GROWTH OF INFECTIOUS BRONCHITIS VIRUS IN SUSPENDED CHORIOALLANTOIC MEMBRANES

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THESIS

GROWTH OF INFECTIOUS BRONCHITIS VIRUS IN SUSPENDED CHORIOALLANTOIC MEMBRANES

By

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This work is

respectfully dedicated

to

MY FAMILY

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INTRODUCTION

The refinement and expansion of tissue culture methods in virology have led to the discovery of heretofore unrecognizable viruses. The embryonating chicken egg affords an excellent medium for the cultivation of viruses and study of the host-parasite relationship, but the complexity of developing tissues, organs and systems does not permit a precise evaluation of a virus-cell interaction which may be accomplished better through cell culture. The cultivation of infectious bronchitis virus (IBV) on chorioallantoic membranes (CAM) suspended in a medium permits the study of: (1) the metabolism of tissue without the complication of the egg; (2) viral multiplication in a single tissue and (3) a medium free of embryonic urates and inhibitors. With the increasing knowledge of the action of different viruses in specific tissues, the compiling of information on definite viruses is becoming essential.

LITERATURE REVIEW

Infectious bronchitis (IB) is of particular economic importance in adult laying flocks, although chickens of all ages may be infected. The course of the disease is usually one to two weeks, but in some instances it may persist for as long as 4 to 6 weeks. Egg production will fall off along with softened and irregular egg shells.[31] After infection of adult birds long periods of time are required before egg production returns to normal. In young chicks the most characteristic symptoms are nasal discharge, gasping, rales, and coughing.[31] Tarpeia pulli[46] is the proposed name of the etiologic agent.

The virus can be isolated from tissue or exudate from the respiratory tract throughout the respiratory phase of the disease, and is estimated to be from 65 to 135 mu in size.[31, 38] Domermuth and Edwards[16] stated that by electron microscopy of infected CAM the virus appeared to be 200 mu in diameter, and was found separately, in pairs, in chains or in clumps of varying sizes.

Cultivation of IBV has been done in embryonating chicken eggs where initially it is not lethal, but develops this property with subsequent passages according to Loomis et al.[36] Early embryo passage virus produces certain characteristic gross pathological lesions of the embryo consisting of: (1) CAM adherent to shell membrane and thinner than normal; (2) amnionic membrane thickened, dry, fibrotic, resisting removal from embryo, and restricted movement of the embryo; (3) living infected embryos sluggish and weak; (4) embryos dwarfed as

much as one-half size and having a firm ball-like shape characterized by curling with a wry neck and feet deformed and compressed over head and (5) feather development immature and dry.

As embryo lethality increases with egg passages, host infectivity decreases.[31, 15] The allantoic cavity has been cited as the most desirable route of inoculation for primary isolation of IBV due to the appearance of pathological lesions in the first passage and the more simple inoculation.[36] This route is the easiest from a technical standpoint.

Cunningham and El Dardiry[13] found that following the allantoic cavity route of inoculation the greatest concentration of the virus was in the CAM followed in decreasing order in the allantoic fluid, amnionic fluid and liver. Yolk material was innocuous.

Hitchner and White[30] found the maximum titer of the virus in embryos to be at the 24th to 30th hour after inoculation via the allantoic cavity.

Cultivation of IBV in tissue culture has proved to be very rare. Only two articles[10, 19] on tissue culture of this virus have been reported and both of them in the last two years.

Buthala and Mathews[10] studied the use of the chicken embryo kidney as a medium for growth of various viruses. A strain of IBV which was in the 170th passage in chicken embryos was used. Trypsinized embryonic kidney cells, 1:200 dilution, were cultivated in a nutrient medium consisting of 0.5% lactalbumen enzyme hydrolysate, 2% calf serum and 97.5% Hank's balanced salt solution (BSS) at pH 7.8 failed to support growth of virus. Cytopathogenic effects were not observed.

The Beaudette and Connaught strains of IBV were cultivated in whole CAM suspended in medium 597 (medium 199 minus purines and pyrimidines plus Hank's BSS) by Fahey and Crawley.[19] Maximum infectivity, infectious dose 50 (ID_{50}) per ml, as determined by chicken embryo inoculation was demonstrated between 48 to 54 hours with both strains. In initial serial passages, harvests were made at 48 hour intervals and high titers were observed after the third passage. The latent period for the Beaudette strain occurred over a period of from 12 to 18 hours post inoculation while the Connaught strain required as long as 20 to 26 hours. There were no changes in the pH of the control or infected cultures which indicated that cellular metabolism did not occur. Both strains were grown in monkey kidney and chicken embryo heart cells. The Beaudette strain grew very little in whole embryo and chick liver cells, whereas, the Connaught strain could not be propagated in either. An attempt was made to grow both strains in Earle's L strain cells, but growth could not be demonstrated in either case.

Ackermann[1] found that when 14-to 15-day old CAM suspended in Simm's solution was infected cellular respiration remained approximately the same. Sodium malonate 0.02 to 0.06 M exhibited no inhibitory effect on the uptake of glucose by the membrane but showed a partial inhibition of endogenous respiration of the tissue. Amounts of this compound that reduce oxygen uptake inhibit viral propagation, yet it is not virucidal. Variations in oxygen tension influence the rate of virus propagation indicating aerobic oxidative processes taking place. The action of malonate indicates at least one reaction is a metabolic step in the Kreb's cycle. Succinic dehydrogenase is the enzyme

affected in this reaction and without the continuous supply of di- and tricarboxylic acids, the blocking of the whole cycle will result.

Ackermann and Johnson[2] concluded that the energy derived or required for viral synthesis from 200 mgm. of CAM in Simm's solution was obtained from the oxidative phosphorylation activity of the host tissue.

Ackermann and Maassab[3, 4, 5] reported that sulfonic acid, p-fluorophenylalanine, methoxinine and alpha-amino-alpha-p-methoxyphenyl-methanesulfonic acid inhibited the growth of influenza virus in whole CAM and Simm's solution.

Ackermann et al.[6] and Ishida and Ackermann[32] demonstrated the binding of influenza virus to the CAM suspended in a modified Simm's solution and concluded that binding is of two types: (1) sensitivity to the receptor destroying enzyme action and (2) sensitivity to the blocking effect of alpha-p-methoxyphenylmethanesulfonic acid.

Colville et al.[11] grew influenza virus on 11- and 19- day old CAM, lung, heart, liver, gut, skin and muscle. The best growth was demonstrated in lung and CAM. The CAM culture also grew equally well at 37 and 41 C.

Bernkopf[8] cultivated influenza virus in 14- to 15- day old CAM of deembryonated eggs. The embryo was removed leaving the CAM intact along the wall of the shell. Tyrode's solution was placed in the shell, inoculated with virus and covered with sterile rubber caps.

According to Daniels et al.[14] virus production was minimal in glucose deficient undefined balanced salt solution when employed with the CAM adherent to the shell of deembryonated eggs. When glucose was

added virus production was stimulated. Reduced oxygen consumption, 60 to 70 per cent of the control system, was characteristic of the glucose deficient medium.

According to Levine et al.[33,34] adsorption of influenza virus to the CAM of 10- to 11- day old embryos in modified Hank's BSS was not found to be affected by the deficiency of potassium ions in the medium. Respiration of the cells was decreased by 10 to 30 per cent. When the virus was grown in potassium deficient media there was a suppresion of: (1) growth of the CAM epithelium and fibroblasts, (2) multiplication of virus, and (3) respiration by 10 per cent. When potassium was added to the system the results were reversed. The lack of potassium also affected the accessibility of the virus attached to tissue to neutralization by immune serum and also intracellular viral synthesis.

Shulls and Rights[44] demonstrated the effect of influenza virus on the free amino acid pools of the CAM suspended in 1 part glucosol solution of Fulton and Armitage and 1 part phosphate buffer solution. Using membranes from 14- to 15- day old embryos it was shown after 17 hours of infection that slightly higher amounts of alanine, proline, glycine and glutamic acids were detected in the tissue.

The Roakin strain of Newcastle disease virus was grown in isolated CAM membrane preparations maintained in Tyrode's solution by Scott et al.[43] The strain was cultured through 30 serial passages and it was concluded that multiplication of the virus occurred because: (1) a significant increase in the quantity of virus inoculated occurred within a single culture and (2) the material from the last of a series of preparations contained the virus and represented a dilution far in

excess of the original titer.

Finter et al.,[20] Liu et al.,[35] and Henle et al.,[28, 29] studied the deembryonating techniques prior to and after infection of CAM with influenza virus using a mixture of modified glucosol and phosphate buffer solution. Phosphate buffered saline, Tyrode's solution, 10 per cent chicken serum in saline, normal allantoic fluid or even physiological salt solution gave similar results. Deembryonation after infection gave the better and more reproducible results. Release of the virus from the CAM occurred at constant rates for periods of 30 to 36 hours before yields decreased rapidly. In subsequent work, they hypothesized that hemagglutinins are produced in allantoic membranes. A greater concentration of non-infectious hemagglutinins were present in the CAM than in the suspending fluid and more infectious virus particles were in the fluid than in the tissue. In further work it was found non-infectious hemagglutinins were not released from the CAM following infection. Addition of large amounts of seed virus after initial infection resulted in decreased infectious dose 50 (ID_{50}) per hemagglutinin ratios up to 2 to 4 hours. Virus added after this time had no influence. The distribution of external cell receptors played little, if any, role in non-infectious hemagglutinin production.

A review of the multiplication of influenza virus in the entodermal cells of the allantois of the chicken embryo suspended in any BSS was conducted by Henle.[27] The tissue remained viable and supported viral propagation for at least 24 hours. The medium was of no relative importance unless long incubation periods were used. In the case of long incubation periods glucose was essential for maximal titers. The

virus could be controlled in such a manner that it would be permitted to undergo one cycle of multiplication and the time required for the first cycle extended over a period of 8 hours for influenza A and a period of 8 to 12 hours for influenza B virus. The conversion from incomplete virus to infectious virus was suggested as having been achieved by some limited material in the host cell. Multiplicity reactivation occurred when a cell adsorbed several inactivated virus particles and production and liberation of these particles by lysis occurred. Genetic recombination was suggested if host cells were infected with two different mutants. The offspring would contain not only the parent types but a combination of the two and the wild type, in which case all four types would retain their characteristics when infection of host tissue organ or cell with one virus prevented or partially inhibited simultaneous propagation of another viral agent.

Zuschek *et al.*[50, 51] studied factors that influenced the growth of Newcastle disease virus in CAM and Tyrode's solution. A deficiency of potassium suppressed the growth of the virus. As the concentration of glucose increased the amount of viral synthesis decreased. The maximum titer occurred when the medium was deficient in glucose. Maximum growth occurred in the medium with 0.1 g. of Mg Cl_2/liter, the usual amount present in Tyrode's solution. Concentrations of 0.2 g. calcium chloride per liter were necessary for maximal titers. Greater concentrations had an inhibitory effect on viral growth. Growth of the virus over a temperature range from 33 to 42 C occurred best at 42 C.

The growth of influenza virus was inhibited when cultured in CAM suspended in inorganic salts and glucose or pyruvate diluted 1:2

or 1:4 according to Eaton and Scala.[18] Restoration of the tenicity of the BSS with sodium chloride as late as 42 hours post inoculation resulted in normal growth of the virus. A 1:4 dilution of BSS sustained normal multiplication with 0.14 M of glucose but the effect was not observed until 24 hours had elapsed.

Gohd and Huang[23] hypothesized that phosphate in excess in a CAM-BSS system led to depletion of intracellular calcium, thereby denying the cells a necessary substance for the synthesis of virus. This was demonstrated by allowing influenza virus to be adsorbed and penetration to occur before removing the tissue and placing it in phosphate buffer. Viral multiplication did not occur. The addition of calcium to the same culture permitted production of virus.

Studies by Burr et al.[9] on the propagation of influenza virus in CAM in Hank's-Simm's serum ultrafiltrate, Earle's BSS and Medium 199 employed 9, 11, 13 and 16-day old membranes. As the age of the membranes increased the yield of virus decreased. The best results were obtained with membranes 9-days old but due to the small amount of tissue it was necessary to use 11-day old membranes. The different media yielded approximately the same amount of virus. Virus propagation occurred mainly at the expense of the intracellular material and was independent of the nature of the extracellular medium. Influenza virus could be grown equally well in healthy surviving cells, in actively growing cells or in cells depleted of intracellular nutrients and undergoing degeneration.

Gohd[24] observed influenza virus synthesis in CAM suspended in a suitable medium at 25, 30, and 35 C. Some growth was detected at

22.5 C. No growth occurred at 20 C, but the tissue retained the ability to synthesize virus at 25 C or higher. The adsorption and penetration phases occurred at lower temperatures but no free virus was found within the cells, which led to the conclusion that after penetration the virus went into a latent period. The latent periods were from 2 to 3 hours at 37 C and 3 weeks at 20 C. The differences in the periods seemed to be related to the nutrition of the cell.

Schlesinger and Karr,[41, 42] using CAM and Tyrode's solution, concluded that the maintainance or restoration of a normal supply of mucoprotein substrate is a function of the ability of cells of the allantois to maintain homostatic conditions under stress. In another report, they concluded that virus multiplied in cycles rather than continuously.

In a study of the pyruvate metabolism of CAM infected with influenza virus, Wielgosz[48] used modified Simm's solution and found that pyruvate uptake was increased by infecting the CAM with the virus as compared with controls.

Green and Beard[26] conducted experiments to determine the enzyme properties associated with the dephosphorylation of adenosine triphosphate. They found that potassium, sodium, calcium and magnesium activated the enzyme at approximately the same rates, but sodium proved slightly better than potassium. The enzyme was enhanced by these four ions acting alone or together. The divalent ions, separately or together, in the presence of either or both of the monovalent ions, strongly activated the reaction. The pH optimum was 7.16.

Work conducted by Ackermann[7] showed that the oxidative activity

of the mitochondria of the CAM was very low but the glycolytic activity was substantial. He reported that the endogenous supply of glucose and other essential nitrogenous requirements needed for influenza virus was completely adequate. He also found adenosine triphosphatase in minces of CAM.

The CAM, which is highly vascular and constitutes the respiratory organ of the embryo, is formed from the fusion of the chorion and allantois. It reaches maximum growth at the 10th to 12th day of development and a maximum weight of 2 gm.[40]

The CAM has proved to be an excellent medium for the abundant growth and culture of a large number of human and animal viruses.[37] Inoculation of this membrane in embryonating chick eggs has been beneficial in the titration of viruses, isolation of viruses and for the production of vaccines. IBV has been found to multiply on the CAM but as yet has not been reported to produce specific lesions on it.[10, 19]

Virus multiplication is said to be a cyclic phenomenon which consists of four phases: (1) the adsorption of viral particles to a susceptible host, (2) penetration of infecting particles into these cells, (3) synthesis of new viral particles, and (4) the release of new particles from benefactor cells. There are many factors that influence the growth of virus in embryonating chicken eggs. These are: (1) the age of the embryo; (2) the route of inoculation; (3) the volume and dilution of inoculum; (4) the time and temperature of incubation and (5) the physiological and nutritional state. These same factors apparently would have the same degree of effect in tissue culture.[22]

In the use of tissue culture there are many factors involving

homogeneity of the tissues used, to be considered. If the cells are heterogeneous one would expect that the virus eluted from these cells would not be eluted at the same time but over a period of time. If the cells are homogeneous, virus would be released at approximately the same time, over a period of time.[7]

Dulbecco[17] conducted work that showed in multicellular cultures the amount of virus released by single cells increased exponentially with time and that the number of cells releasing virus increased linearly with time.

There are at least three different types of cells contained in the CAM and it may be considered heterogeneous for two reasons: (1) because there are different types of cells and (2) cells of the same type may be in different physiological states.[7]

MATERIALS AND METHODS

The Beaudette egg-adapted strain, North Central Infectious Bronchitis Virus Repository, Code Number 42, was used. This strain is commonly used in serum neutralization tests and exhibits the ability to kill embryos within 36 hours post inoculation via the allantoic cavity. There are no records pertaining to the exact number of egg passages of this virus, but it has been through hundreds of passages. The passages for this study will be enumerated. The virus was passaged in eggs at the beginning of the experiment to establish a stock culture, and then used in the CAM system where it was maintained in serial passage with Hank's BSS. Harvests of the infected fluid were collected and stored at -30 C until used.

Hank's BSS contained the following ingredients on a grams per liter basis: 8.0 gm NaCl; 0.4 gm KCl; 0.14 gm $CaCl_2$; 0.1 gm $MgSO_4 \cdot 7H_2O$; 0.1 gm $MgCl_2 \cdot 6H_2O$; 0.12 gm $Na_2HPO_4 \cdot 12H_2O$; 0.06 gm KH_2PO_4; 1.0 gm glucose; 0.02 gm phenol red; 0.35 gm $NaHCO_3$ and 1 liter double distilled water.

Modification of the amounts of the ingredients of this solution was made to enable the same amounts present in 20 ml to also be present in 18.2 ml. This was accomplished by using the following formula:

Example using NaCl

$$\begin{array}{l} 8.0 \text{ gm NaCl/liter} \\ 0.16 \text{ " " } 20 \text{ ml} \end{array} \qquad \frac{0.16 \text{ gm NaCl}}{18.2 \text{ ml}} = \frac{X \text{ gm NaCl}}{20 \text{ ml}}$$

$$X = 0.17582 \text{ gm/20 ml}$$

$$8.7910 \text{ gm/liter} = \text{the amount needed to enable 0.16 gm to be present in 18.2 ml.}$$

Modification of the amounts of the ingredients of Hank's BSS on a grams per liter basis would then be: 8.7910 gm NaCl; 0.4395 gm KCl; 0.1538 gm $CaCl_2$; 0.1099 gm $MgSO_4 \cdot 7H_2O$; 0.1099 gm $MgCl_2 \cdot 6H_2O$; 0.1219 gm $Na_2HPO_4 \cdot 12H$ 0; 0.0659 gm KH_2PO_4; 1.0990 glucose; 0.0220 gm phenol red; 0.3843 gm $NaHCO_3$ and 1 liter of double distilled water.

Each ingredient of the BSS was prepared separately in a 10X concentration in 100 ml of double distilled water. In this manner the complete BSS could be prepared or certain ingredients could be excluded for preparation of deficient media. No attempt was made to adjust the pH of the deficient media. The pH of the complete BSS was 7.3. The media were sterilized in an autoclave for 10 minutes at 10 lbs. pressure.

Embryonating chicken eggs from a commercial, disease-free flock were incubated in a Jamesway model 252 incubator at 99-99.5 C. When the embryos were 10 to 11 days old they were candled. Tincture of metaphen was applied to the shell over the air cell. The shell over the air cell was cracked with forceps and removed. The shell membrane at the base of the air cell was peeled from the CAM. The CAM was cut in a straight line with scissors from one side to the other and the endogenous mass, excluding the CAM, was emptied into a sterile petri dish. The CAM was then removed, washed in three 500 ml flasks each containing 200 ml of physiological saline, minced into small fragments with scissors and placed in an 8 oz. prescription bottle containing 18.2 ml of Hank's BSS in which the total amount of the ingredients normally contained in 20 ml would be present in 18.2 ml. The membrane was then inoculated with 1 ml of virus and 0.8 ml of antibiotics which brought the total fluid in the bottle to 20 ml. The cultures were incubated at

37 C. At 12 hour intervals throughout a 60-hour period 1.5 ml samples were removed and stored at -30 C until used for titrations.

Infectivity titrations were performed using serial 10-fold dilution of virus and 5 eggs per dilution, 0.1 cc per egg, with inoculation via the allantoic cavity.[12] Calculation of infectivity was made by the method of Reed and Muench.[39] When used for titration the samples were thawed and centrifuged lightly to sediment fragments of tissue which were present. Following inoculation the eggs were returned to the incubator and candled once a day. Embryo mortality during the first 24 hours was considered to be due to nonspecific causes and was not included in the final calculations. A post inoculation observation period of 6 days was used. Criteria for viral infectivity were: (1) embryo mortality and (2) gross pathological lesions. Embryo mortality occurred more frequently because this is an egg adapted strain which characteristically produces death. Gross pathological lesions considered to be characteristic were balling, curling and dwarfing of the embryo to approximately one-half the size. These lesions were only observed at the limiting dilutions and, therefore, did not occur with regularity.

The diluent used in all titrations was sterile nutrient broth. Bacterial sterility of all materials was determined by inoculating thioglycollate broth.

RESULTS

There was no special emphasis on pH during the period of the experiment, but some observations were made concerning the relationship between the control and infected cultures at 0 and 60 hours.[21] The pH of Hank's solution was 7.68 but after the addition of antibiotics, membranes and virus the pH decreased approximately 0.3 units. The pH of the controls decreased about 0.2 units after 60 hours in contrast to the infected cultures in which there were slight fluctuations above or below the initial pH value (Table I). The hydrogen ion concentration of deficient media and complete media differed, but no attempt was made to adjust to any definite value.

Previous work by Spring[45] showed that IBV yielded higher titers when propagated in minced CAM than in whole CAM. The differences were most marked after several serial passages of the virus. To corroborate these findings growth curves of IBV in the 1st CAM passage were conducted using whole CAM and minced CAM with Hank's BSS. As shown in Table II and Figure 1, virus was rapidly adsorbed during the 1st 24 hours with both preparations but with the whole CAM there was a continous decline of infectivity of the suspending medium for the next 48 hours. With minced CAM, virus was released from the cells between the 12th and 24th hours. The titer remained fairly stationary from this time until the 60th hour but did not yield the original titer of 7.5. These results were similar to those obtained by Spring[45] and indicated a slight advantage of using minced CAM as compared to whole CAM.

Through serial passage of the virus in minced CAM the pattern of growth demonstrated adsorption of virus to the cells within 24 hours as indicated by the precipitous decline of titer. Release of virus occurred as shown by the continuous increase in infectivity up to the 60th hour at which time the titer was as high as or higher than that of the seed virus. This is evident when comparing the growth curves of the 6th, 9th and 12th passages where the periods of adsorption of virus and penetration into cells with subsequent liberation into the suspending medium are essentially the same for certain time periods (Table III, Figures 2, 3 and 4).

With 6th passage virus the typical cycle of infection occurred when Hank's BSS was used. At the 60th hour the titer of the virus was essentially the same as that of the seed virus used to initiate infection. The rate of adsorption of virus with calcium, magnesium and glucose deficient medium was the same as with the complete medium, but after the 12th hour release of virus from cells could not be detected by infectivity tests of the medium. Adsorption occurred with the potassium deficient medium but was not complete until 24 hours. This was followed by evidence of release of the virus from the 24th through the 60th hour (Table III, Figure 2).

Viral infectivity with the 9th passage virus of the deficient media followed essentially the same growth curve patterns, but at different numerical levels. There were lag, log, and decline phases with the exception of the potassium deficient medium. In this case, there was a stationary phase after the log phase rather than a definite decline phase. Viral infectivity of the glucose deficient medium

showed that the lag phase was at a slower rate than with the other media. Maximum infectivity, 2.8, of calcium deficient medium occurred at 48 hours and was 0.2 units less than the seed virus. Glucose and magnesium deficient media had a maximum infectivity at 36 hours. With glucose this was 0.4 units higher than the seed virus, but that containing magnesium was the same as the seed virus. Viral infectivity of the potassium deficient medium showed an infectivity titer peak, 2.4, at 60 hours, but it was essentially at a stationary phase of 0.6 units below the initial titer of the seed virus. With Hank's BSS lag and log phases only were detected. The rate of the log phase was most prominent from the 12th through the 36th hours followed by a slower rate to the termination of the experiment at the 60th hour. At the 60th hour the titer of the virus was 2.1 units higher than the seed virus (Table III, Figure 3).

With 12th passage virus, infectivity of glucose and potassium deficient media followed essentially the same growth curves with the highest titer occurring at the 48th hour along with magnesium and calcium. There was a sharp decline for these media by the 60th hour. In the log phase at the 24th and 36th hours the numerical value of infectivities was the same for all deficient media. All deficient media exhibited a viral log growth phase extending over a period of 36 hours. Maximum infectivities of the deficient media were all above the value of the seed virus, potassium 0.2, glucose 0.3, calcium 0.7 and magnesium 0.9 units. The maximum titer with Hank's BSS medium occurred at 60 hours but a stationary phase apparently began at the 36th hour. Viral infectivity had a net increase of 2.1 units in Hank's

BSS which was the same as with the 9th passage virus.

TABLE I

THE EFFECT OF VIRAL GROWTH ON THE pH OF HANK'S BSS WITH CAM

Serial passage	Culture at 0 time pH	Culture at 60 hours pH	Culture at 60 hours pH	Control pH
8	7.35	7.27	7.37	-
9	7.35	7.30	7.40	7.17
10	7.35	7.35	7.45	7.13
11	7.30	7.31	7.35	6.97

TABLE II

1ST PASSAGE COMPARISON OF GROWTH OF IBV IN WHOLE AND MINCED CAM SUSPENDED IN HANK'S BSS IN TERMS OF LOG ID_{50}/0.1 CC

CAM	TIME IN HOURS					
	01	12	24	36	48	60
Whole	7.5	2.5	2.0	1.7	0.8	1.3
Minced	7.5	1.0	2.2	2.0	1.7	1.5

TABLE III

THE EFFECT OF HANK'S BSS AND CERTAIN ION AND GLUCOSE DEFICIENCIES IN THE CULTIVATION OF IBV IN MINCED CAM IN TERMS OF LOG ID_{50}/0.1 CC

	Time in Hours					
	0	12	24	36	48	60
1st Passage						
Hank's BSS	7.5	1.0	2.2	2.0	1.7	1.5
6th Passage						
Hank's BSS	4.4	0	2.5	3.5	3.8	4.2
Deficient of						
calcium	4.4	0	0	0	0	0
glucose	4.4	0	0	0	0	0
magnesium	4.4	0	0	0	0	0
potassium	4.4	0.6	0	0.7	0.3	0.6
9th Passage						
Hank's BSS	3.0	0	1.8	4.0	4.6	5.1
Deficient of						
calcium	3.0	0.3	0.7	2.2	2.8	2.0
glucose	3.0	1.6	1.6	3.4	2.7	2.2
magnesium	3.0	0.4	1.0	3.0	2.4	2.0
potassium	3.0	0.6	10.	2.2	2.2	2.4
12th Passage						
Hank's BSS	3.5	1.8	4.3	5.4	5.5	5.6
Deficient of						
calcium	3.5	1.4	2.5	3.5	4.2	2.0
glucose	3.5	0.5	2.5	3.5	3.8	3.0
magnesium	3.5	2.0	2.5	3.5	4.4	3.4
potassium	3.5	0.5	2.5	3.5	3.7	2.5

FIGURE 1

GROWTH CURVES OF IBV IN WHOLE AND MINCED CAM SUSPENDED IN HANK'S BSS IN THE 1ST PASSAGE

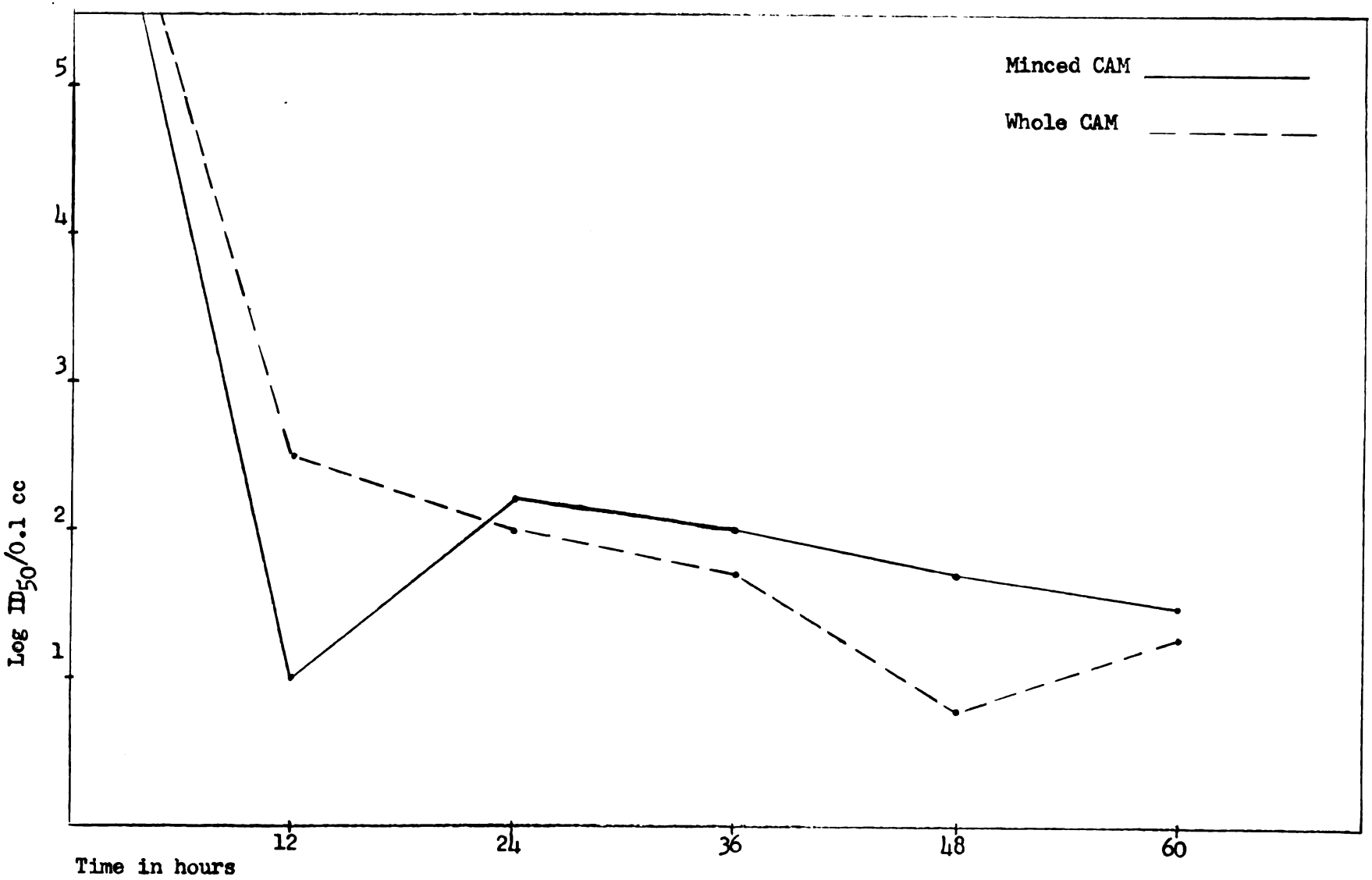


FIGURE 2

GROWTH CURVES OF THE 6TH PASSAGE OF IBV IN CAM SUSPENDED IN HANK'S BSS AND CERTAIN ION AND GLUCOSE DEFICIENCIES

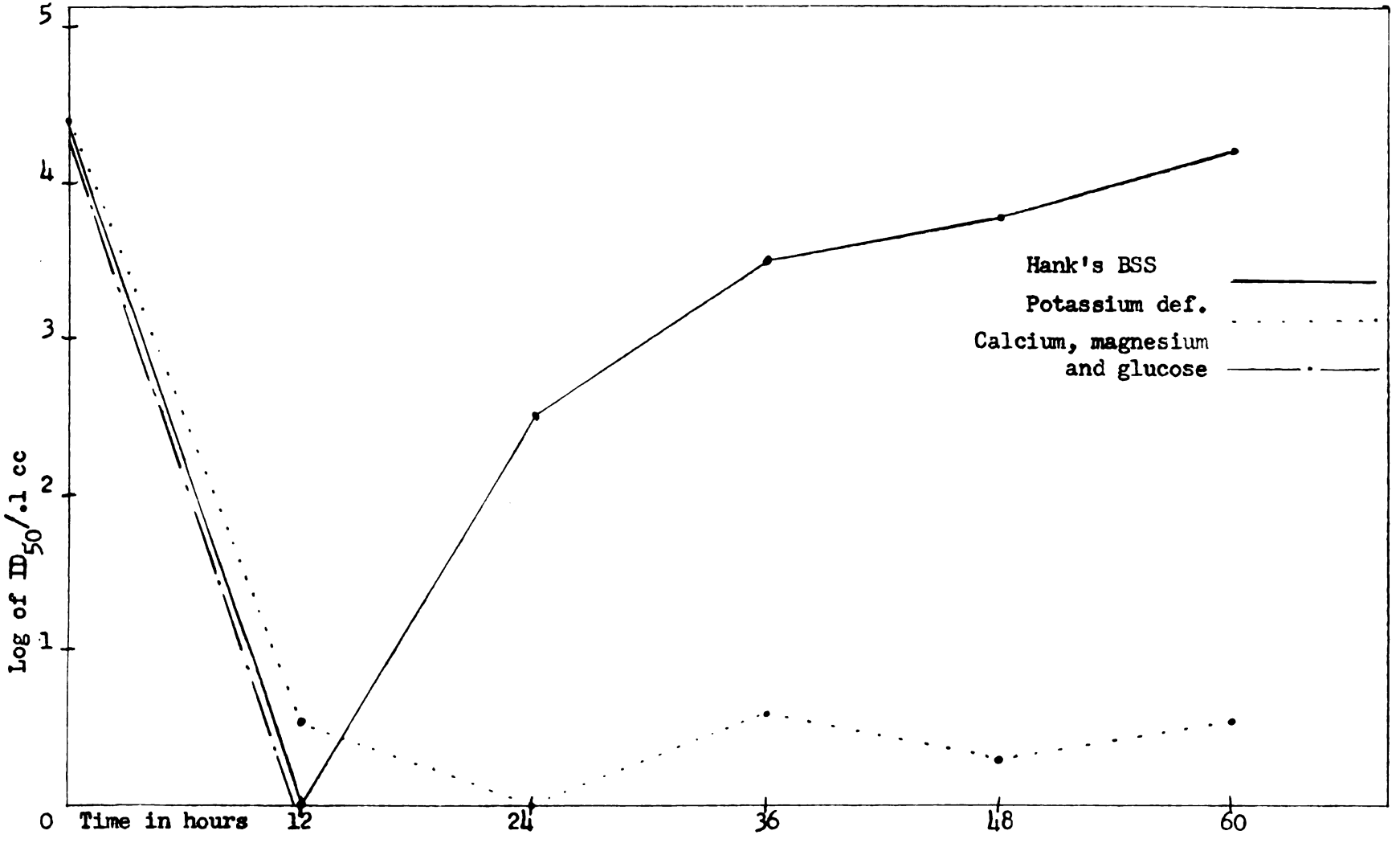


FIGURE 3

GROWTH CURVES OF THE 9TH PASSAGE OF IBV IN CAM SUSPENDED IN HANK'S BSS AND CERTAIN ION AND GLUCOSE DEFICIENCIES

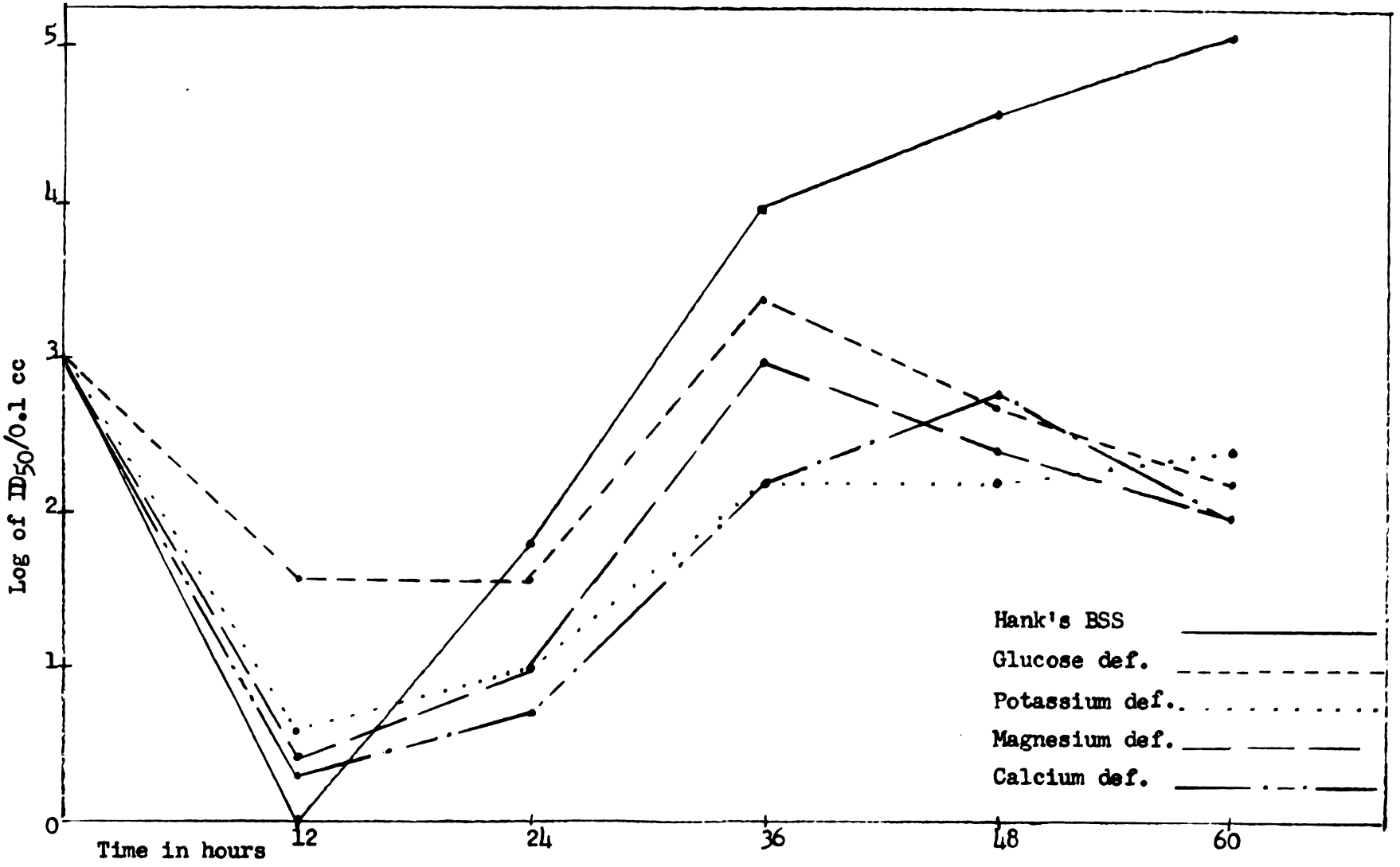
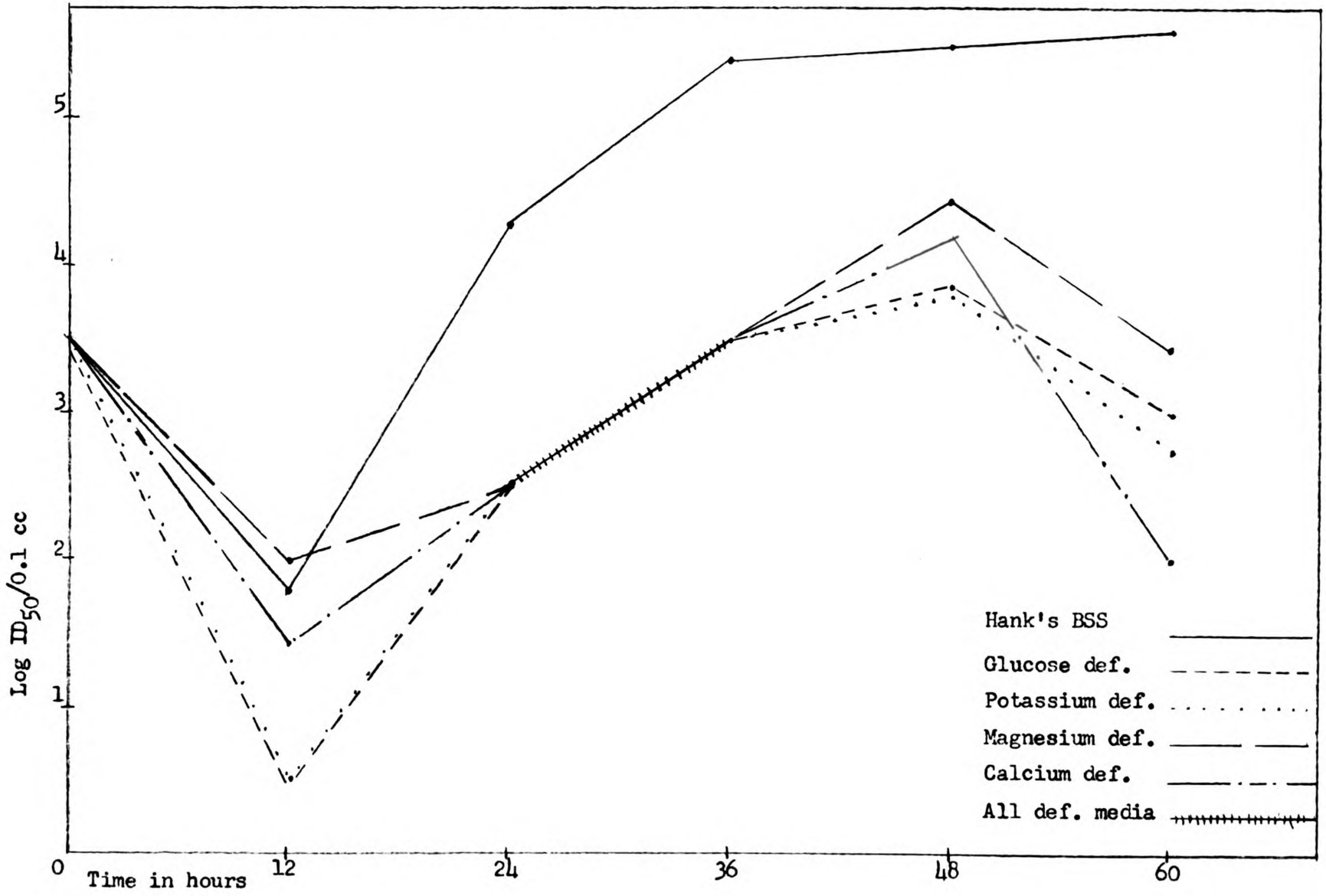


FIGURE 4

GROWTH CURVES OF THE 12TH PASSAGE OF IBV IN CAM SUSPENDED IN HANK'S BSS AND CERTAIN ION AND GLUCOSE DEFICIENCIES



DISCUSSION

Virus propagation is possible only when viable cells are employed as the substrate. This indicates that the virus lacks some physiological systems, which in all probability include enzyme systems, that are provided by the cell in the host-parasite relationship.[37]

A host-virus system, such as was used in the present study, allows an opportunity to measure viral propagation without the interference of defense mechanisms which would be present if the host were used.

Growth curves of virus indicate that either one of two conditions of the host system exists: (1) the virus is released over certain periods of time because of the preferential infection of different types of cells at different times, or (2) the infection of homogeneous cells at the same time which would lead to the release of virus at the same time over a period of time.[7] The present study seemingly supports the first theory because the CAM is composed of three types of epithelial cells, those cells lining the allantoic cavity, the surface of the chorion, and connective tissue. Although these three types of cells are different, each type is homogeneous, but individual cells may differ physiologically and in the state of development. The CAM is apparently entirely heterogeneous with respect to cell types and viral liberations may be continuous over a period of time.

IBV multiplies readily in cells of the CAM but there is no

definite cytopathogenic effect that can be recognized microscopically as evidence of the cellular infection. Other cell types in which this has been observed are monkey kidney cells, whole embryo cells, chicken embryo heart cells and chicken liver cells.[19] Plaque formation by IBV with chicken embryo kidney cells has been reported and indicates that cytopathogenic effects may be induced with these cells.[49] Apparently IBV does not disrupt the normal metabolic process of the cell to an extent that necrosis or other pathological responses may be observed.[7]

The significance of a dilution factor when seed virus was introduced into the CAM system should not be overlooked. At each passage, one ml of virus was added to 20 ml of medium which resulted in a further 1:20 or a 1.3 log unit dilution. It is quite evident that viral multiplication had occurred in the 12 tissue culture passages, as the material harvested from the 12th passage contained virus and represented a dilution far in excess of the titer of the original seed virus. With some cultures the titer of virus after 60 hours incubation was higher than that of the seed virus before introduction into the medium. Consideration of the 1.3 log dilution emphasizes the higher magnitude of virus multiplication. In only the first passage was there evidence that virus could not be recovered at a concentration equal to that of the seed virus. Consideration of this and the increases observed with continued passage would indicate the possibility of an adaptive process being required before the virus could be established in a system of this type. This is not unusual as serial passage is required for adaptation of the virus to the embryonating chicken egg[15] and perhaps is a reflection of a selective adaptation to the host system.

With subsequent passage of virus, the lag phase or adsorption phase occurred in a shorter period accompanied by an increasingly rapid production of virus. This could be considered to be another reflection of adaptation to the time for one single synthesis cycle to take place.

The presence of magnesium, calcium and potassium in the allantoic fluid[40] suggests that they are also present in the CAM. In this case, the endogenous supply of these ions and cellular glycogen would in all probability be sufficient for viral propagation to occur for at least a brief period of time. The presence of these materials in the extracellular medium would have the primary purpose of restoring the salt balance of the cell upon depletion during cellular metabolism.

This fact, along with adsorption of glucose by the cell to replenish its carbohydrate store, would in part explain the importance of the extracellular balanced salt and glucose medium. It would also correspond with the results obtained using deficient media and detection of viral synthesis at the lower rate and shorter log phase as compared to complete Hank's BSS.

Since, glucose is the only energy supplying substrate present in Hank's BSS and magnesium, potassium and calcium all play a role in the carbohydrate metabolism consisting of the anaerobic glycolysis and the Kreb's cycles, deficiencies of any of these ions and glucose have an inhibitory effect on viral synthesis. Adsorption apparently occurs according to a decrease in the amount of virus at the 12th hour interval. If the cells supposedly supply enzymes essential for carbohydrate metabolism and the major difference exhibited by the viral growth curves between the various deficient media and Hank's BSS is

directly related to carbohydrate metabolism, then these ions function as enzyme activators and glucose is the energy supplying substrate.

It is apparent that carbohydrate metabolism does not produce the only substrates needed for viral synthesis. Therefore, there are probably other limiting factors such as nucleic acids, amino acids and specific proteins supplied by the cells which complete the metabolism of substrates essential to viral propagation. In this study these factors were constant, therefore, they have no apparent effect on growth curve differences.

Magnesium[25, 47] is essential for viral synthesis to proceed at its maximum and is comparable in effect to the other ions. It is primarily concerned with the activation of certain enzymes in the anaerobic glycolysis cycle; such enzymes would be phosphorylase, enolase and phosphoglucomutase. In high concentration, magnesium will inhibit the action of adenosine triphosphatase but small amounts of this ion are essential for the maximum action of this enzyme.

With the absence of this ion in the extracellular medium the metabolism of carbohydrate in the medium would be substantially slowed down, which apparently is the case according to the results obtained. Glucose in the medium is the only energy source supplied to the cell, but in order for the cell to utilize this energy substance it must break it down by means of the anaerobic glycolysis and Kreb's cycles. The cell has enough magnesium to initiate these cycles for a limited amount of time, but if no magnesium is present in the extracellular medium this cycle will soon begin to decrease in activity and finally stop. This in turn can be demonstrated by the reduction in the amount

of virus synthesized. Magnesium is also needed for the induction of the tissue glycogen into the anaerobic glycolysis cycle. So, in theory, some of the magnesium contained within the cell is used for the anaerobic breakdown of the tissue glycogen. Such enzyme systems as phosphorylase, and phosphoglucomutase are required to take tissue glycogen and initiate it into the glycolysis cycle. Glucokinase is essential along with ATP to phosphorylate glucose and all these enzyme systems need magnesium to activate them.

If there is enough intracellular magnesium to accomplish the foregoing reactions, still more magnesium is needed to complete the anaerobic glycolysis cycle. The enzyme enolase requires magnesium for the step leading to the production of 2 phosphoenolpyruvic acid, which in turn requires magnesium to be transformed to pyruvic acid. This is the point of entrance to Kreb's cycle. Adenoside triphosphate is the main energy souce produced by this system and, therefore, it might be feasible to state that IBV requires adenosine triphosphate for its production.

The Kreb's cycle is an abundant source of adenosine triphosphate, but in order for the continuation of the metabolism of pyruvic acid in this cycle, more magnesium is needed in conjunction with pyruvic oxidase to enter the cycle.

Calcium[25, 47] is a required ion for the maximum synthesis of virus as has been shown by the results obtained. This ion is a known activator of succinic dehydrogenase and adenosine triphosphatase and is concerned with membrane permeability. Green and Beard[26] have demonstrated the activity of calcium ions with adenosine triphosphatase

and concluded that it was essential for the maximum activity of this enzyme. Succinic dehydrogenase is present on the Kreb's cycle which has been shown to be present in certain host-virus systems. Ackermann[1] found a decrease in respiration and further evidence to show that influenza virus in suspended CAM was involved with the Kreb's cycle. Gohd and Huang[23] found that calcium was essential for the maximum growth of influenza and after the depletion of the intracellular calcium viral synthesis ceased.

Although calcium is not essential in a large number of reactions, apparently it plays a role as important as the other ions. Calcium is essential in only one reaction in the Kreb's cycle and seemingly would not have any great effect on the amount of adenosine triphosphate produced. In the reaction involving succinic dehydrogenase adenosine triphosphate is produced. The absence of the reaction which involves this enzyme and which calcium is required to activate would completely block the action of the Kreb's cycle from going any farther. If adenosine triphosphatase is needed for the production of adenosine diphosphate from adenosine triphosphate, and the release of energy in the presence of calcium, then the absence of this ion would stop the release of energy and, in turn, inhibit the production of virus. However, complete inhibition of virus production did not occur in the absence of this ion and, therefore, it would be feasible to state that viral production in this instance would result from the intracellular ions normally present in the CAM.

It is commonly known that potassium[25, 47] plays some role in the permeability of membranes and may, therefore, have some effect on the

penetration of IBV into the CAM cells. If this is the case, it would correlate favorably with the results thus far obtained in this study. Certain enzyme systems also require this ion for their activation. An example is the dephosphorylation of adenosine triphosphate (ATP). The absence of this ion in the extracellular medium would theoretically slow down the enzyme activity and thereby limit the amount of virus produced in such a system. If CAM cells contain this ion, it would be feasible that the deficiency would not entirely inhibit the enzyme systems. Viral synthesis, as a result of enzyme activity, would be reduced, because of the limited supply of this ion and after a certain period of time it would cease due to the depletion of the intracellular ion. If potassium does effect penetration then it would reduce the production of virus also. Levine et al.[33] found potassium deficient BSS did not have any effect on the adsorption of influenza virus.

Potassium is essential in the reaction involving the enzyme phosphokinase which leads to the production of pyruvic acid. If this reaction was inhibited, then entrance to the Kreb's cycle would be eliminated and the production of adenosine triphosphate virtually stopped. This would lead to the production of a limited amount of adenosine triphosphate produced by the anaerobic glycolysis cycle which in all probability would be insufficient to stimulate viral growth. Minimal amounts of this ion are apparently present in the intracellular system and would then, of course, be available to allow this reaction to take place. If this is the case, the reaction would be limited by the amount of intracellular potassium ions available and when this supply is exhausted, viral synthesis would terminate.

Glucose,[25, 47] the only energy source supplied by Hank's BSS, should be essential for viral growth. In high concentration, according to Zuschek et al.,[50] glucose inhibits viral multiplication. The results of experiments conducted on the deficiency of glucose showed that an inhibition of viral multiplication occurred. Viral growth did occur in this deficient medium which could be explained by intracellular glycogen which inevitably must be present in the cells of the CAM. Being that all the ions in the medium were present, maximum enzymatic activity would be characteristic and, therefore, the only limiting factor would be the amount of carbohydrate present. If the glucose were utilized in conjunction with tissue glycogen, a growth curve, the same as Hank's BSS, would occur. This is not the case since the only source of carbohydrate in glucose deficient medium is the tissue glycogen. It must, therefore, supply enough energy for the early stages of viral synthesis.

Results obtained in this experiment are in direct contrast to the results obtained by Zuschek et al.[50] who showed that the maximum growth curves exhibited were in glucose deficient medium (Tyrode's solution). Although it appears that glucose is not the most essential constituent of the medium because of the supply of glycogen in the tissue, it is necessary to obtain maximum growth of the virus in Hank's BSS.

SUMMARY

1. IBV can be readily grown in serial passage in whole or minced CAM suspended in Hank's BSS but with a higher yield from the latter. Growth curves with Hank's BSS show a lag and a log phase during the time interval from 0 to 60 hours. At 60 hours, growth curves were increasing, but by 36 hours the major portion of the log phase had been completed.
2. Several serial passages of virus were necessary for adaptation of IBV to this tissue culture system. Selective adaptation of IBV was indicated by (1) the increasing titers in subsequent passages, (2) the inability to recover the virus in the first passage at a concentration equal to the seed virus and (3) a shortening of the lag phase with subsequent passage.
3. Growth of IBV in this system was deleteriously affected by the absence of potassium, calcium, magnesium and glucose in the extracellular medium. Growth curves in these cases showed a longer lag phase and a decline phase which differed from Hank's BSS. The log phase was the same but at a lower numerical level. The decline phases were prominent at 36 and 48 hours.
4. Potassium, calcium, and magnesium are known activators of certain enzyme systems present in the anaerobic glycolysis and Kreb's cycles and may be responsible for inhibiting viral production by a lack of activation of such enzymes. Intracellular supplies of these ions and glucose are sufficient to

support viral synthesis at a reduced rate and shorter time.

5. The absence of glucose, the only energy-supplying constituent of Hank's BSS, inhibited maximum production of IBV. Intracellular glycogen being responsible for the energy produced through the metabolic pathways of the anaerobic glycolysis and Kreb's cycles.

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