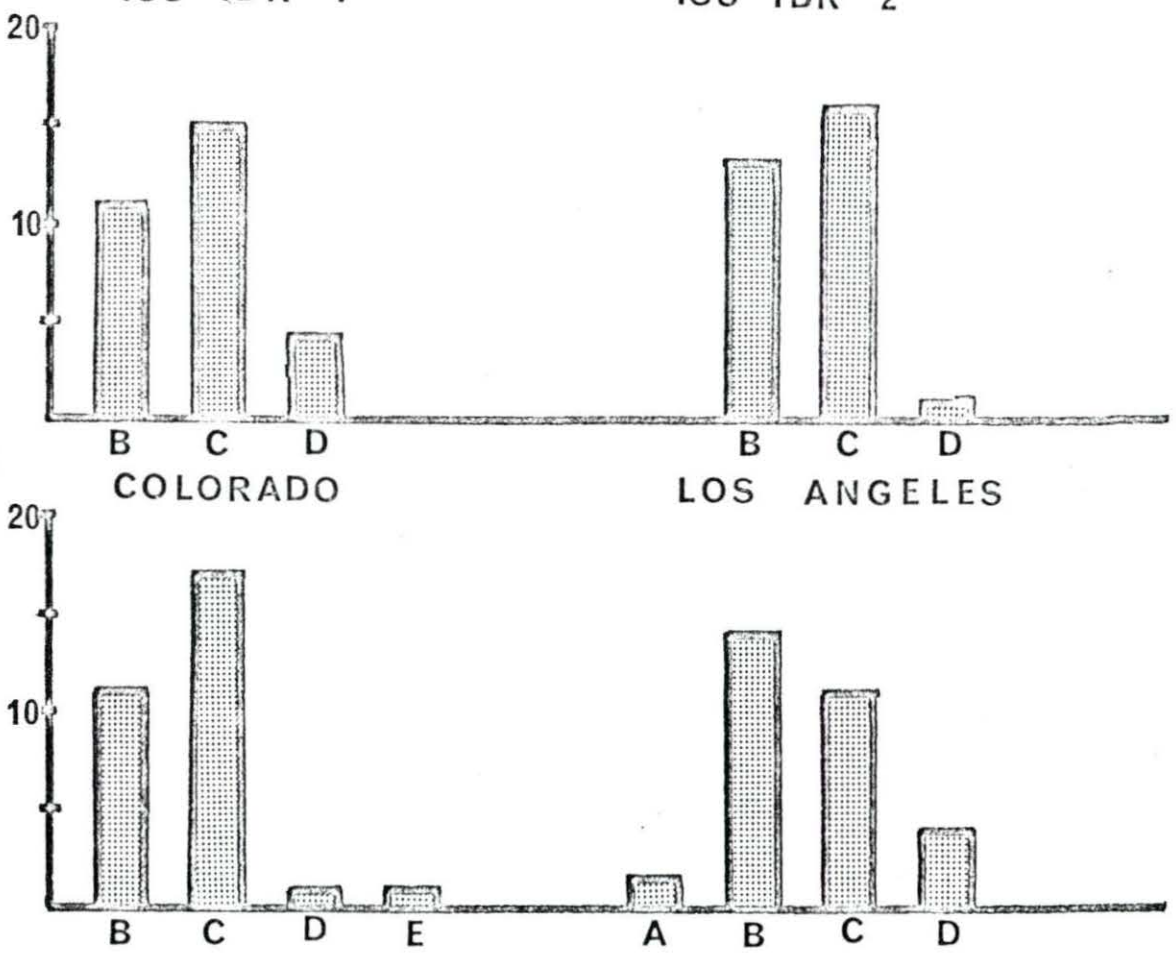


Figure 3. Histogram of plaque sizes of four IBR strains on the fifth day after inoculation

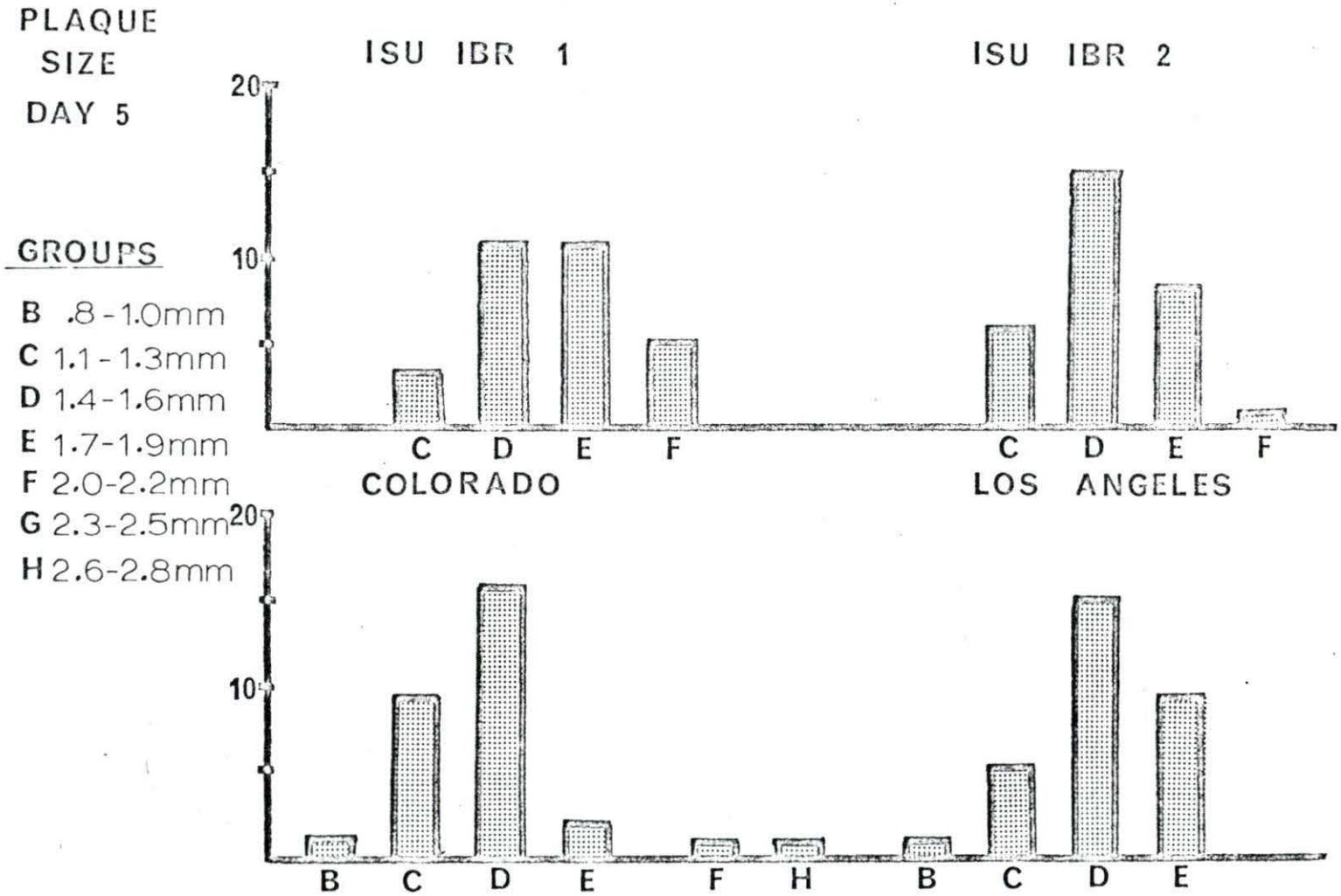

PLAQUE SIZE
DAY 5
GROUPS
B .8-1.0mm
C 1.1-1.3mm
D 1.4-1.6mm
E 1.7-1.9mm
F 2.0-2.2mm
G 2.3-2.5mm
H 2.6-2.8mm
ISU IBR 1
ISU IBR 2
20
10
C D E F
C D E F
COLORADO
LOS ANGELES
20
10
B C D E F H
B C D E

Figure 4. Histogram of plaque size of four IBR strains on the sixth day after inoculation

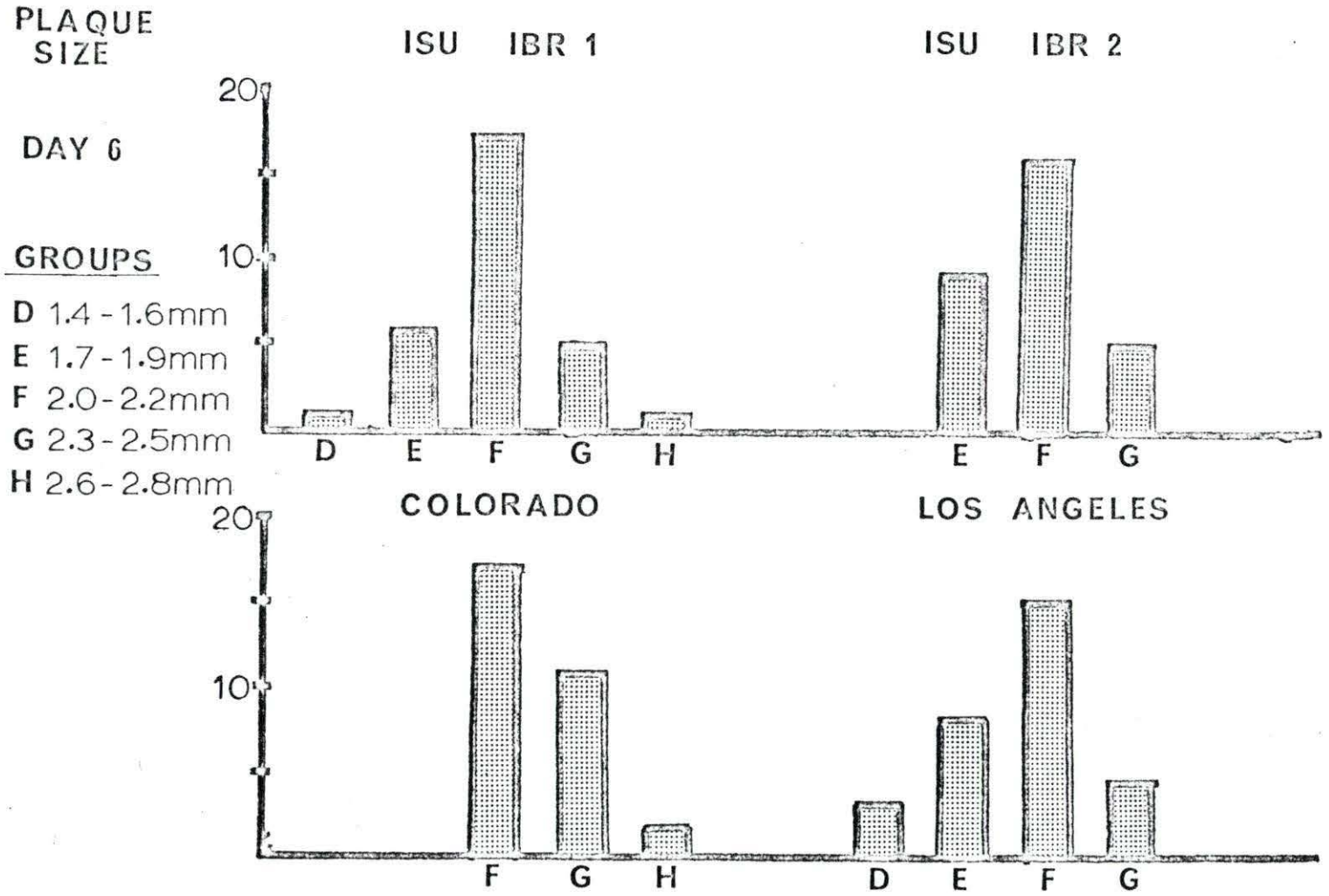

PLAQUE SIZE
DAY 6
GROUPS
D 1.4 - 1.6mm
E 1.7 - 1.9mm
F 2.0 - 2.2mm
G 2.3 - 2.5mm
H 2.6 - 2.8mm
ISU IBR 1
ISU IBR 2
COLORADO
LOS ANGELES
20
10
D E F G H
E F G
F G H
D E F G

Figure 5. Histogram of plaque size of four IBR strains the seventh day after inoculation

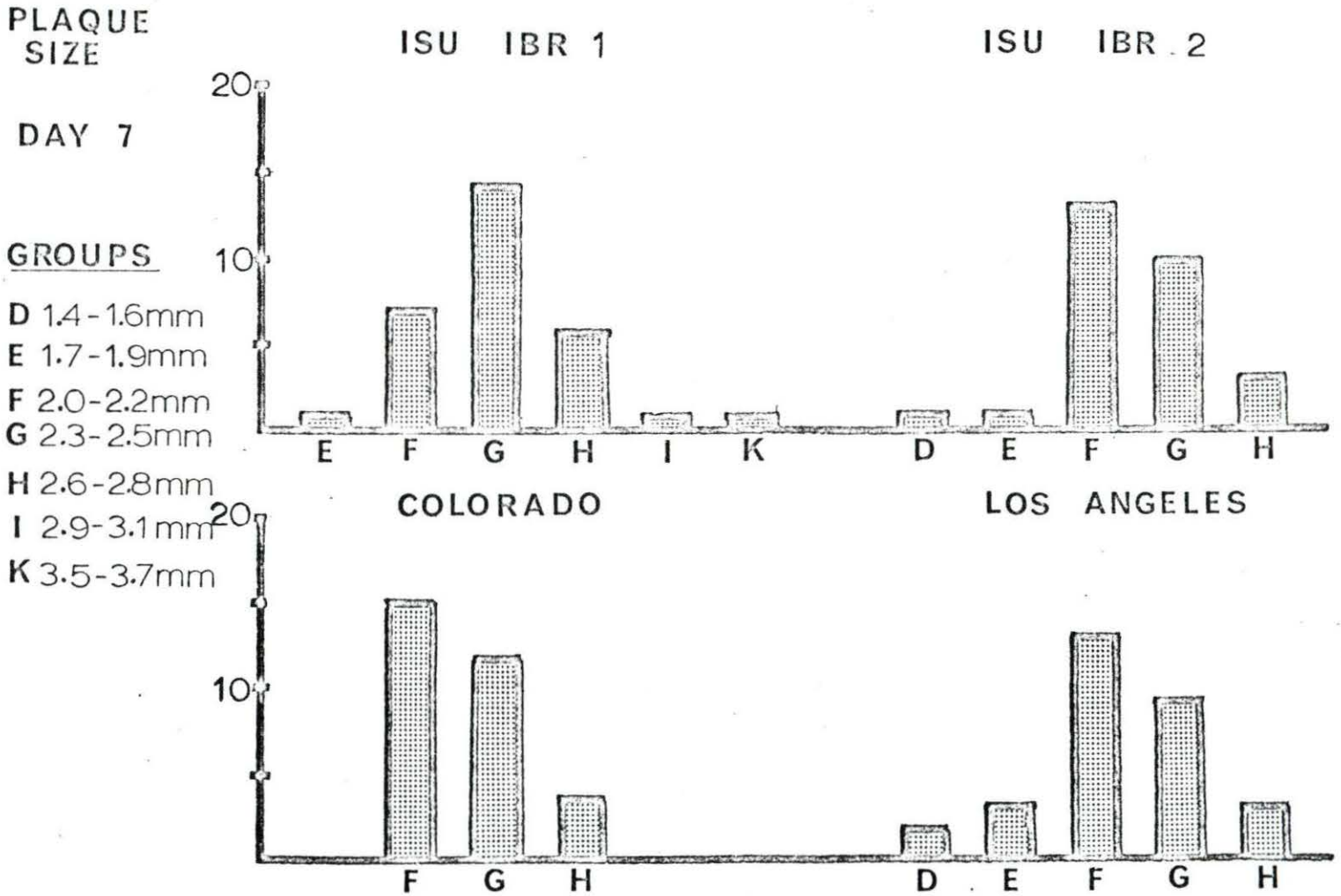

PLAQUE SIZE
DAY 7
GROUPS
D 1.4-1.6mm
E 1.7-1.9mm
F 2.0-2.2mm
G 2.3-2.5mm
H 2.6-2.8mm
I 2.9-3.1mm
K 3.5-3.7mm
ISU IBR 1
ISU IBR 2
COLORADO
LOS ANGELES
20
10
E F G H I K
D E F G H
F G H
D E F G H

Figure 6. Curve of mean plaque progression of the four strains of IBR

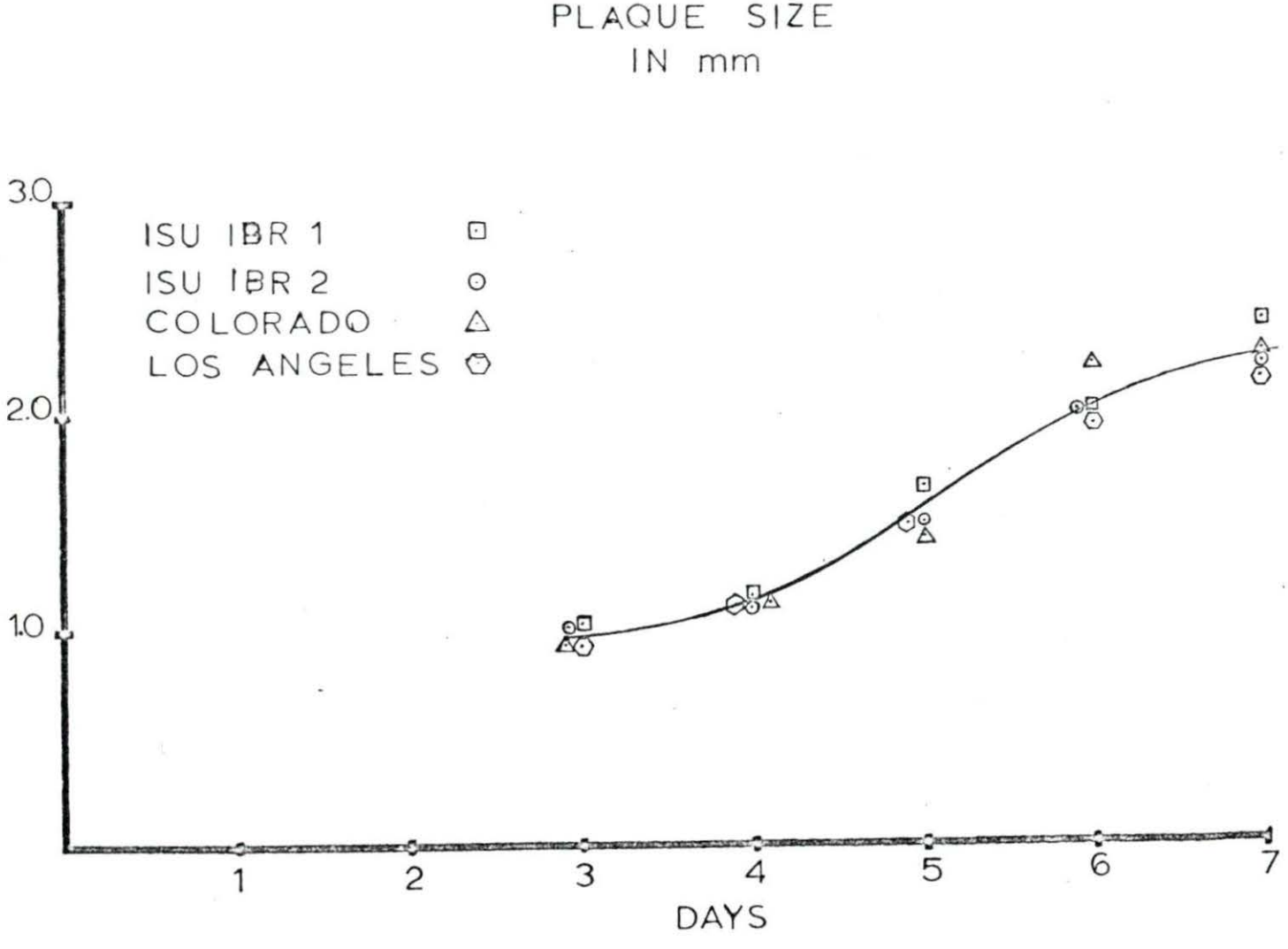

PLAQUE SIZE
IN mm
3.0
2.0
1.0
ISU IBR 1
ISU IBR 2
COLORADO
LOS ANGELES
1
2
3
4
5
6
7
DAYS

Figure 7. Plaque size of ISU-IBR 1 four days after inoculation

Figure 8. Plaque size of ISU-IBR 2 four days after inoculation

Figure 9. Plaque size of Colorado strain four days after inoculation

Figure 10. Plaque size of Los Angeles strain four days after inoculation

were determined by calculating the per cent of surviving infective virus at various time intervals. Since thermal inactivation has been shown to follow first order kinetics by plotting experimental points for surviving virus on a logarithmic scale versus time on a linear scale, these points should delineate a straight line.

Percentage of surviving virus at time intervals for each IBR strain at 37 C are presented in Table 6, and at 42 C in Table 7. Graphs constructed from this data are presented in Figures 11 and 12. Lines which best fitted the experimental points were drawn. In Figure 11 it will be observed that the ISU-IBR 2, Colorado and Los Angeles strains had similar inactivation rates. However, ISU-IBR 1 appeared to be more thermolabile at 37 C. In Figure 12 it can be observed that all of the IBR strains are inactivated at similar rates at 42 C. Therefore, only one line was fitted to the experimental points, since no individual strain was consistently low or high.

Single-Step Growth Curves

Samples which had been stored at -69 C were assayed using the plaque technique. In order to minimize variation, all of the monolayers used were derived from the same batch of trypsinized cells, and samples of all strains taken at a

Table 6. Percentage of surviving virus at various time intervals at 37 C

Hours	IBR strains			
	ISU-IBR 1 %	ISU-IBR 2 %	Colorado %	Los Angeles %
6	80.68	85.84	95.24	87.86
12	64.11	73.12	73.99	75.72
18	50.92	65.89	66.67	55.56
24	42.64	58.09	57.14	52.26

Table 7. Percentage of surviving virus at various time intervals at 42 C

Hours	IBR strains			
	ISU-IBR 1 %	ISU-IBR 2 %	Colorado %	Los Angeles %
3	75.00	55.78	91.78	63.24
6	37.50	34.63	48.89	39.71
9	25.00	27.89	21.07	28.68
12	15.63	14.97	20.25	20.59

Figure 11. Thermal inactivation of four IBR strains at 37 C

ISU-IBR 1 □

ISU-IBR 2 ○

Colorado △

Los Angeles ⬡

HEAT INACTIVATION AT 37 C

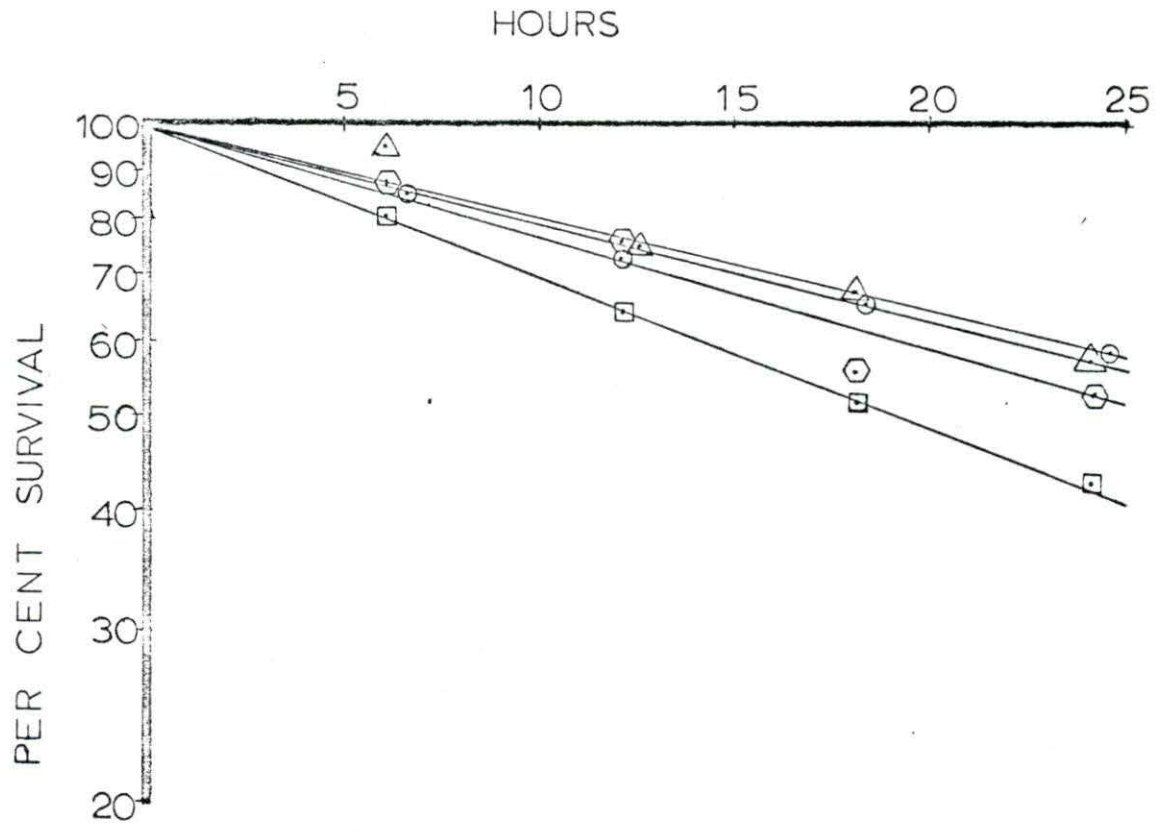


Figure 12. Thermal inactivation of four IBR strains at 42 C

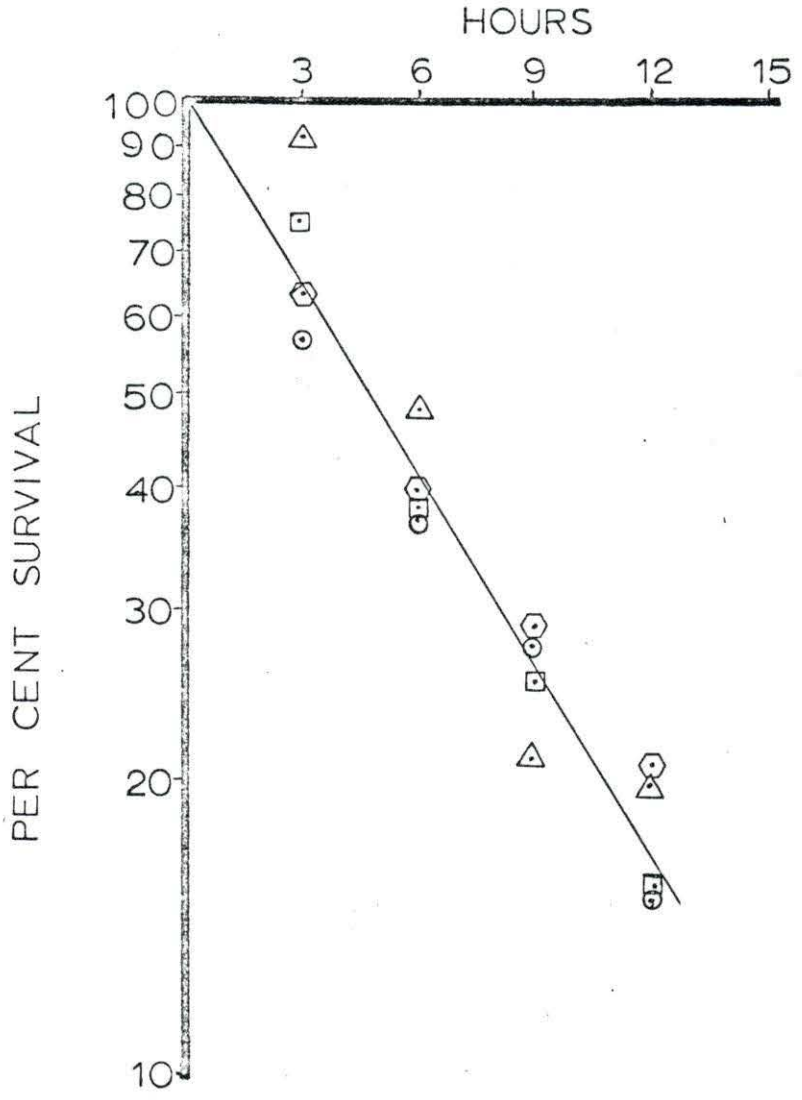
ISU-IBR 1 □

ISU-IBR 2 ○

Colorado △

Los Angeles ⬡

HEAT INACTIVATION AT 42 C



given hour were assayed simultaneously. Because of the large number of samples and dilutions to be assayed, a single monolayer per dilution was used. Results of the dilution which produced between 10 and 150 plaques were recorded and used in the calculations.

Data representing the rate of multiplication of ISU-IBR 1 virus during a 24 hour period is presented in Table 8. A graphical representation of this data is given in Figure 13. It can be concluded from Figure 13 that the eclipse phase of the growth cycle lasted approximately 7 hours. This was followed by a rapid increase of intracellular virus until a peak at 16 hours. Thereafter, the concentration of intracellular virus gradually decreased. At 20 hours the concentration of extracellular virus surpassed the intracellular concentration.

Data compiled for the one-step growth curve of ISU-IBR 2 strain over a 24 hour period is presented in Table 9. The graphical representation is given in Figure 14. In this case the eclipse phase terminated at approximately 7 hours. This was followed by a rapid increase in intracellular virus until a peak was reached at approximately 20 hours. Extracellular virus was detected at 8 hours with the peak occurring at 24 hours. Extracellular virus concentration did not equal intracellular concentration at that time.

Table 8. Results[a] obtained from a one-step growth study of ISU-IBR 1

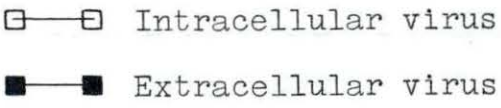
Hours	Intracellular virus	Extracellular virus
2	2.10×10^5	2.21×10^6 [b]
4	2.40×10^4	6.32×10^3
6	1.95×10^4	5.96×10^3
8	2.76×10^6	9.30×10^3
10	7.41×10^7	1.16×10^6
12	1.56×10^8	1.04×10^7
16	3.71×10^8	6.60×10^7
20	1.90×10^8	4.28×10^8
24	3.10×10^8	3.52×10^8

[a]Expressed as PFU per monolayer.

[b]Unabsorbed inoculum, which was approximately 16.8 per cent of the total.

Data for a one-step growth curve for Colorado IBR virus is presented in Table 10. Using this data, a graph was constructed which is presented in Figure 15. Intracellular viral multiplication was initiated at approximately 7 hours increasing rapidly until a peak was reached at 18 hours after which time it slowly decreased. Extracellular virus was released beginning at approximately 9 hours reaching a peak concentra-

Figure 13. Growth curve of ISU-IBR 1 virus over a period of 24 hours



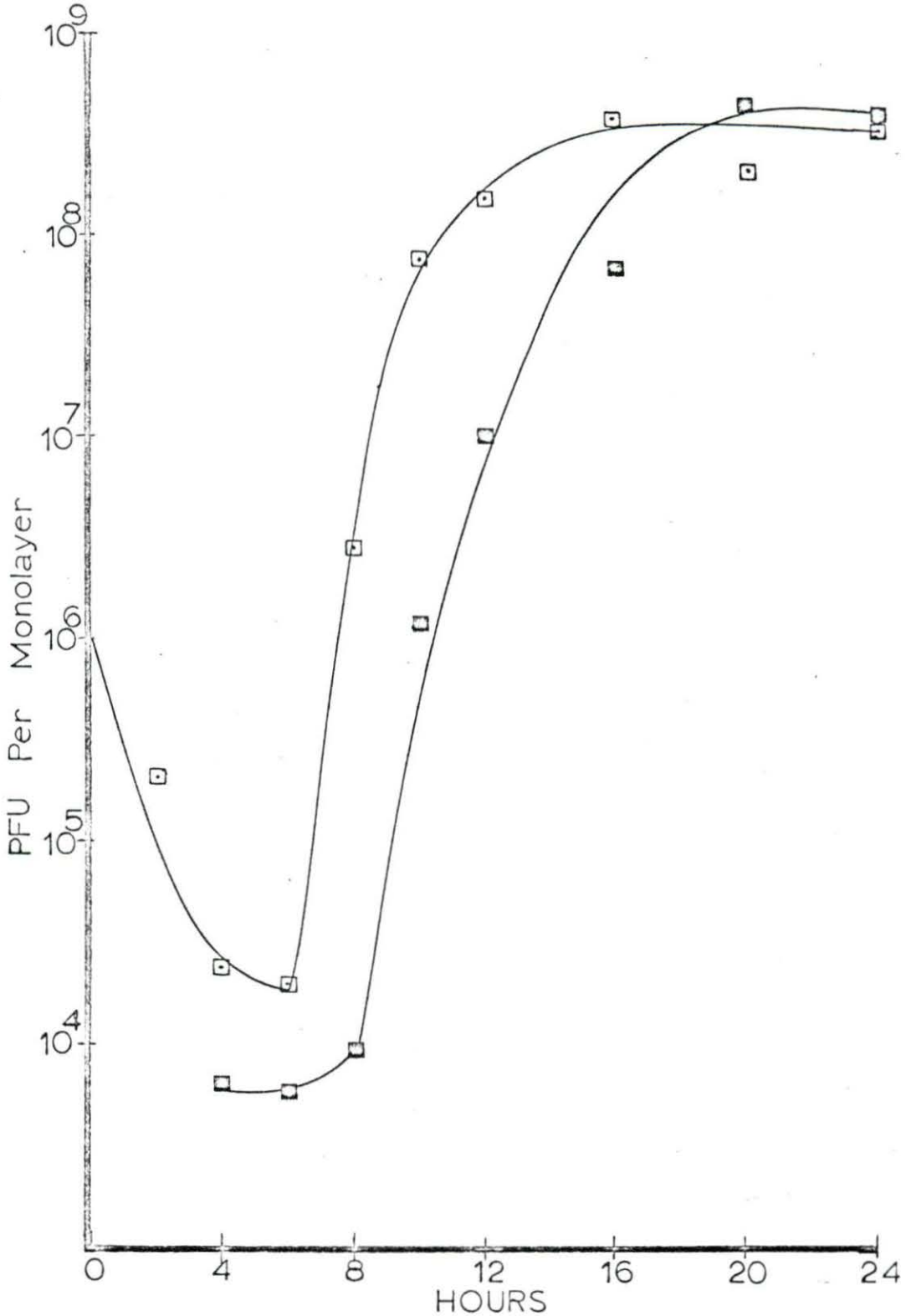

PFU Per Monolayer
10^9
10^8
10^7
10^6
10^5
10^4
0
4
8
12
16
20
24
HOURS

Table 9. Results[a] obtained from a one-step growth study of ISU-IBR 2

Hours	Intracellular virus	Extracellular virus
2	6.00×10^4	2.04×10^{6} [b]
4	2.10×10^4	4.18×10^3
6	2.10×10^4	2.96×10^3
8	6.90×10^6	6.40×10^5
10	6.58×10^7	2.80×10^5
12	2.53×10^8	1.60×10^6
16	7.82×10^8	3.76×10^7
20	9.47×10^8	2.04×10^8
24.5	9.00×10^8	4.36×10^8

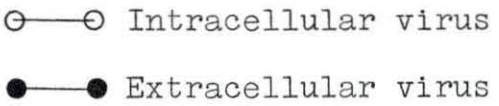
[a]Expressed as PFU per monolayer.

[b]Unabsorbed inoculum, which was approximately 9.5 per cent of the total.

tion at 24 hours. At this time the concentration of intracellular and extracellular virus were about equal.

Data for a one-step growth curve of the Los Angeles IBR strain is presented in Table 11 and graphically represented in Figure 16. From this graph it may be concluded that the eclipse phase is approximately 7 hours. This is followed by a rapid multiplication of intracellular virus which reaches a peak at 20 hours. At about 8 hours extracellular virus

Figure 14. Growth curve of ISU-IBR 2 virus over a period of 24 hours



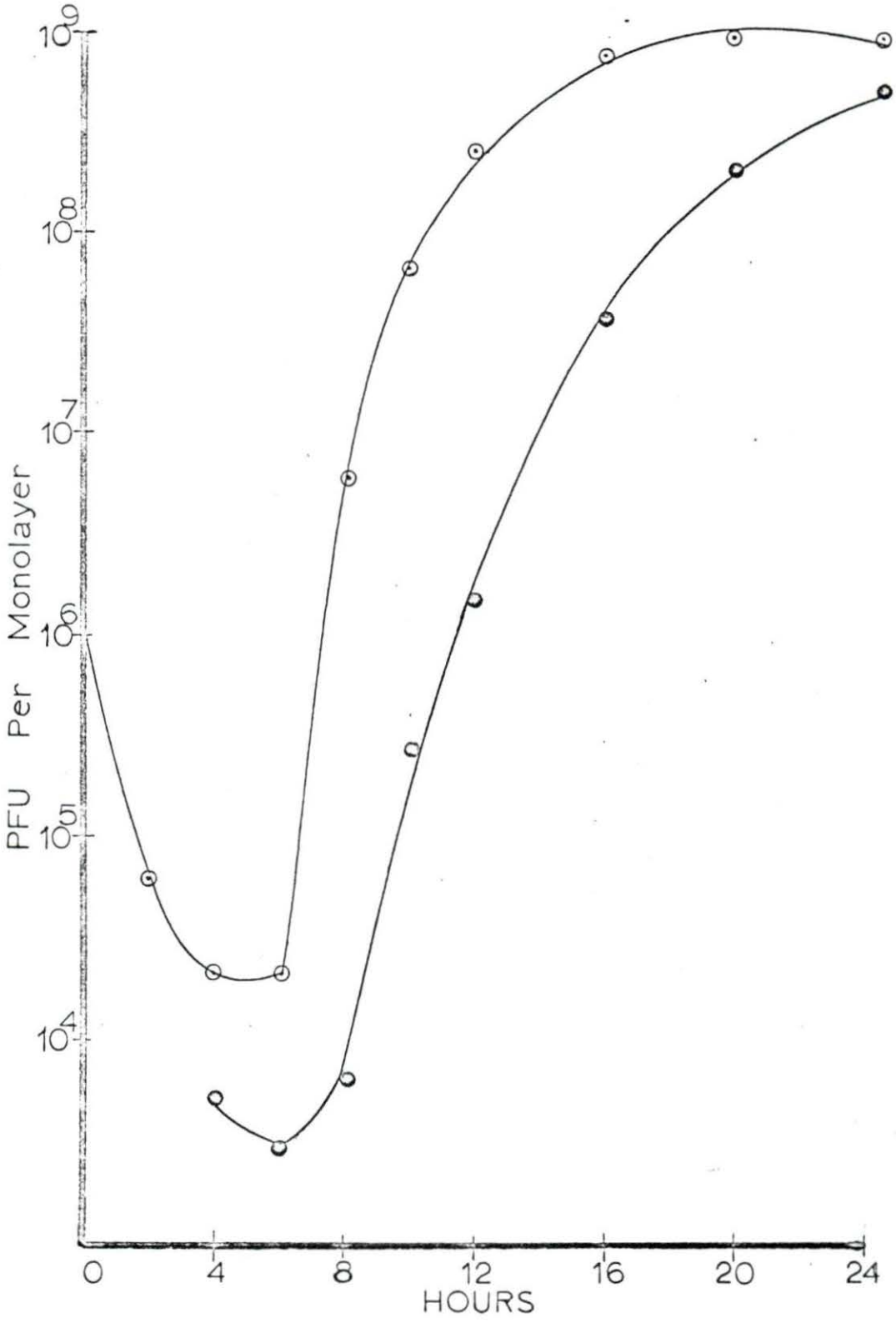

PFU Per Monolayer
10^9
10^8
10^7
10^6
10^5
10^4
0
4
8
12
16
20
24
HOURS

Figure 15. Growth curve of Colorado virus over a period of 24 hours

△——△ Intracellular virus

▲——▲ Extracellular virus

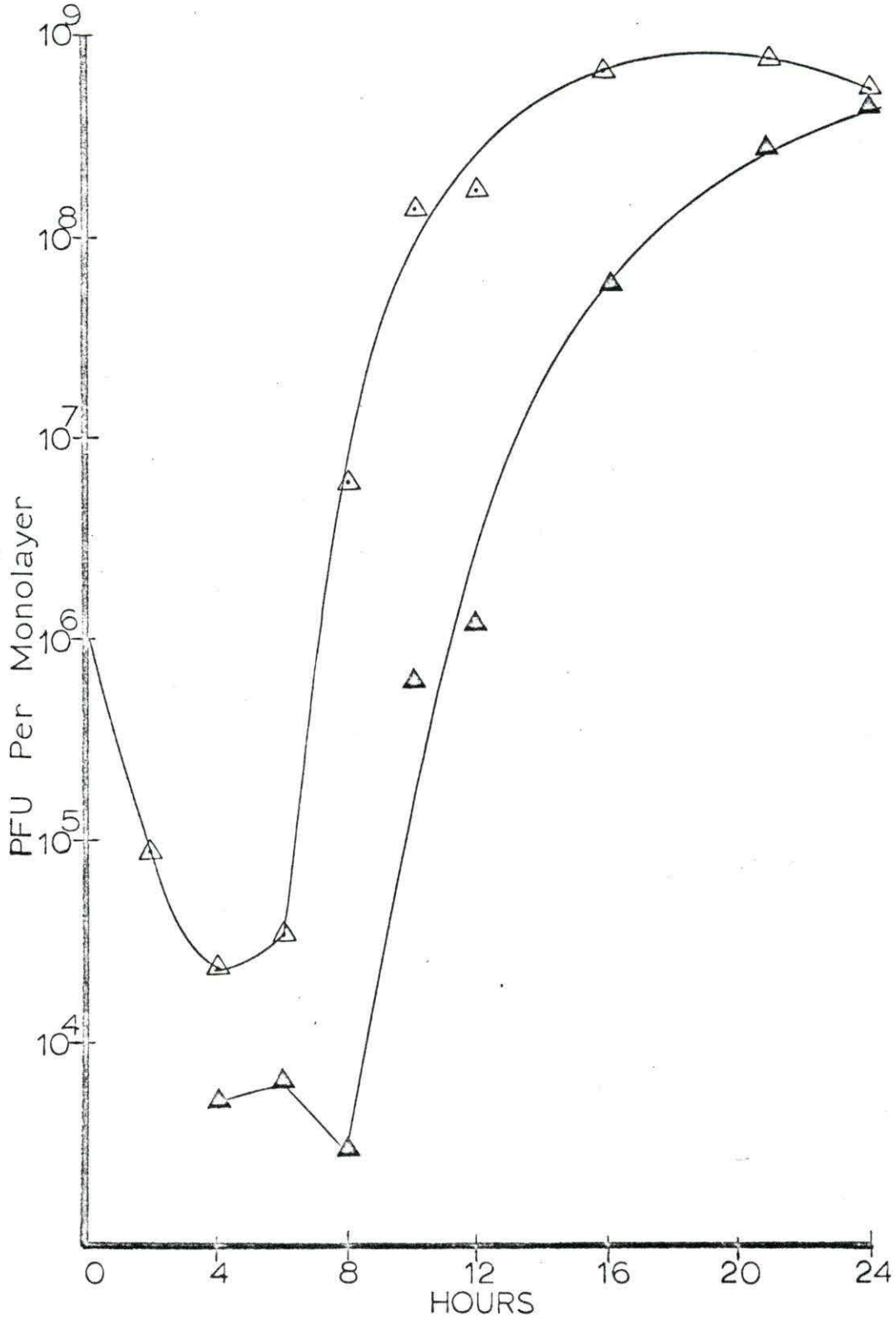

PFU Per Monolayer
10^9
10^8
10^7
10^6
10^5
10^4
0
4
8
12
16
20
24
HOURS

Table 10. Results[a] obtained from a one-step growth study of Colorado virus

Hours	Intracellular virus	Extracellular virus
2	9.00×10^4	3.74×10^6 [b]
4	2.40×10^4	5.32×10^3
6	3.51×10^4	6.56×10^3
8	6.00×10^6	2.80×10^3
10	1.44×10^8	6.00×10^5
12	1.61×10^8	1.20×10^6
16	6.77×10^8	5.68×10^7
21	7.44×10^8	2.88×10^8
24	5.20×10^8	4.68×10^8

[a]Expressed as PFU per monolayer.

[b]Unabsorbed inoculum, which was approximately 12.9 per cent of the total.

can initially be detected reaching a peak concentration at 24 hours after inoculation.

Comparisons of the growth curves of the four IBR strains reveals that the period between maturation and release for the ISU-IBR 1 was shorter than that noted for the other

Table 11. Results[a] obtained from a one-step growth study of Los Angeles virus

Hours	Intracellular virus	Extracellular virus
2	1.50×10^5	2.55×10^6 [b]
4	9.00×10^3	3.72×10^3
6	2.10×10^5	4.08×10^3
8	2.40×10^7	6.80×10^3
10	2.19×10^8	1.60×10^5
12	2.22×10^8	4.00×10^6
16	6.04×10^8	2.60×10^7
20	1.36×10^9	1.48×10^8
24	8.60×10^8	2.48×10^8

[a]Expressed as PFU per monolayer.

[b]Represents unabsorbed inoculum, which was approximately 12.6 per cent of the total.

three strains. By reference to Table 12 and Figure 17, it will be noted that the ISU-IBR 1 strain gave less total virus yield per cell than the other three strains.

Figure 16. Growth curve of Los Angeles virus over a period of 24 hours


Intracellular virus


Extracellular virus

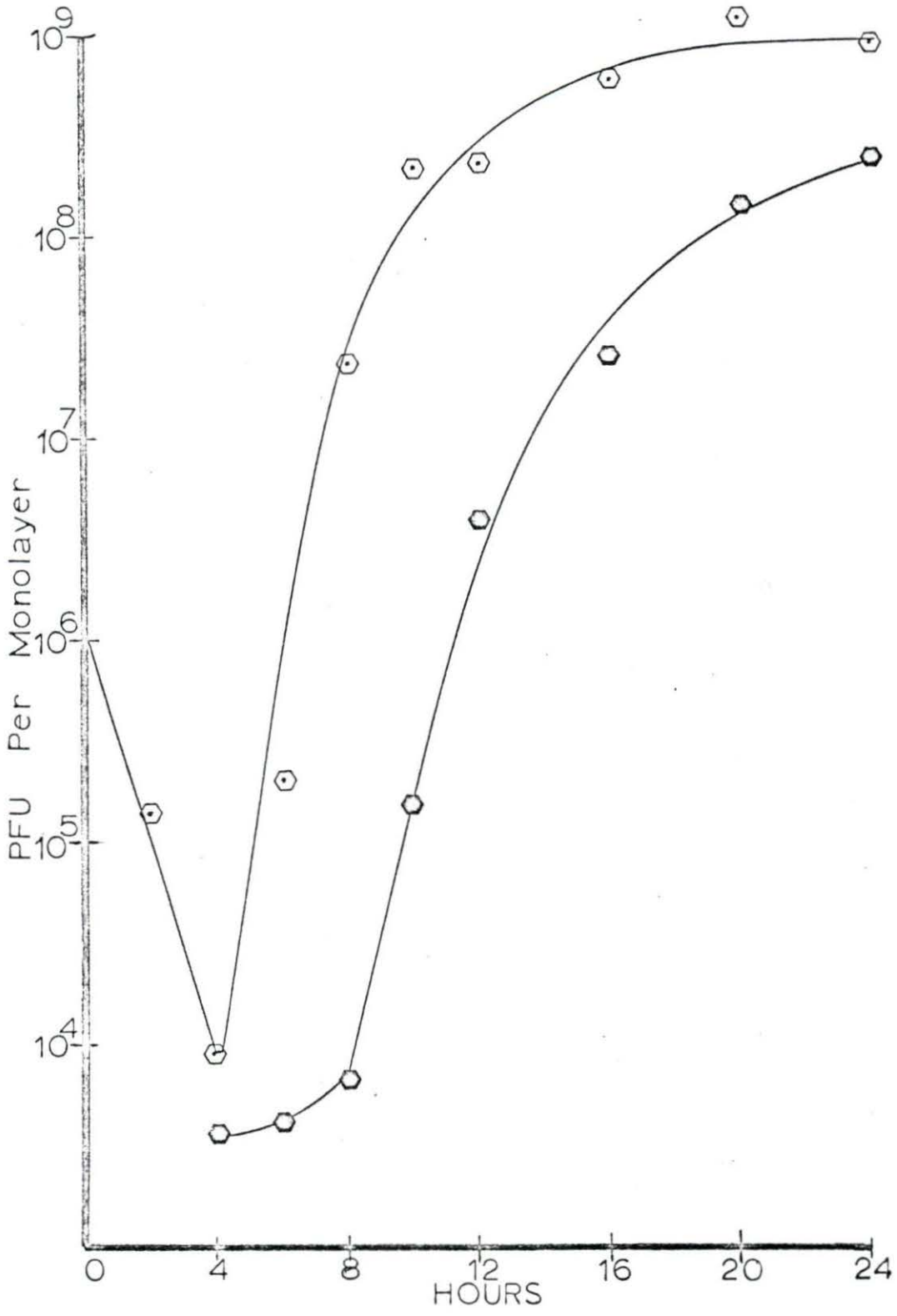

PFU Per Monolayer
10^9
10^8
10^7
10^6
10^5
10^4
0
4
8
12
16
20
24
HOURS

Table 12. Comparison of infectious virus produced per cell by the four IBR virus strains

Hours	IBR strains			
	ISU-IBR 1	ISU-IBR 2	Colorado	Los Angeles
2	.21[a]	.06	.09	.15
4	.03	.025	.029	.013
6	.026	.024	.042	.021
8	2.76	6.90	6.00	24.00
10	75.30	66.10	145.00	219.00
12	166.00	254.00	162.00	226.00
16	437.00	820.00	734.00	630.00
20	618.00	1150.00	1030.00[b]	1510.00
24	662.00	1340.00[c]	988.00	1110.00

[a]Numbers represent the total PFU per cell and were estimated by dividing total virus yield by the estimated number of total cells in a monolayer.

[b]This sample was taken at 21 hours post inoculation.

[c]This sample was taken at 24.5 hours post inoculation.

Neutralization Kinetics

The procedure used for antigenic analysis by neutralization kinetics has been described previously. The dilution of strain specific antiserum which neutralized 1.5 to 2.0

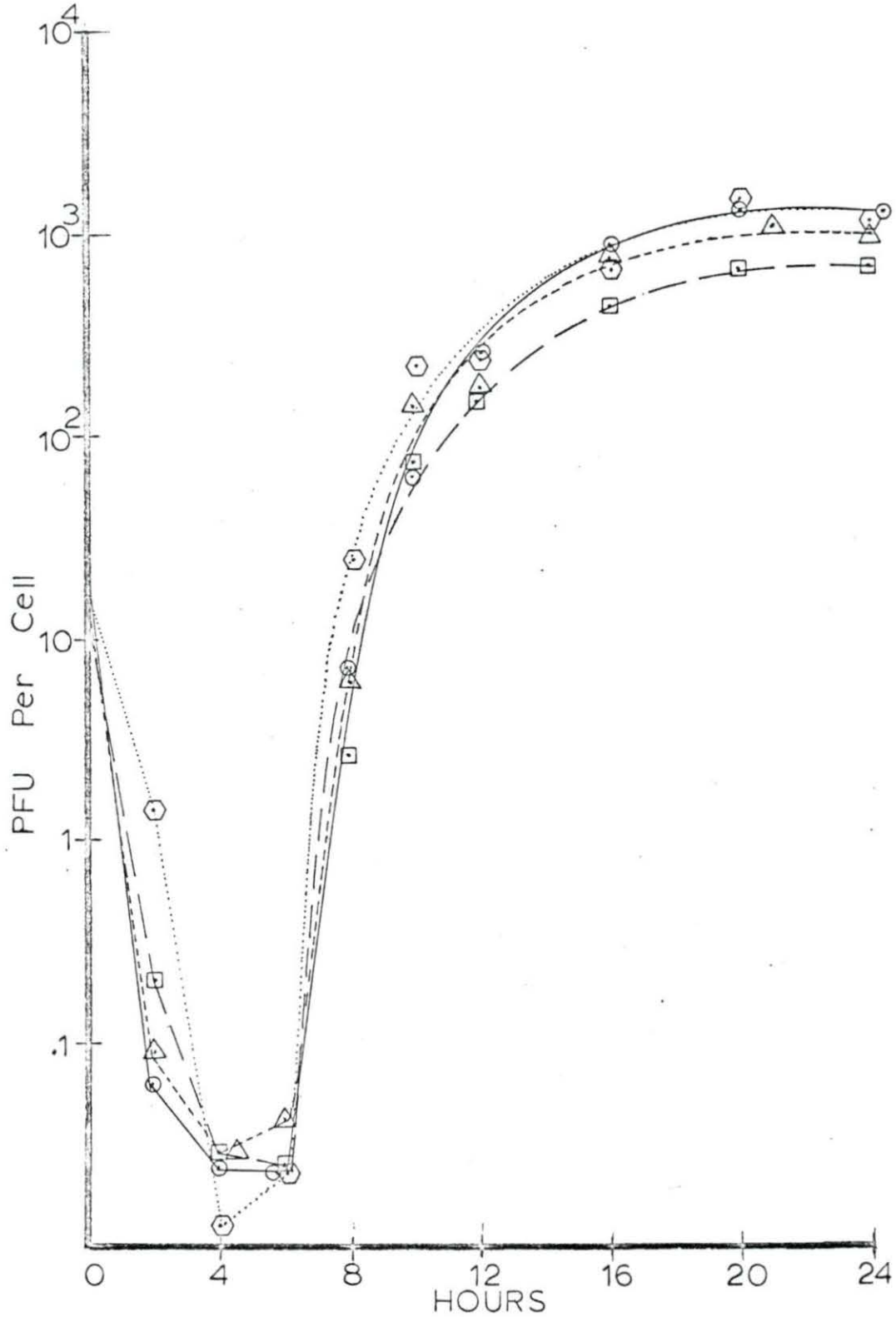
Figure 17. Comparison of infectious virus produced per cell by the four IBR strains over a 24 hour period

ISU-IBR 1 □

ISU-IBR 2 ○

Colorado △

Los Angeles ⬡


10^4
10^3
10^2
10
1
.1
PFU Per Cell
0
4
8
12
16
20
24
HOURS

logs of the homologous virus strain in 30 minutes was determined by preliminary experimentation. These results are recorded in Table 13.

Results derived from the experiments were transformed to $\log_{10} V_o/V_t$ values. The inclinations of the slopes were calculated using this data and are presented in Table 14. Estimated values for $\log_{10} V_o/V_t$ were calculated by using regression analysis. This data for strain specific antiserum is given in Tables 15 through 18.

In order to statistically compare the slopes of the homologous virus-antiserum system to heterologous systems the pooled standard deviation of the slopes had to be calculated. Results of such calculations for each antiserum are given in Table 19.

Graphs constructed from the regression analysis data for each strain specific antiserum are presented in Figures 18 through 21. The assumption is usually made that the homologous virus-antiserum system neutralizes faster than other systems. However, in Figure 18 it is apparent that both the ISU-IBR 2 and Colorado viruses were neutralized considerably faster than the homologous system. This seemed to be a reflection of the antigenic nature of the ISU-IBR 1 virus since antiserum prepared by hyperimmunizing another rabbit demonstrated a similar effect.

Table 13. Dilution of strain specific antiserum used in neutralization kinetic experiments

Antiserum	Dilution[a]
Anti-ISU-IBR 1	1:64
Anti-ISU-IBR 2	1:128
Anti-Colorado	1:260
Anti-Los Angeles	1:40

[a]Referred to as D.

Table 14. Calculated value of the slope[a] of the regression line for the various virus-antiserum systems

	IBR virus			
Antiserum	ISU-IBR 1	ISU-IBR 2	Colorado	Los Angeles
ISU-IBR 1	.0427	.04555	.07124	.05118
ISU-IBR 2	.0729	.08188	.09173	.08964
Colorado	.0691	.04787	.08499	.07849
Los Angeles	.0224	.06215	.06649	.09631

[a]The slope is equal to K/2.3D.

Table 15. Data from neutralization kinetic experiments and regression analysis for ISU-IBR 1 antiserum against four IBR strains

Minutes	IBR virus strains							
	ISU-IBR 1		ISU-IBR 2		Colorado		Los Angeles	
	Y^a	$\hat{Y}^b$	Y	$\hat{Y}$	Y	$\hat{Y}$	Y	$\hat{Y}$
5	.13490	.2344	.22475	.1789	.22621	.4188	.11919	.0322
10	.49894	.4679	.53018	.5434	.76447	.7643	.11919	.1442
15	.77728	.6814	.81531	.9079	1.26211	1.1098	.16749	.2562
20	.98726	.8949	1.32331	1.2724	1.70997	1.4553	.33723	.3682
30	1.22185	1.3219	2.01055	2.0014	1.93181	2.1463	.64975	.5922

[a]This term represents $\text{Log}_{10}\ V_o/V_t$.

[b]This term represents the estimated value of $\log_{10}\ V_oV_t$ calculated from regression analysis.

Table 16. Data from neutralization kinetics experiments and regression analysis for ISU-IBR 2 antiserum against four IBR strains

Minutes	IBR virus strains							
	ISU-IBR 1		ISU-IBR 2		Colorado		Los Angeles	
	Y^a	$\hat{Y}^b$	Y	$\hat{Y}$	Y	$\hat{Y}$	Y	$\hat{Y}$
5	.62120	.55266	.32331	.25821	.20512	.17611	.17653	.11111
10	.81816	.78041	.59688	.66761	.33913	.41546	.40561	.42186
15	.75945	1.00816	1.04144	1.07701	.69897	.65481	.66354	.73261
20	1.36151	1.23591	1.51856	1.48641	.90319	.89416	1.00000	1.04336
30	1.70774	1.69141	2.30521	2.30521	1.36351	1.37286	1.71670	1.66486

[a]This term represents $\log_{10} V_o/V_t$.

[b]This term represents the estimated value of $\log_{10} V_o/V_t$ calculated from regression analysis.

Table 17. Data from neutralization kinetics experiments and regression analysis for Colorado antiserum against four IBR strains

Minutes	IBR virus strains							
	ISU-IBR 1		ISU-IBR 2		Colorado		Los Angeles	
	Y^a	$\hat{Y}^b$	Y	$\hat{Y}$	Y	$\hat{Y}$	Y	$\hat{Y}$
5	.14691	.13457	.40450	.39360	.27572	.34415	.22841	.18769
10	.55440	.49077	.89620	.35225	.74473	.76960	.44855	.52014
15	.67161	.84697	1.31336	1.31090	1.20412	1.19405	.84164	.85259
20	1.30803	1.20317	1.66154	1.76955	1.82391	1.61900	1.23518	1.18504
30	1.91009	1.91557	2.73755	2.68685	2.34679	2.73755	1.84164	1.84994

[a]This term represents $\log_{10} V_o V_t$.

[b]This term represents the estimated value of $\log_{10} V_o V_t$ calculated from regression analysis.

Table 18. Data from neutralization kinetics experiments and regression analysis for Los Angeles antiserum against four IBR strains

Minutes	IBR virus strains							
	ISU-IBR 1		ISU-IBR 2		Colorado		Los Angeles	
	Y^a	$\hat{Y}^b$	Y	$\hat{Y}$	Y	$\hat{Y}$	Y	$\hat{Y}$
5	.35262	.43962	.27737	.26439	.35262	.38168	.27084	.41932
10	.77728	.69552	.74473	.71259	.74958	.77393	1.03621	.90087
15	.99140	.95142	1.03621	1.16079	1.23807	1.16658	1.49485	1.38242
20	1.20135	1.20732	1.69897	1.60899	1.57349	1.55903	1.79588	1.86397
30	1.69037	1.71912	2.49485	2.50539	2.31158	2.34393	2.79588	2.82707

[a]This term represents $\log_{10} V_o/V_t$.

[b]This term represents the estimated value of $\log_{10} V_o/V_t$ calculated from regression analysis.

Table 19. Pooled standard deviation of slopes compared to homologous virus-antiserum systems

Heterologous system		
Virus	Antiserum	Standard deviation[a]
ISU-IBR 2	ISU-UBR 1	.00485
Colorado	ISU-IBR 1	.00971
Los Angeles	ISU-IBR 1	.00516
ISU-IBR 1	ISU-IBR 2	.00725
Colorado	ISU-IBR 2	.00304
Los Angeles	ISU-IBR 2	.00340
ISU-IBR 1	Colorado	.00700
ISU-IBR 2	Colorado	.00588
Los Angeles	Colorado	.00578
ISU-IBR 1	Los Angeles	.00584
ISU-IBR 2	Los Angeles	.00616
Colorado	Los Angeles	.00549

[a]Pooled value of homologous and heterologous systems compared, and used in calculating t values for significant tests.

Figure 18. Neutralization kinetic slopes for ISU-IBR 1 antiserum

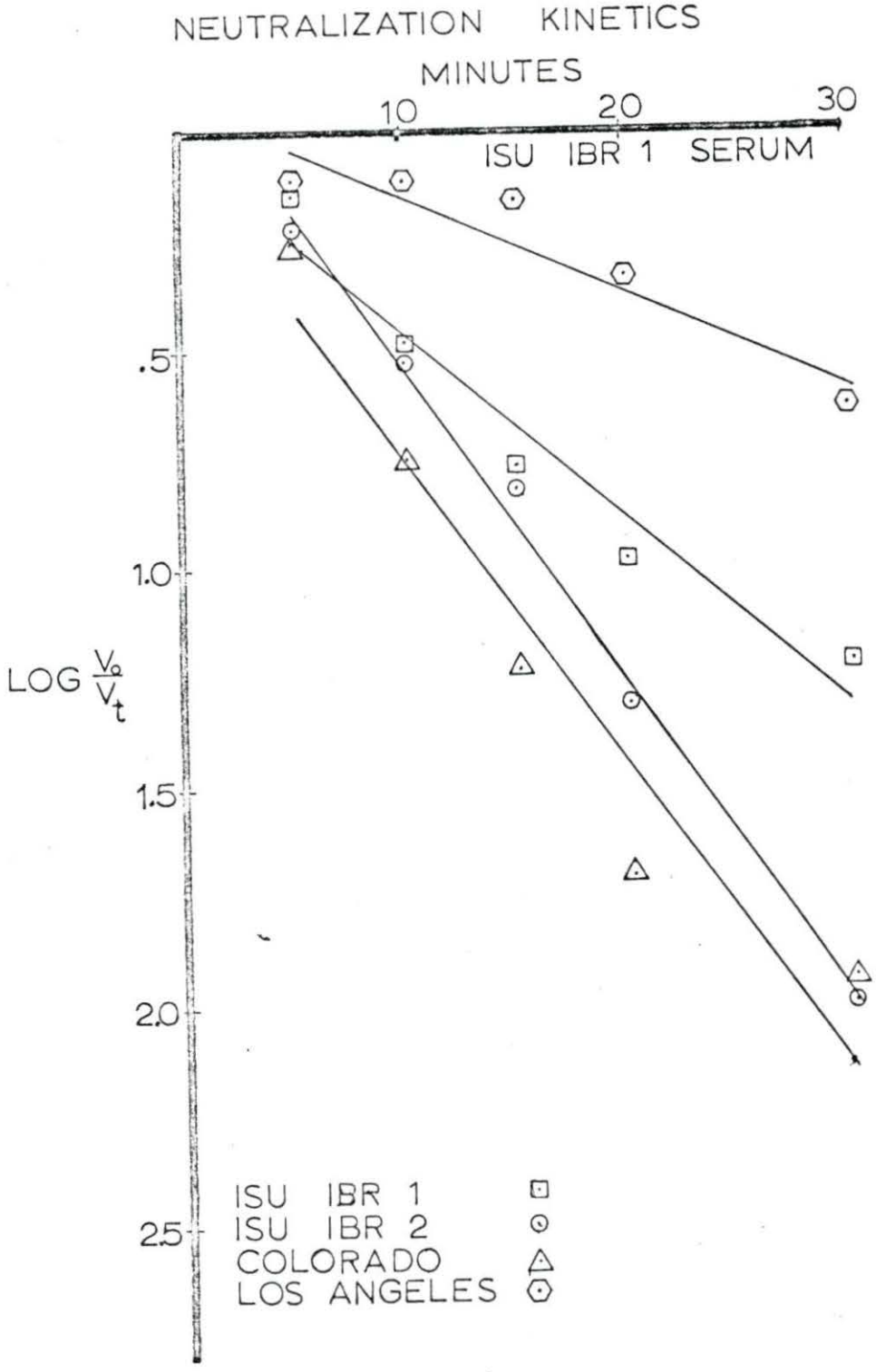

NEUTRALIZATION KINETICS
MINUTES
10
20
30
ISU IBR 1 SERUM
.5
1.0
1.5
2.0
2.5
LOG $\frac{V_o}{V_t}$
ISU IBR 1
ISU IBR 2
COLORADO
LOS ANGELES

Figure 19. Neutralization kinetics slopes for ISU-IBR 2 antiserum

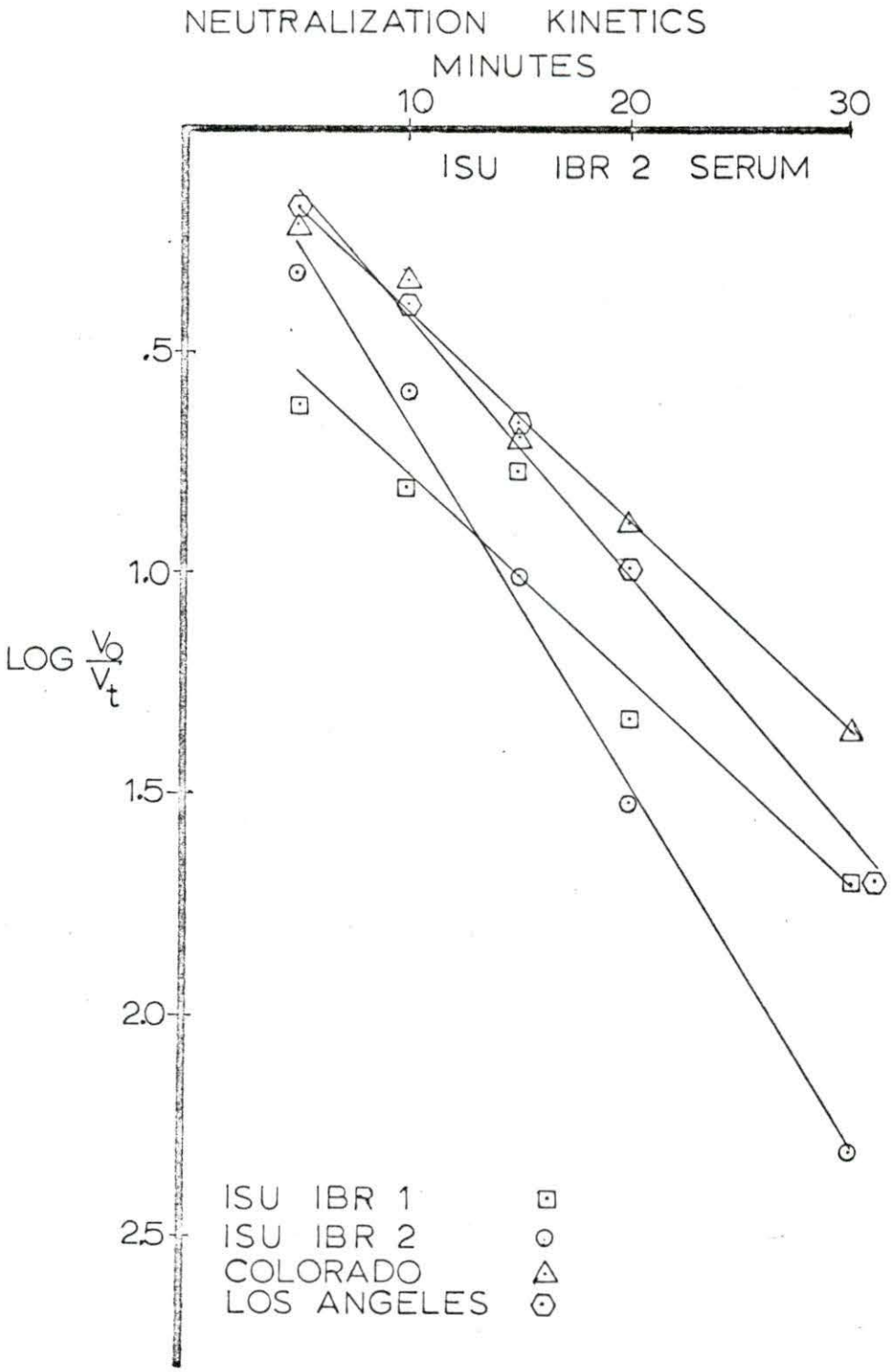

NEUTRALIZATION KINETICS
MINUTES
10
20
30
ISU IBR 2 SERUM
.5
1.0
1.5
2.0
2.5
LOG $\frac{V_0}{V_t}$
ISU IBR 1
ISU IBR 2
COLORADO
LOS ANGELES

Figure 20. Neutralization kinetic slopes for Colorado antiserum

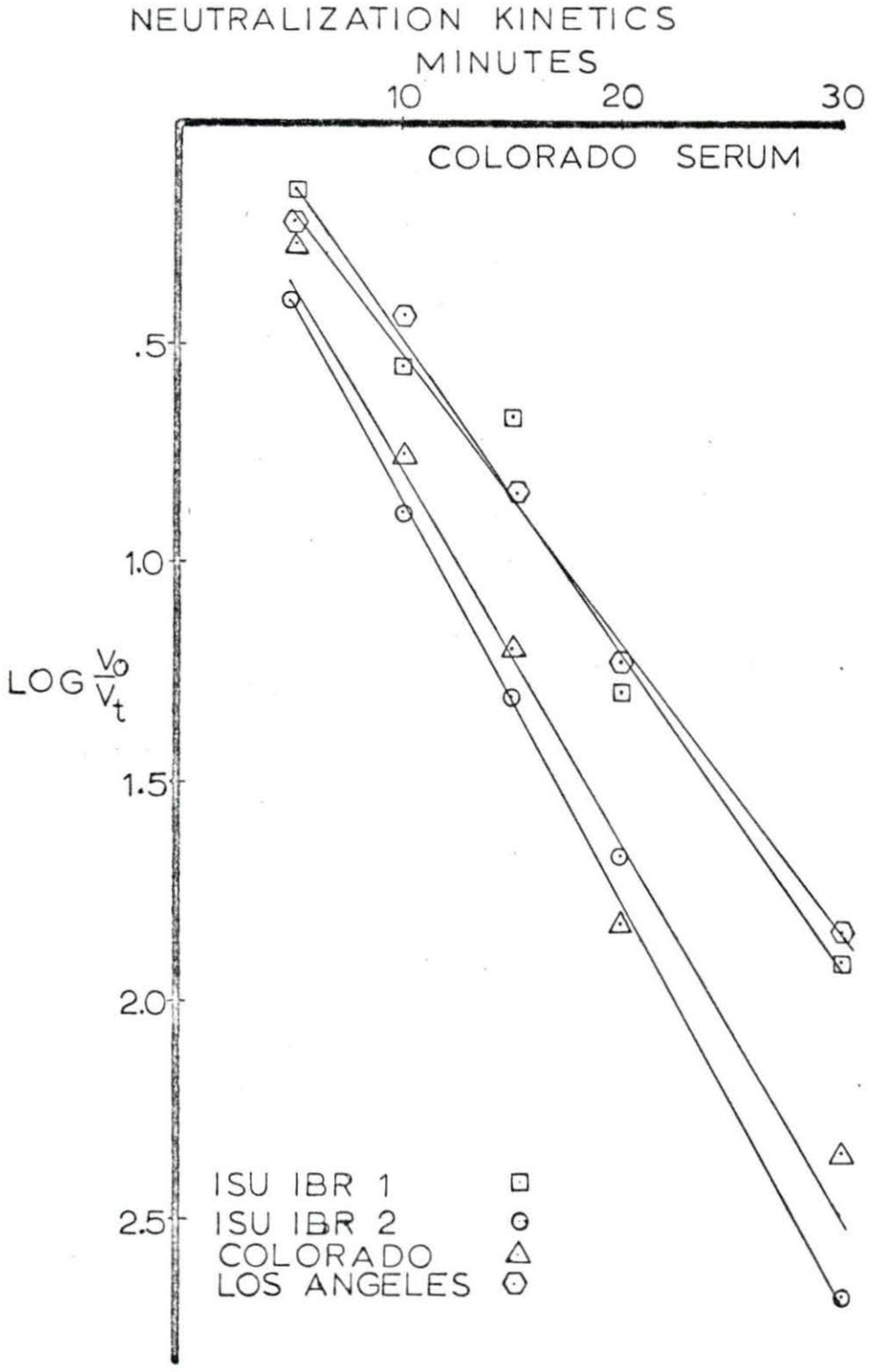
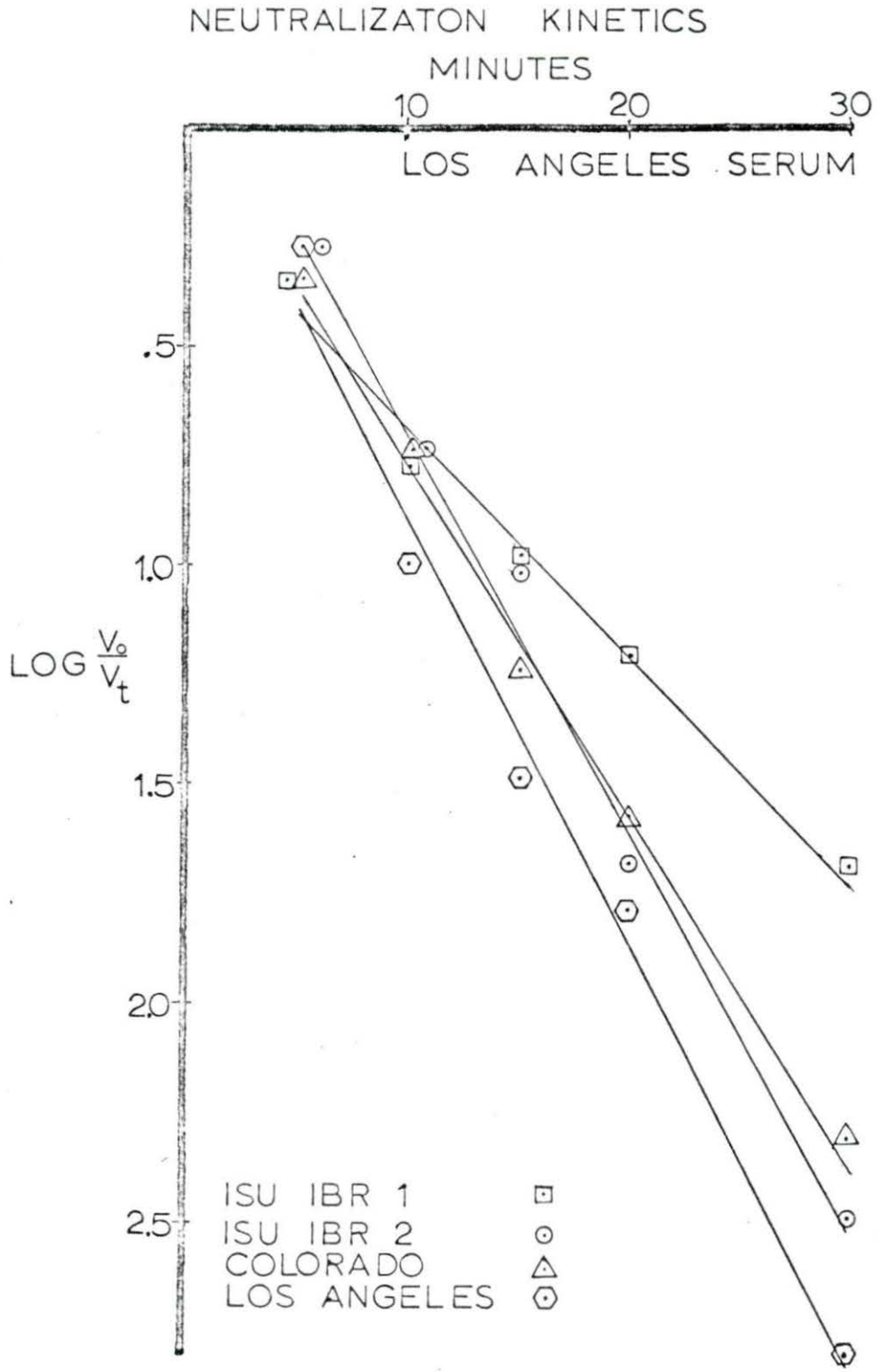

NEUTRALIZATION KINETICS
MINUTES
10
20
30
COLORADO SERUM
.5
1.0
1.5
2.0
2.5
LOG Vo/Vt
ISU IBR 1
ISU IBR 2
COLORADO
LOS ANGELES

Figure 21. Neutralization kinetic slopes for Los Angeles antiserum


NEUTRALIZATON KINETICS
MINUTES
10
20
30
LOS ANGELES SERUM
.5
1.0
1.5
2.0
2.5
LOG $\frac{V_o}{V_t}$
ISU IBR 1
ISU IBR 2
COLORADO
LOS ANGELES

Statistical comparisons of the reciprocal neutralization slopes are summarized in Figure 22. It will be noted that a non-reciprocal relationship existed between the ISU-IBR 2 and Colorado IBR strain and between ISU-IBR 2 and the Los Angeles IBR strain.

A comparison of reciprocal neutralization rates by calculating the K and NK values is summarized in Figure 23. This method of analysis correlated very closely with the other procedure. This method has the added advantage of being able to compare various antiserums. The NK values for ISU-IBR 2 and the Colorado strain were very similar except in the reciprocal cross of the Colorado strain with ISU-IBR 2 antiserum. This would tend to indicate a close antigenic relationship between these strains. There is also a non-reciprocal relationship shown between the Los Angeles strain and the ISU-IBR 2 strain. This would indicate an overlapping of minor antigenic components by these three strains.

The ISU-IBR 1 strain appeared to express more antigenic individuality than the other three strains.

Figure 22. Comparison of reciprocal neutralization slopes

COMPARISON OF RECIPROCAL NEUTRALIZATION SLOPES

NEUTRALIZATION SLOPES	SERUM			
	ANTI ISU IBR 1	ANTI ISU IBR 2	ANTI COLORADO	ANTI LOS ANGELES
VIRUS	t - value	t - value	t - value	t - value
ISU IBR 1	--------	5.011 *	1.972 *	7.728 *
ISU IBR 2	6.227 *	--------	1.136	1.083
COLORADO	2.719 *	11.188 *	----------	3.246 *
LOS ANGELES	3.934 *	5.803 *	3.200 *	--------

* = SIGNIFICANTLY DIFFERENT SLOPE AT THE 5% LEVEL

Figure 23. Comparison of reciprocal neutralization rates

COMPARISON OF RECIPROCAL NEUTRALIZATION RATES

VIRUS	SERUM							
	ANTI ISU IBR 1		ANTI ISU IBR 2		ANTI COLORADO		ANTI LOS ANGELES	
	K	NK	K	NK	K	NK	K	NK
ISU IBR 1	6.6	100	16.6	73.5	38.4	77.6	5.3	60.7
ISU IBR 2	10.0	152	22.6	100	54.0	109	7.7	88.5
COLORADO	10.7	162	13.5	60	49.5	100	7.2	82.7
LOS ANGELES	3.0	45.5	16.3	72	37.1	75	8.7	100

$$K = \frac{D}{t} 2.3 \text{ LOG } \frac{V_o}{V_t}$$

DISCUSSION

Since plaque size has been observed to be a relatively constant characteristic of a plaque purified strain under defined conditions, it was used as a parameter of virus characterization in this study. It had been previously demonstrated that plaque size variation is often correlated with the virulence of a strain of virus. Clones derived from small plaques of a strain are frequently less virulent than those derived from large plaques (42).

In 1963 Rouhandeh and Werder (43) reported plaque formation of IBR virus in primary bovine embryonic kidney cells. They observed plaques 5-7 mm in diameter after 7 days incubation. Sabina and Parker (46) reported that plaque sizes of the Colorado strain of IBR varied with the nutrients incorporated in the overlay medium. They reported observing plaques 1.0 -2.0 mm in diameter in 3 days on monolayers of a bovine kidney cell line. Two other strains of IBR gave similar results.

McKercher (35) compared plaques produced by IBR and IPV virus. He observed both small and large plaques in the IBR and IPV strains. An IBR vaccine strain produced only large plaques. Plaques of different sizes were cloned and when passed retained the size characteristic. However, both the small and large plaques were neutralized equally well by

homologous and heterologous antiserum. Plaque sizes of 5-7 mm in 7 to 9 days on primary bovine kidney cell monolayers were reported.

In this study plaque sizes of the strains studied were very similar. The size varied from approximately 1.0 mm at day 3 to 2.4 mm on day 7. Occurrence of two plaque sizes was not observed prior to or after plaque selection. The marked differences in plaque size with strains used in this study compared with those reported by Rouhandeh and Werder (43) and McKercher (35) most probably reflect differences in the assay system. Results of this study are consistent with those reported by Sabina and Parker (46) who observed a single plaque size produced by three strains of IBR.

The variability of plaque sizes obtained in this study as determined by the coefficient of variation tended to decrease as incubation progressed. A range in coefficient variation from 8.8 per cent to 20.8 per cent was noted for all the data, and these values compare favorably with the accepted values for other biological systems.

Thermal stability characteristics of the four strains were studied primarily to more accurately interpret the results obtained from 24 hour growth studies. Variations in thermal stability of virus strains may reflect differences in the protein configuration of the viral capsid and may be related to other virus-cell interactions.

Thermal stability has been used as a marker for strains of poliovirus by Wallis et al. (56). They reported that attenuated type 1 poliovirus could be differentiated from virulent strains by the rate of inactivation at 50 C in the presence of 12.5 mM $AlCl_3$. Virulent strains demonstrated an increased thermolability compared to vaccine strains.

Concerning the thermal stability of IBR virus at 37 C, Griffin et al. (16) reported a half-life of approximately 22 hours. Sabina and Parker (46) indicated that the half-life of the strain which they studied was 16 hours. Stevens and Groman (50) reported a half-life of 10 hours for an IBR strain. A half-life of approximately 8 hours was reported for a strain by Bläschencusschlages (Coital Vesicular Exanthema) by Hahnefeld et al. (18).

Sabina and Parker (46), Stevens and Groman (50), and Hahnefeld (18) indicated that thermal inactivation at 37 C was a first order reaction during the inactivation period.

Strains ISU-IBR 2, Colorado, and Los Angeles used in this study had similar inactivation rates (Figure 11) with a half-life calculated to be approximately 30 hours. Strain ISU-IBR 1 had a half life of approximately 20 hours.

The differences in inactivation rates for the IBR strains reported in the literature may represent true differences of thermal stability or they may represent differences in the test system. For example, Hahnefeld et al. (18) used a

diluent free of serum, whereas other researchers incorporated serum in the diluent. The protective effect of colloids on thermal stability of viruses has been well established.

In 1963 Sabina and Parker (46) reported that the inactivation of an IBR strain at 42 C also followed first order kinetics over the period studied with a half-life of approximately 3.5 hours. When the four IBR strains used in this study were heated at 42 C, all appeared to have similar inactivation rates with a half-life of approximately 4.5 hours (Figure 12).

Results of the present study on the thermal inactivation rates of the four IBR strains at 37 C indicate that the ISU-IBR 1 strain is slightly more thermal labile than the other strains; however, at 42 C all of the strains had similar inactivation rates. Although only the data from one experiment was reported, preliminary experiments using fewer replicates indicated similar trends.

The trend toward increased thermolability of the ISU-IBR 1 strain at 37 C suggested the presence of minor configurational differences in the capsid protein. These slight differences appeared insufficient to affect growth curve results obtained over a 24 hour period.

Since the rate of virus replication and release may be related to virulence, single-step growth curves of each respective virus strain were compared.

One-step growth curves for IBR virus in an established bovine kidney cell line were reported by Sabina and Parker (46) and Stevens and Groman (50). Sabina and Parker (46) used multiplicities of .1 PFU per cell and 1.0 PFU per cell. In this system a latent period of 6 hours was followed by a logarithmic increase of total virus until the twelfth to fourteenth hour. A yield of 30 PFU per cell for the lower multiplicity and 210 PFU per cell for the higher multiplicity was reported.

Stevens and Groman (50) used a high multiplicity of IBR virus and determined that an average of 6 PFU per cell was absorbed. They reported a lag phase of 4.5 hours, followed by intracellular virus increase for the next 7 hours which remained stationary for the next 10 hours. Extracellular virus was detected at 7 hours and reached a peak at 18 hours. Virus yields per cell varied between 115 to 270 PFU. In later experiments they reported (49) obtaining yields of 500 PFU per cell with maximum virus yields at 20 hours after infection.

Graphical representations of one step growth curves obtained for the four IBR strains used in this study are presented in Figures 13 through 16. Duration of the eclipse phase for the ISU-IBR 2, Colorado, Los Angeles strains was approximately 7 hours. Rapid multiplication of intracellular

virus followed until a peak was reached at 18 to 20 hours. Extracellular virus was detected at 8 hours and peaked at 24 hours.

One-step growth for the ISU-IBR 1 strain appeared to deviate from the others in that intracellular virus replication reached a peak sooner, and extracellular virus was detected sooner indicating accelerated release after virus maturation. In addition, at 20 hours the concentration of extracellular virus surpassed intracellular virus. This finding was unique for the ISU-IBR 1 strain and suggests accelerated cell lysis as compared to the other three strains. The fact that peak virus yields per cell were considerably less than for the other strains would support this view. The accelerated lysis of cells by ISU-IBR 1 strain as compared to the other strains studied is probably under the control of the viral genome in the form of toxic accumulative products or inhibitor and is probably related to the increased virulence of this strain as demonstrated by Peter (41).

As previously mentioned, the ISU-IBR 1 strain is slightly more thermolabile at 37 C than the other strains. However, the difference between its growth curve and those of the other strains could not be attributable to this factor.

In comparing the data of this study to the one-step growth curves reported for IBR virus by others (46, 50), differences were noted. Stevens and Groman (50) using bovine

kidney cell line monolayers established that IBR virus peaked at 14 hours with a virus per cell yield of about 240 PFU. In this study the growth cycle of the strains studied lasted from 18 to 20 hours with a much higher PFU yield per cell. Since the relationship between cell size and virus yield is well established (8), it is difficult to compare studies where different cell systems were used.

ISU-IBR 2, Colorado and Los Angeles strains produced over 1,000 PFU per cell, while ISU-IBR 1 produced approximately 650 PFU per cell. These results would indicate that the ISU-IBR 1 strain differs significantly in its replicative cycle from the other strains studied.

Serum neutralization kinetics have been used to reveal fine antigenic differences between strains of bacteriophages (2) and animal viruses (4, 25).

McBride (25) in 1959 demonstrated that serum neutralization kinetics afforded a very sensitive method of antigenic analysis for poliovirus strains. Nakano et al. (39) in 1963 compared a large number of poliovirus type 1 strains, and they were able to distinguish vaccine-like strains from non-vaccine strains. In 1963 Ashe and Scherp (4) reported that 15 strains of herpes simplex virus could be classified into four groups using serum neutralization kinetics.

Sabina and Parker (46) reported that the rate of serum neutralization of IBR virus by anti-IBR bovine and anti-IBR

rabbit serum were the same. The reaction was observed to follow first order kinetics. Later Sabina (45) reported serum neutralization kinetic experiments with a strain of IBR virus and analogue-resistant variants. Antigenic differences between the parental strain and one analogue-resistant strain were noted.

Results from the reciprocal serum neutralization kinetic studies of the four IBR strains are summarized in Figures 21 and 22. While close serological relationships were detected between the Colorado strain and ISU-IBR 2 strain and between the Los Angeles strain and ISU-IBR 2 strain, the ISU-IBR 1 strain was unique in that the neutralization constants differed from all other strains. These findings suggest that the ISU-IBR 1 strain differs from the other strains in minor antigenic components. This finding is supported by the fact that antiserum produced against the ISU-IBR 1 strain neutralized the Colorado and ISU-IBR 2 strains much faster than the homologous system. This was not an idiosyncrasy of the individual rabbit's response, but was shown to be a property of the virus because antiserum produced in another rabbit acted similarly. Although this was not an expected result, a similar phenomenon using other virus-antiserum systems have been reported. For example, Wallis and Melnick (55) reported in 1965 that two serologically related type 4 echoviruses behaved quite differently in reciprocal serum

neutralization experiments. Either antiserum readily neutralized the Dutoit strain, whereas, the Pesascek strain was not readily neutralized.

An overall conclusion can be made that, of the four strains compared, the ISU-IBR 1 strain possesses distinct characteristics. Such distinct characteristics could be related to the pathogensis of this strain. Peter (41) demonstrated that this strain generalized following experimental inoculation of calves. It is noteworthy that the original isolation of this strain by Gratzek et al. (14) was from the deep scrapings of a Peyers patch from a calf which had died of mucosal disease.

A mild upper respiratory disease was produced in calves following the intranasal inoculation of the ISU-IBR 2 virus. These results are reported in Part II. Prototype strains in this study were the Colorado (vaccine) strain and Los Angeles strain. The Los Angeles strain had been isolated from upper respiratory disease in cattle.

Taken collectively these differences in experimental disease induced by the various strains and from the differences obtained by neutralization kinetic studies, would suggest that the factor or factors responsible for antigenic individuality is related to a virulence factor.

This hypothesis is supported by the work of Straub et al. (51) who reported that IBR virus and IPV virus, which

appeared to be identical by the serological method they used, could be differentiated by carrier-free virus zone electrophoresis in a glucose density gradient. These findings indicate that minor surface differences exist between IBR and IPV strains and may account for the differences in the disease syndrome which they produce.

Lukas et al. (22) suggested that IBR virus isolated from aborted fetuses "may possess infective and antigenic properties not shared by the virus of IBR." McKercher and Wada (32) reported that IBR isolates from aborted fetuses when inoculated intranasally could readily be recovered from the blood, whereas the respiratory isolate (L.A.) in parallel studies could not be recovered from the blood. They suggested that the virus had acquired new properties, but that these were not antigenic in nature because they could not be detected by standard neutralization tests.

Considering the above reports and the results of the present study it can be postulated that the IBR virus strains may vary slightly in their antigenic make-up and that these antigenic variations may be related to the type of disease syndrome produced.

The relationship between slight antigenic differences and virulence remains speculative; however, several possibilities exist. Since NK values measure differences in surface antigenic configuration, a speculation can be made

that such differences are related to an altered affinity for receptor sites on diverse cell types within the body. This could result in increased virulence. Another possibility is that these antigenic differences are eventually attributable to the nucleotide sequence in the viral DNA. This cannot be disputed in the light of modern concepts of nucleic acid and protein synthesis. Thus, if nucleotide sequences code for minor antigenic determinants, the question concerning other fine differences arise. For instance, is the accelerated growth potential and the decreased virus per cell yield seen with the ISU-IBR 1 strain directly or indirectly related to these antigenic differences? Does a particular antigenic mosaic of an ISU-IBR 1 virus subunit exhibit increased allosteric inhibition to host DNA? Is the antigenic difference only a convenient marker which has a casual relationship to the virulence factor?

From these results, a theory may be constructed which holds that observed differences in virulence and tropism patterns as seen in field cases are referable to basic virus-cell mechanisms. These would consist of receptor affinity and accelerated growth rates.

SUMMARY

Selected characteristics of four IBR strains were compared in a semi-standardized cell culture system. All of the strains studied produced plaques of similar size. The ISU-IBR 2 Colorado and Los Angeles strains had a similar thermal inactivation rates at 37 C and 42 C and similar growth curves. Non-reciprocal relationships were detected between these strains by serum neutralization kinetics studies.

The ISU-IBR 1 strain was slightly more thermal labile at 37 C. In comparing single-step growth experiments, it was found that with the ISU-IBR 1 strain both intracellular and extracellular virus reached a peak sooner in the growth cycle than did the other strains. Extracellular virus surpassed intracellular virus at 20 hours suggesting an accelerated cell lysis as compared to the other strain. Virus yield per cell was approximately 650 PFU per cell for ISU-IBR 1 compared to over 1000 PFU per cell for the other strains.

The relationship between these basic differences and observed differences in the experimental disease which these strains produce are discussed.

PART II. ANIMAL INOCULATION

MATERIALS AND METHODS

The ISU-IBR 2 virus was isolated from nasal exudate of beef cattle exhibiting signs of a mild upper respiratory disease. After the initial isolation, the isolate was passed several times and identified as IBR by the neutralization of CPE in cell culture by specific IBR antiserum. Cell culture fluids of the fourteenth passage were used for animal inoculations.

The purpose of this study was to determine the virulence of this isolate for calves exposed by the intranasal and oral routes.

Source of Animals

Three calves between the ages of 4 and 6 months were used. These calves were obtained by Caesarean section from cows in the dairy herd maintained by the Department of Veterinary Clinical Sciences and were housed in modified Rockfeller type isolation units. All calves were negative for serum neutralizing antibodies to IBR virus before inoculation.

Virus Inoculation

One week prior to inoculation daily temperature and total leucocyte counts were recorded. Calves were inoculated

intranasally using an 18 gauge needle attached to a 5 ml syringe. The head was raised and the infective cell culture fluid sprayed into the external nares. Calf A was inoculated with 5×10^5 PFU, calves B and C were initially inoculated orally with 3×10^3 PFU, and reinoculated thirteen days later by the intranasal route with 1×10^6 PFU.

Calves were observed twice daily at which time rectal temperatures were taken. Blood samples for total leucocyte counts and virus reisolation were taken every other day. Nasal and fecal swabs were also collected for virus reisolation every other day. Serum samples for assay of antibody titers were collected at various intervals after inoculation.

Virus Isolation

Nasal and fecal swabs were placed in tubes containing 5 ml of saline G diluent plus 25,000 units of penicillin and 25,000 µg of streptomycin. These tubes were incubated for 30 minutes at 37 C and then centrifuged at 2,000 RPM for 20 minutes. Approximately 2.5 ml of the supernatant was withdrawn and recentrifuged, and approximately 1.5 ml of this supernatant was removed and 0.1 ml inoculated into each of four tubes containing monolayers of primary bovine testicle cells.

Blood samples were collected in sterile vacuum tubes[1] containing EDTA as the anti-coagulant. These tubes were centrifuged at 2,000 RPM for 15 minutes and the serum withdrawn. The buffy coat was removed with a sterile Pasteur pipette and inoculated into four cell culture monolayer tubes.

Samples tested for virus isolation were allowed to absorb onto monolayers for one hour at room temperature. Maintenance medium consisting of Eagles' medium with 2 per cent horse serum was added to each tube. All tubes were observed daily for cytopathic changes and cell culture fluids from suspected cultures were passed a second time.

Samples which produced typical cytopathic changes in cell cultures were regarded as positive. Also, at least once during the reisolation study on each calf, a virus isolate was plaqued and neutralized using specific IBR antiserum.

Serum Neutralization

Serum samples collected before and after inoculation were assayed for the presence of IBR neutralizing antibodies. The neutralization test employed constant virus and variable serum using plaque reduction as an indicator system. Serial

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[1]Becton, Dickson and Company, Rutherford, New Jersey.

2-fold dilutions of heat inactivated serum were mixed with an equal volume of a virus dilution containing 200 PFU per ml. The virus-serum mixtures were incubated for 30 minutes at room temperature. One ml aliquots were inoculated onto each monolayer in 60 mm petri dishes. After adsorption for one hour at room temperature, the inoculum was decanted and the monolayers overlayed with the overlay medium. When the agar had solidified, the plates were returned to the CO_2 incubator.

At 3 days after overlay, the plates were stained with a second overlay of neutral red solution. Plaques were counted and the number per dilution recorded. The neutralizing end point of the serum was determined as the reciprocal of the serum dilution which caused a 90 per cent reduction in plaque number as compared to virus control plates.

RESULTS

Clinical Observations

When initially inoculated by the oral route calves B and C showed no clinical signs or febrile response. These calves were later exposed by the intranasal route, and again no febrile response was noted (Figures 25 and 26). Six days after inoculation both calves were slightly off feed. Calf B also exhibited a serous nasal discharge at this time. On closer examination, the nasal mucosa of both calves appeared inflamed and discrete white foci were observed. Two weeks after inoculation the areas were completely healed.

Calf A responded with a biphasic febrile response starting approximately 24 hours after inoculation (Figure 24). The peak of the febrile response occurred 4 days after inoculation. At this time a serous nasal discharge was observed. The nasal mucosa was inflamed, and numerous areas of focal necrosis were seen. During this period the calf was weak and inappetent. Two weeks after inoculation the calf had completely recovered.

Hematologic Observation

The total leucocyte count of calf A fell approximately 20 per cent during the febrile response (Figure 24). Total

Figure 24. Clinical and laboratory data obtained on calf A exposed to ISU-IBR 2 virus

———— Temperature

------ Leucocytes per cmm

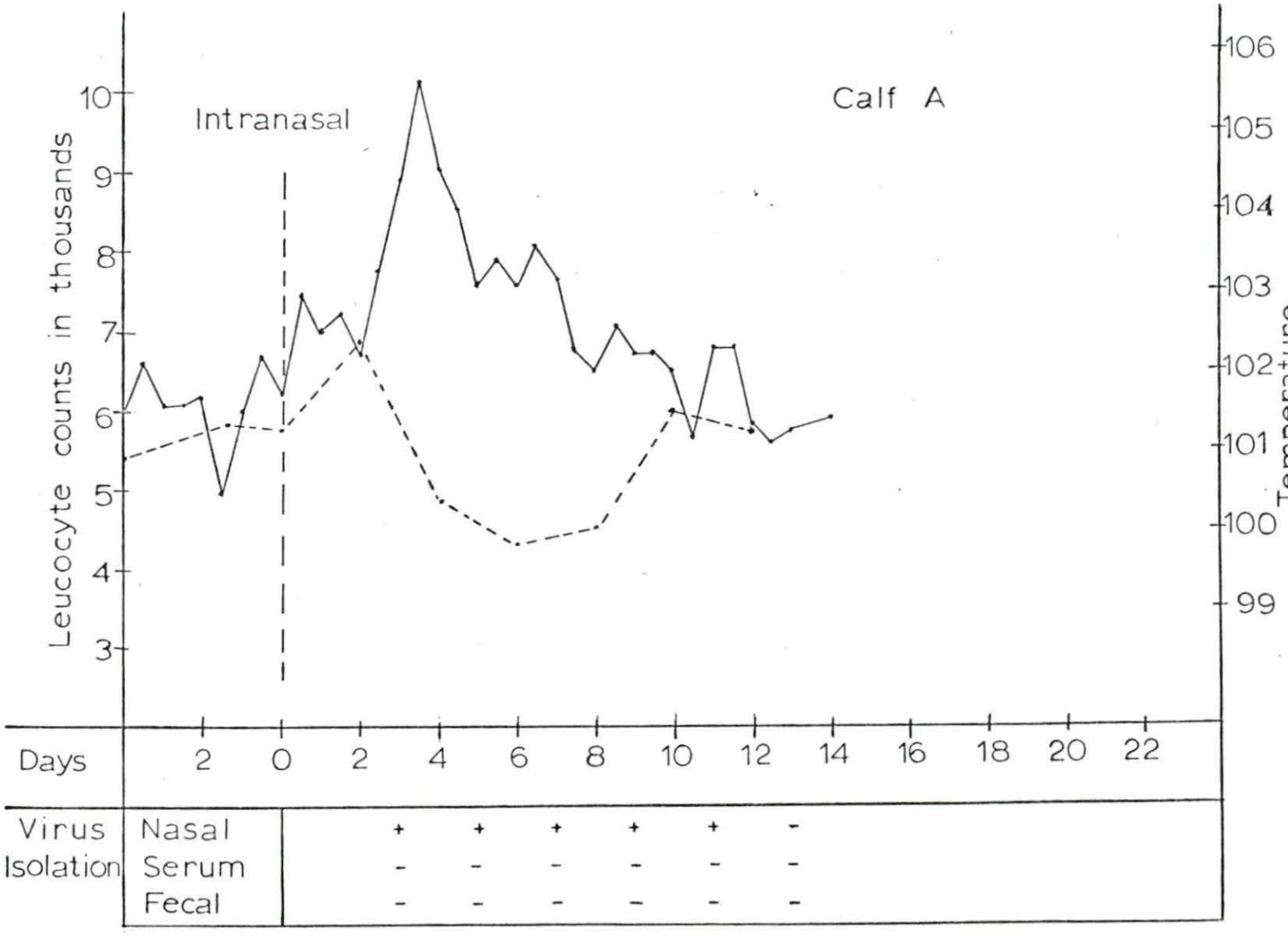

Calf A
Intranasal
Leucocyte counts in thousands
Temperature
10
9
8
7
6
5
4
3
106
105
104
103
102
101
100
99
Days
2
0
2
4
6
8
10
12
14
16
18
20
22
Virus Isolation
Nasal
Serum
Fecal
+ + + + + -
- - - - - -
- - - - - -

Figure 25. Clinical and laboratory data obtained on calf B exposed to ISU-IBR 2 virus

———— Temperature

------ Leucocytes per cmm

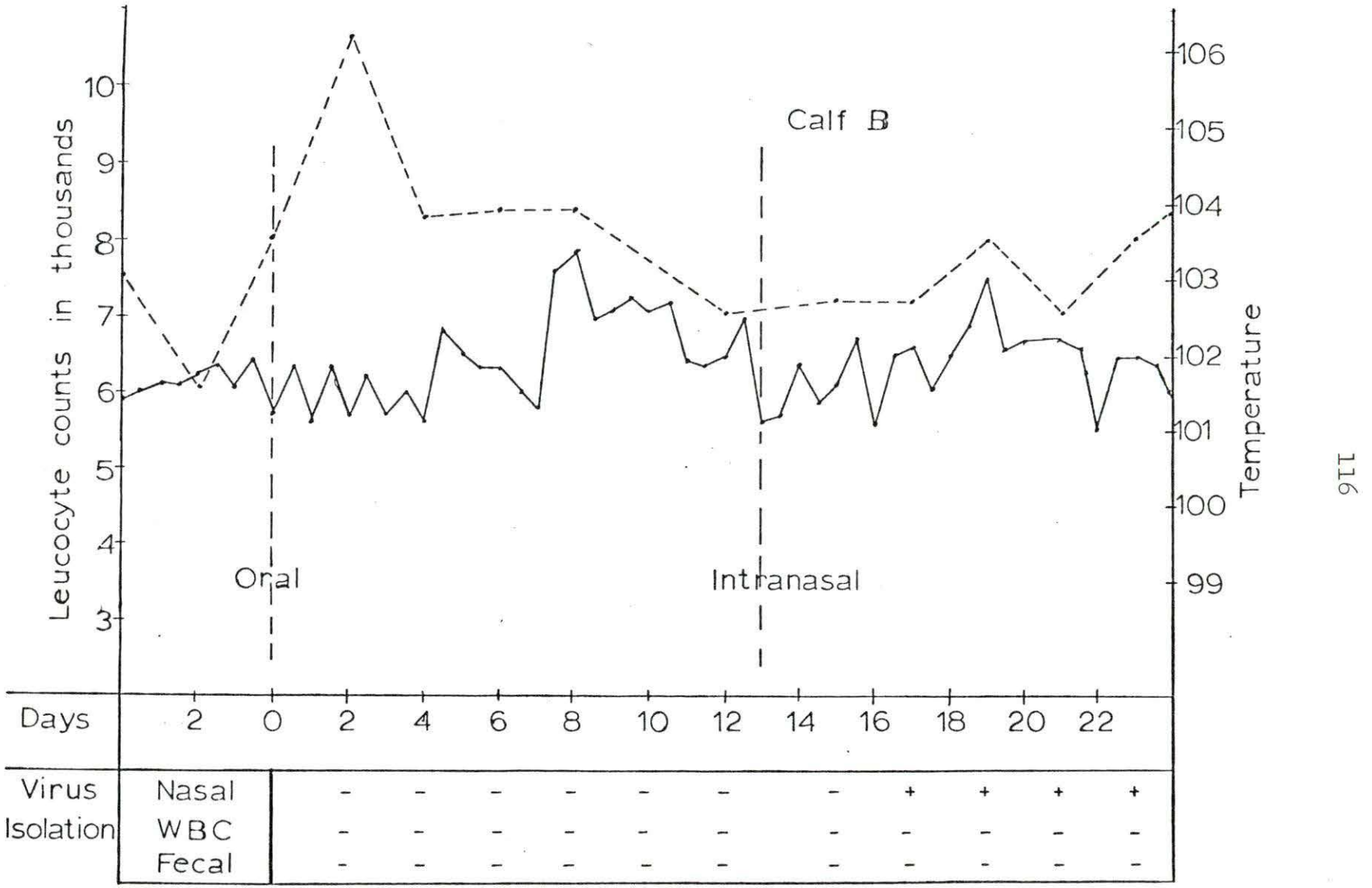
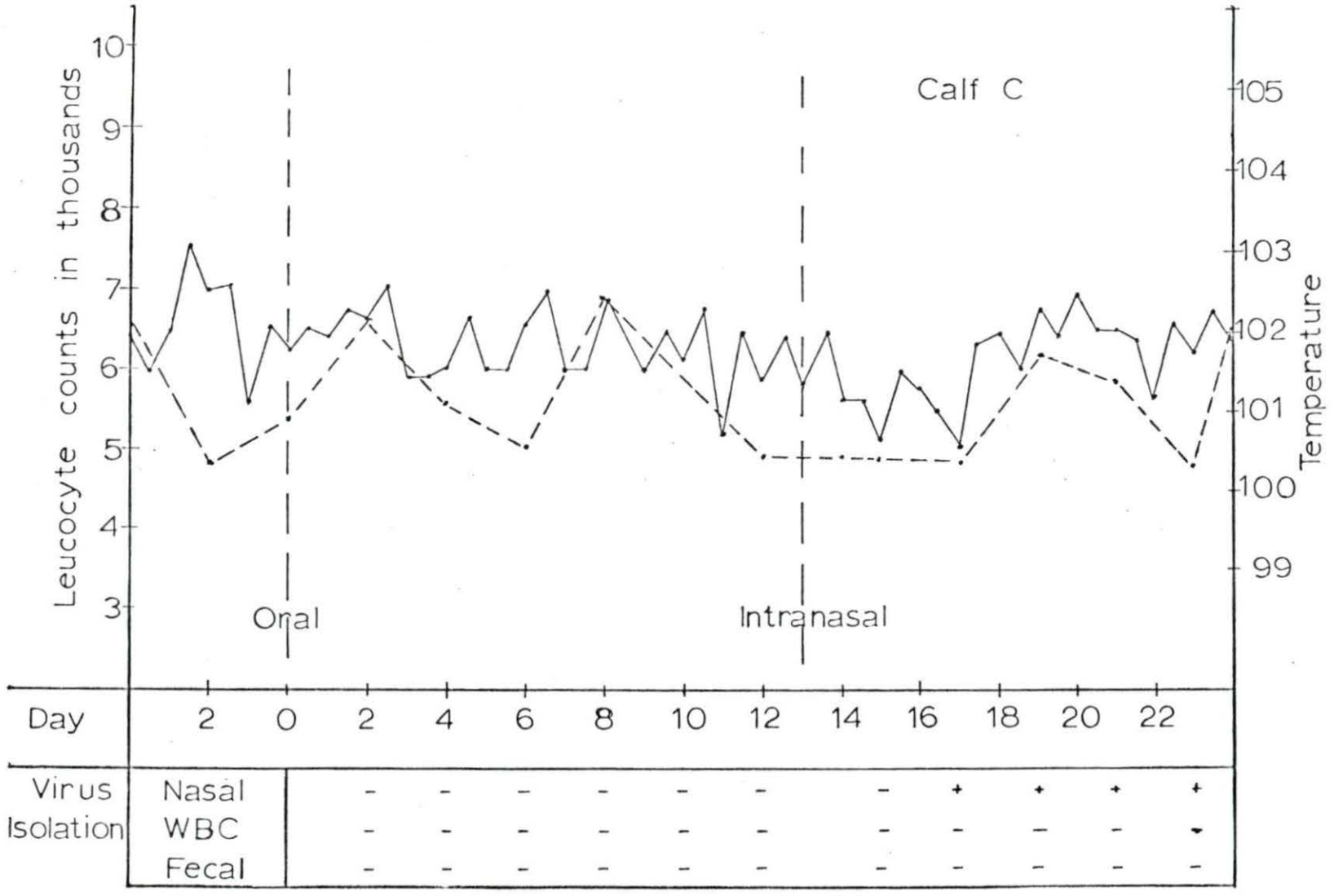

Calf B
Leucocyte counts in thousands
Temperature
Oral
Intranasal
Days
2 0 2 4 6 8 10 12 14 16 18 20 22
Virus Isolation
Nasal
WBC
Fecal

Figure 26. Clinical and laboratory data obtained on calf C exposed to ISU-IBR 2 virus

———— Temperature

- - - - - - Leucocytes per cmm


Calf C
Leucocyte counts in thousands
Temperature
10
9
8
7
6
5
4
3
105
104
103
102
101
100
99
Oral
Intranasal
Day
2
0
2
4
6
8
10
12
14
16
18
20
22
Virus Isolation
Nasal
WBC
Fecal

leucocyte counts on calves B and C did not follow a consistent pattern. Differential leucocyte counts were made and were found to be within normal limits throughout the study.

Virus Isolation

Virus was reisolated from the third through the eleventh day from nasal swabs of calf A following intranasal inoculation. The virus was not reisolated from nasal and fecal swabs or from the leucocytes of calves B and C following oral administration of the virus (Figures 25 and 26). However, when these calves were reinoculated by the intranasal route, virus was reisolated from nasal swabs from the fourth through the twelfth day after inoculation as illustrated in Table 20. At no time was the virus isolated from fecal swabs, leucocytes or the serum of the 3 calves after intranasal inoculation.

Serum Neutralization

The presence of IBR neutralizing antibodies in the serum of calf A were detected on the eleventh day after inoculation. Antibodies appeared on the twelfth day after intranasal inoculation in calf C and on the fourteenth day after intranasal inoculation in calf B. The correlation between the

Table 20. Correlation of virus reisolation with serum neutralizing antibody titers

Calf number	Sample	Day after inoculation 0	2	4	6	8	10	12	14	16	18	20
A	Reisolation		+	+	+	+		+	-			
	SN titer[a]		0	0	0	0		2	8	16	16	16
B	Reisolation	-	-	+	+	+	+	+	-	-		
	SN titer	0			0	0	0	0	2	4	8	8
C	Reisolation	-	-	+	+	+	+	-	-	-		
	SN titer	0			0	0	0	2	4	8	16	16

[a]Serum neutralization titer expressed as the reciprocal of the serum dilution with neutralization of 90 per cent of the plaques.

disappearance of virus in the nasal secretion and the appearance of antibodies in the serum is presented in Table 20. None of the calves developed serum neutralizing antibodies over 1:16.

DISCUSSION

Experimental reproduction of IBR with a field isolate by oral and intranasal inoculation of susceptible calves was studied. The isolate (ISU-IBR 2) was isolated from nasal exudate of cattle exhibiting signs of a mild upper respiratory disease.

When inoculated by the oral route calves B and C were not infected as judged by clinical signs, reisolation studies, and subsequent successful challenge. Baker et al. (5) produced a fatal disease in newborn calves by feeding high concentrations of IBR-IPV virus. Peter (41) reported a marked diarrhea in calves following the oral administration of ISU-IBR 1 strain.

It would appear that this strain (ISU-IBR 2) was unable to infect these calves by the oral route. The dose administered to these calves should have induced an experimental reaction. The possibility remains that the inability of this strain to infect by the oral route is due to a relatively narrow receptor tropism which it possesses for cells of the respiratory tract.

Various workers (23, 11, 58, 30, 6, 11) have inoculated cattle intranasally with cell culture fluids of IBR virus and have produced clinical signs of the disease. The mild clinical signs which they describe were consistent with those

observed for calf A (Figure 24). Calves B and C exhibited a very mild form of the disease following intranasal inoculation in that no febrile responses were observed (Figures 25 and 26).

In contrast to these milder syndromes, a more severe form of the disease has been reported by Webster and Manktelow (57) and French (9) which involved the central nervous system. McKercher and Wada (32) reported abortion in pregnant cattle following intranasal inoculation.

Total leucocyte counts of calves A, B, and C did not vary more than 30 per cent from the normal preinoculation level. No marked or prolonged decrease or increase was observed. These findings are consistent with those reported by McKercher et al. (29) and Gillespie et al. (11) following intranasal inoculation.

Variation in the clinical IBR signs following intranasal inoculation with various strains could be due to minor antigenic variations in the virus strains. For example, it was reported (32) that the Australian IBR strain has been isolated from the leucocytes of cattle following intranasal inoculation. Such data would indicate that the virus would be able to spread to many tissues. The concept of tropism variability of virus strains is well established. Differences in tissue tropism by virulent and avirulent poliovirus type 1 strains was shown by Sabin (44) in 1957 and confirmed

by Holland and Hoyer (20) in 1962. The avirulent strains did absorb to cells of the gastrointestinal tract, but not to cells of the nervous system. However, the virulent strain absorbed equally well to both cells of the gastrointestinal tract and nervous system.

Virus was reisolated only from nasal swabs. Swabs from calf A were taken from the same nostril which had been inoculated with the virus, whereas, with calves B and C the virus was recovered from the "uninoculated" nostril. Virus was isolated from the third through the twelfth day after intranasal inoculation. These findings are consistent with those reported by Gillespie et al. (11), McKercher et al. (30), and Brown and Bjornson (6).

In this study detectable serum neutralizing antibodies were detected between 11 and 14 days after inoculation. These results agree closely with those of other workers (11, 30, 6).

Correlation between the presence of serum neutralizing antibodies and the disappearance of virus from the nasal secretions are illustrated in Table 20. IBR neutralizing antibodies could be detected beginning at day 11 in calf A, day 14 in calf B, and day 12 in calf C. Failure in reisolation attempts correlated closely with the presence of detectable serum neutralizing antibodies.

McKercher and Wada (32) in 1964 reported that 2 isolates from aborted bovine fetuses could readily be recovered from the blood following intranasal inoculation. However, a respiratory IBR isolate could not be recovered from the blood in parallel studies. It was also reported that the Australian strain which is associated with encephalitis could be isolated from leucocytes following nasal exposure of cattle. These reports would tend to indicate that the ability of a particular strain to cause a viremia is a strain characteristic.

The general conclusion can be drawn from the clinical observations, virus isolations, and serological data that a mild upper respiratory disease was produced by the IBR isolate (ISU-IBR 2) in susceptible calves. Since virus was not reisolated from leucocytes or form fecal swabs, the isolate is considered to be relatively non-virulent as compared to the ISU-IBR 1 strain.

It is interesting to note that on the basis of NK and regression values, the "avirulent" Colorado strain and Los Angeles strains are closely related to the ISU-IBR 2 strain. The relationships between antigenic differences, growth characteristics, and virulent mechanisms have been discussed above.

SUMMARY

A field isolate (ISU-IBR 2) of IBR virus experimentally produced a mild upper respiratory disease in susceptible calves following intranasal inoculation. Efforts to infect calves by the oral route were unsuccessful.

The virus was readily reisolated from nasal swabs, but could not be detected in fecal swabs, blood or leucocytes. Nasal swabs were positive for virus until IBR neutralizing antibodies could be detected in the serum.

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