SOME PROPERTIES OF INFECTIOUS BRONCHITIS VIRUS PROPAGATED IN THE ISOLATED CHORIOALLANTOIC MEMBRANE

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SOME PROPERTIES OF INFECTIOUS BRONCHITIS VIRUS PROPAGATED IN THE ISOLATED CHORIOALLANTOIC MEMBRANE

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ABSTRACT

Infectious bronchitis of chickens is an acute, contagious respiratory disease which is of economic importance to the poultry industry.

The isolated chorioallantoic membrane maintained in Hank's balanced salt solution nas been found to be suitable for the propagation of infectious bronchitis virus, Tarpeia pulli.

The purpose of the present study was to investigate some properties of the Beaudette embryo adapted strain of infectious bronchitis virus which was adapted to and propagated through 36 serial passages in isolated chorioallantoic membrane cultures.

The growth curve of the virus exhibited five phases: a variable lag phase of 8 hours, a logarithmic phase during the next 52 hours, a primary decline phase over the following 60 hours, a stationary phase of 72 hours, and a secondary decline phase of 48 hours.

The optimum incubation temperature was found to be 37°C at pH 7.0. The maximum yield of virus was obtained from cultures containing 200-600 mg of chorioallantoic membrane. Cultures incubated at 37°C for 24 to 81 hours prior to inoculation yielded the highest concentration of virus. Cultures inoculated immediately after preparation or 105 hours' incubation had a lower yield. The yield was lowest from cultures incubated for 144 and 216 hours.

Viral infectivity decreased at the following rates: log ID_{50} 0.113 per week at -25°C, and log ID_{50} 0.63 per hour at 37°C. During

the first 30 minutes at 45°C, the rate was log ID_{50} 0.154 per minute followed by log ID_{50} 0.0053 per minute for the subsequent 90 minutes. The activation energy for inactivation for the virus was 19.46 X 10^3 calories per mole.

Chemical fractionation failed to reveal association of infectivity with the ribonucleic acid portion of the virus.

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INTRODUCTION

Tissue culture methods permit study of the host-parasite relationship and multiplication of viruses at the cellular level.

The isolated chorioallantoic membrane maintained in a chemically defined medium has been found to be suitable for the propagation of several animal viruses.

Cultivation of infectious bronchitis virus in chorioallantoic membrane suspended in Hank's balanced salt solution offers the following advantages: (1) Ease of preparation; (2) Large volume of culture may be obtained; (3) Serum, tissue extract, and other growth factors are not required; (4) The virus is free from other proteins.

The objective was to study some properties of infectious bronchitis virus propagated in the isolated chorioallantoic membrane suspended in Hank's balanced salt solution.

LITERATURE REVIEW

Infectious bronchitis is caused by the virus, Tarpeia pulli.[60] It is an acute and contagious respiratory disease of chickens first described by Schalk and Hawn[54] in 1931. Symptomatically the disease is similar to Newcastle disease and laryngotracheitis. The mortality may be 70 to 80 per cent in chicks but death losses are negligible in older birds. The course of the disease is usually one to two weeks, but it may persist for several weeks in some instances. Broadfoot et al.[12,13] and Sevoian et al.[56] found that egg production and egg quality were markedly affected. After infection of adult birds several months may elapse before egg production returns to normal.

Infectious bonchitis virus (IBV) can be readily isolated from the lung and trachea of affected chickens. Fabricant and Levine[30] studied the persistence of the virus in eggs laid during and after an experimentally induced infection. The virus was first detected in the yolk two days after inoculation of the birds. The last successful virus isolation was from eggs laid 43 days after infection. The virus was also recovered from the trachea between one and four weeks after infection.

Beaudette and Hudson[9] first cultivated IBV in the chorio-allantoic membrane (CAM) of embryonating chicken eggs. They found that the virus did not produce gross lesions such as those produced by the viruses of laryngotracheitis, fowl pox, and pigeon pox. They also observed that with serial passage, the virus increased in virulence

and became lethal to the embryo.

Delaplane and Stuart[24] confirmed the observations of Beaudette and Hudson[9] and also observed modification of the virus as the result of passage in the CAM. There was a decrease in virulence for chickens accompanied by a loss of immunogenicity.

Delaplane[23] reported that the allantoic cavity route of inoculation was superior to the chorioallantoic membrane route for initial isolation of the virus. Dwarfing of embryos, which is a characteristic of IBV infection, was detected in the first passage via the allantoic cavity.

According to Loomis et al.[44] gross lesions produced in chicken embryos after inoculation of IBV by the allantoic cavity route consist of the following:

1. embryos dwarfed as much as one-half normal size and having a firm ball-like shape characterized by curling with a wry neck and feet deformed and compressed over the head,
2. congestion of the liver and deposition of urates in the kidney,
3. living embryos are sluggish in their movements,
4. the chorioallantoic membrane is thinner than normal and adherent to the inner shell membrane,
5. there are no visible lesions on the chorioallantoic membrane,
6. the amnionic membrane is thickened, dry, fibrotic, resists removal from the embryo, and restricts movements of the embryo.

7. feather development is immature and dry.

According to a study by Cunningham and Jones[21] on the effect of different routes of inoculation on the adaptation of the Van Roekel strain of IBV to the chicken embryo, chorioallantoic membrane inoculation produced mortality rates that did not show marked variation from passage to passage. The mortality rates following amnionic cavity inoculation were uniformly higher for the first five passages than those by other routes. Increasing adaptation of the virus by the allantoic cavity route produced higher mortality rates in the sixth and seventh passages.

The allantoic cavity route is the most desirable for primary isolation of IBV due to the simplicity of operation and the appearance of pathological lesions in the first passage.[21]

Distribution of the Beaudette egg-adapted strain of IBV in the chicken embryos was studied by Cunningham and El Dardiry,[20] who found the greatest concentration of the virus to be in the chorioallantoic membrane followed in decreasing order in the allantoic fluid, amnionic fluid, and liver. Yolk was innocuous. The maximum titer of the virus was obtained at the 36th hour after inoculation. The titer of the virus was greater in materials collected from living embryos than from embryos dead at the same postinoculation interval. Allantoic fluid preparations of the virus stored at -35°C retained the initial titer for 30 days, but storage for 60 days resulted in a ten-fold decrease in titer. The virus was thermolable in eggs subjected to 99°F for 8 to 12 hours after the death of the embryo. The maximum embryo mortality rate occurred between the 24th and 36th hour after inoculation.

Hofstad[40] found differences in the thermostability of 60 different isolates of IBV cultivated in eggs. When virus infected allantoic fluid diluted 1 : 100 in nutrient broth containing 20 per cent horse serum with a final pH of 7.2 was subjected to 56°C, some strains were inactivated within 15 minutes while others were infective after 45 minutes.

Page[48] reported that the Beaudette strain of IBV in the form of infected, undiluted allantoic fluid was inactivated within 10 minutes at 56°C, 60 hours at 37°C, and 25 days at 22-25°C. At the end of 20 weeks at 4°C, the virus was still infective.

Singh[57] studied the thermostability of 13 strains of IBV at various egg passage levels. Most strains of the virus in the form of undiluted infected allantoic fluids were inactivated within 45 to 120 minutes at 56°C. Quantitative studies showed that thermal inactivation of IBV followed a three halves order reaction rate which was indicative of a bimolecular reaction. It was assumed that in an early egg passage the virus existed in two phases: (1) O phase, in which some particles retained their original identity and were relatively thermostable, and (2) D phase, in which some particles were derived as a result of embryo culture and were thermostable.

Bachrach et al.[8] reported that thermal inactivation of type A, strain 119 of foot-and-mouth disease virus in bovine kidney tissue culture did not follow a first order reaction, and that the activation energy for inactivation of the virus culture was 27,200 calories per mole below 43°C.

Bourdillon[10] reported that thermal inactivation of SK strain

of poliomyelitis virus did not obey a first order reaction.

The maximum titer of IBV was obtained in eggs between 18 and 26 hours with Japanese strains[52] and between 24 and 30 hours with the Connaught Laboratory Vaccine Strain.[38]

There are only a few reports on the propagation of IBV in tissue culture. Fahey and Crawley[31] cultivated the Connaught Laboratory Strain and the Beaudette embryo adapted strain[9] in CAM suspended in medium 597 (medium 199 minus purine and pyridine plus Hank's balanced salt solution). Maximum infectivity as determined by the 50 per cent infectivity dose (ID_{50}) in embryonating chicken eggs was obtained between the 48th to 54th hour with both strains. With the Connaught strain the ID_{50} was $10^{5.1}$. The titer of the Beaudette strain ranged from ID_{50} $10^{5.28}$ to $10^{8.28}$. Both strains were grown in monkey kidney and chicken-embryo-heart cells but at a lower titer than with the CAM culture. No cytopathogenic effects were observed in infected cells.

Buthala and Mathews[15] failed to observe cytopathogenic changes in monolayer cultures of chicken-embryo-kidney cells after inoculation with a New York strain of IBV.

Chomiak et al.[16] observed a cytopathogenic effect in chicken-embryo-kidney cells in the second passage following the inoculation with the Beaudette strain. By the tenth culture passage the virus titered at $10^{-4.7}$ in cell cultures and 10^{-7} in embryos. In determining the growth curve in tissue culture it was found that the virus disappeared from the fluid in 4 hours, reappeared in 16 hours, and reached maximum titer at 48 hours, where it remained for 24 hours and

then declined. They also found that undiluted, infected allantoic fluid of both the Connecticut and Massachusetts strains of IBV passed serially five times in embryo kidney cells, failed to infect chicken embryos. The virus passed in embryo kidney cells caused cytopathogenic effects in chicken-embryo-fibroblast cultures. In the kidney cell cultures, the tissue culture propagated Beaudette strain of IBV was neutralized by both the Massachusetts and Connecticut type antiserum, but not by normal serum. However, the same virus, when tested in embryos, was neutralized only by Massachusetts type antiserum and not by normal serum or Connecticut type antiserum.

Wright and Sagik[64] reported plaque formation by the Beaudette strain of IBV in monolayers of chicken-embryo-kidney cells. After a 3-day incubation period, the plaques were from 3.0 to 4.0 mm in diameter.

Minced CAM culture was first used by Weller and Enders[63] for propagation of mumps and influenza A viruses. The nutrient medium consisted of 3 parts of Hank's balanced salt solution (BSS) and 1 part of Simm's oxblood-serum ultrafiltrate. In such media these viruses produced a measurable quantity of hemagglutinin. Fulton and Armitage[35], applying the same method,[63] described a new technique of titrating the infectivity of egg-adapted influenza virus by adding virus dilutions to minced CAM suspensions.

Robind and Enders[51] prepared an extensive review of studies of animal viruses in tissue culture. It was emphasized that many workers had investigated the factors influencing the growth of viruses such as composition of the medium, salt solution, type of tissue, and other

factors.

Ackermann[1] observed cellular respiration of the CAM following infection with the PR8 strain of influenza virus. Sodium malonate, 0.02 to 0.06M was found to have no inhibitory effect on the uptake of glucose by the membrane, but showed a partial inhibition of endogenous respiration of the tissue. Amounts of sodium malonate that reduced oxygen uptake inhibited viral propagation, but were not virucidal. The action of sodium malonate indicates at least one reaction which is a metabolic step in the Kreb's cycle. Succinic dehydrogenase is affected by malonate and without a continuous supply of di-and tricarboxylic acid, the entire cycle will be blocked.

Eaton et al.[29] found that butyl 3,5-diiodo-4-hydroxy-benzoate, which uncouples phosphorylation from oxidation, had effects very similar to dinitrophenol in inhibiting the growth of the PR8 strain of influenza virus in CAM cultures and de-embryonated eggs.

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

Ackermann and Maassab[3,4] studied the growth characteristics of the PR8 strain of influenza virus in CAM culture. The release or liberation of the virus from the CAM occurred without concurrent destruction of the membrane of the infected cell. There was no evidence of a general "burst" phenomenon, and the destruction of the cell membrane did not seem to be essential to, or concomitant with, the release of virus. An early phase in the development of the virus, 2 hours post infection, was described to be sensitive to the action of

α-amino-p-methoxyphenylmethane-sulfonic acid. It was by virtue of this that virus multiplication was prevented. If this phase was allowed to go on to completion, replication of virus occurred even in the presence of the sulfonic acid, but the release of virus from the tissue was impaired. It was suggested that sulfonic acid might interfere with the adsorption or penetration of the virus, and that the initiation of infection and the liberation of new virus might be processed to share the same common character. It was also found that infection could be initiated in the presence of methoxinine (viral inhibitor) under which condition viral increase was disallowed. The function in viral development of one biochemical process which was inhibited by methoxinine was found to be completed at an interval after initiation of infection, and before the appearance of the infectious form of the virus. It was possible to define four stages of viral development in terms of sensitivity to methoxinine and α-amino-p-methoxyphenylmethane-sulfonic acid.

Tamm and Tyrrell[59] studied the kinetics of multiplication of the Lee strain of influenza virus in the chorioallantoic membrane <u>in vitro</u> employing the hemagglutination technic for measurement of virus concentration. A linear relationship was found between the logarithm of virus adsorbed and amount of membrane used. Of the virus adsorbed, less than 10 per cent could be recovered from the membrane. Of the recoverable virus 90 per cent, which in itself constituted only 2.5 per cent of the virus adsorbed, was neutralized by specific immune serum. Lee virus was adsorbed by the allantoic and chorionic layers of the membrane to a similar extent. Virus produced in membranes was

liberated rapidly and continually into the medium.

Ackermann et al.[5] found that under certain conditions, the PR8 strain of influenza virus might bind to the chorioallantoic membrane and the infectious property was retained upon prolonged incubation of the complex. Apparently the bound active virus was not functioning in the initiation of viral increase. The bound infectious virus might be partially removed by extensive washing. The characteristics of the washing were suggestive of a reversible equilibrium type of binding. It was hypothesized that active virus was held by two types of binding at the same site; one type of binding being sensitive to the action of receptor destroying enzyme (RDE) of Vibrio cholerae, the second type being sensitive to the blocking effect of α -amino-p-methoxyphenylmethane sulfonic acid (AMPS). Virus could be held to the receptor site by either type of binding or both.

It was found by Ackermann and Maassab[6] that p-fluorophenyl-alanine was phase-specific, did not interfere with the initiation of infection, but rather acted during the productive period of the infectious sequence.

Eaton et al.[28] investigated the effect of thyroxin on CAM culture, and observed an increase in oxygen consumption of membrane suspended in BSS with 0.01 to 1.0mg/ml after a lag period of about 12 hours. A slight stimulation of growth of the PR8 allantoic passage strain of influenza virus was observed in the presence of thyroxin.

The reversible effect of hypotonic solutions on growth of influenza virus in CAM cultures has been studied by Eaton and Scala[27]. Growth of the virus was inhibited by exposure of the infected CAM

to Hank's BSS diluted 1:2 or 1:4. Sodium chloride added 18 or 24 hours after infection, although it stimulated growth of the virus, did not increase tissue proliferation. Loss of virus from heavily infected tissue occurred on exposure to 50, 35, or 25 per cent BSS. This was prevented by adjusting the solution to near normal osmolarity with glucose.

Ishida and Ackermann[41] investigated the characteristics of the initial cell-virus complex using influenza A virus, and found that the complex was stable to dilution and to simple washing with buffered saline. It was insensitive to the action of RDE, but sensitive to the inhibitory effect of anti-viral serum. It was also found that the cell-virus complex could be formed at 3°C.

Daniels et al.[22] found minimal production of the PR8 strain of influenza A virus in glucose deficient BSS when employed with the CAM adherent to the shell of de-embryonated eggs. When glucose was added virus production was stimulated.

According to Levine et al.[43] potassium deficiency was found to suppress: (1) growth of chorioallantoic epithelium and fibroblasts, (2) multiplication of the PR8 strain of influenza virus, (3) respiration of normal or virus-infected tissue by about 10 per cent. The results also indicated that potassium ion deficiency affected the virus-host interactions in two ways: (1) accessibility of virus attached to tissue to neutralization by immune serum, (2) intracellular synthesis of high energy bonds which was necessary for virus reproduction.

According to Nagata et al.[47] influenza virus and Newcastle

disease virus could be subcultured in minced CAM suspended in medium containing only NaCl and KCl, but viral infectivity diminished after three or four subcultures.

Scott et al.[55] demonstrated that Newcastle disease virus (NDV) grew in isolated CAM suspended in Tyrode's solution. The Roakin-MJ-1946 strain of NDV was propagated at 36°C and maintained through 30 serial passages in isolated CAM cultures. The factors influencing the growth of NDV in CAM culture have been studied by Zuschek et al.[65,66,67]. The maximum titer was obtained in cultures of CAM suspended in Tyrode's solution at pH 9.5. The smallest yield of virus was obtained at pH 2.7. The maximum titer was also obtained with Tyrode's solution that contained 0.2 gm of $CaCl_2$ per liter. Greater concentrations of $CaCl_2$ appeared to deter growth of the virus. Potassium was also found to be essential. Maximum yields of virus were obtained from the CAM suspended in Tyrode's solution that contained 0.4 gm of KCl per liter. Virus growth was inhibited in Tyrode's solution that was deficient in potassium. Magnesium ions did not overcome the inhibition of growth of the virus in Tyrode's solution deficient in potassium. An excess concentration of magnesium and glucose produced inhibitory effects on the virus. No significant inhibition of NDV was demonstrable in Tyrode's solution that contained 0.01 M of urethane, sodium malonate, or sodium fluoroacetate, but the growth decreased as the concentration of 2,4-dinitrophenol was increased. Proflavin and sodium azide, at concentrations of 5 to 40 gm per milliliter were effective in inhibiting the production of NDV. Growth of the virus was limited to incubation between 33°C and 42°C but it grew best at 42°C. Production of the virus

followed a linear response after 36 hours of incubation.

Brandt[11] cultivated twelve strains of NDV in roller cultures of minced CAM. Intracytoplasmic inclusion body formation in epithelial cells grown on cover slips was demonstrated with NDV and high-egg-passage mumps virus.

Numerous attempts by Dunham and Ewing[25] to establish Type 2 poliovirus in the CAM membrane of chicken embryos were unsuccessful. Strains of cells derived from the CAM and grown in vitro were readily infected after 5 transfers with not only Type 2 poliovirus but also Types 1 and 3. Multiplication of these viruses and cytopathogenic effects on the cultured cells were observed until the series were terminated after 10 or 20 passages.

In general, all viruses appear to consist mainly, and some entirely, of proteins and nucleic acids. The nucleic acids of plant and animal viruses can be either of the ribonucleic acid (RNA) or desoxyribonucleic acid (DNA) type, while bacterial viruses contain only DNA. Animal viruses appear to contain either DNA or RNA but probably not both. The viruses which are known to contain RNA are those of foot-and-mouth disease,[14] eastern and western equine encephalomyelitis, poliomyelitis, influenza, mumps,[50] fowl plague,[53] and Newcastle disease.[11] Viruses which are known to contain DNA are those of vaccinia, psittacosis, herpes, adeno, and rabbit papilloma.[50]

The infective RNA of Type 1 and 2 poliovirus was isolated by Alexander et al.[7] by the method of Gierer and Schramm who isolated infectious RNA from tobacco mosaic virus (TMV) with water saturated phenol in 1956.

Colter and Bird[17] isolated an active RNA fraction of Meningoencephalitis virus from infected Ehrlich ascites tumor cells.

Infective RNA was also isolated from tissues infected with eastern equine encephalomyelitis virus by Wecker and Schäfer[62] by treatment with phenol. Previous to this work, Schäfer[53] found no infectivity of RNA obtained after phenol treatment of fowl plague virus. The reason for this was not clear. It was speculated that the RNA of fowl plague virus was relatively unstable or that, in contrast to tobacco mosaic virus, two units of RNA were needed for the formation of new infectious particles; one for the production of g-antigen and the other for the production of hemagglutinin.

Infective RNA has been isolated from the viruses of mouse encephalomyelitis[34] and foot-and-mouth disease[14] by treatment with phenol.

Furthermore, recent experiments by Kaper and Steere[43] have demonstrated the infectivity of tobacco ringspot virus nucleic acid prepared by a modification of the heat-denaturation method, which uses hot NaCl for extraction.

Wecker[61] extracted infectious RNA from tobacco mosaic virus with hot phenol.

These facts suggest that the nucleic acid fractions of animal viruses might be the infectious units and the carriers of genetic information as in tobacco mosaic virus and also as in DNA of bacteriophage.

MATERIAL AND METHODS

Virus:

The Beaudette strain of IBV, North Central Infectious Bronchitis Virus Repository, Code 42, was used throughout the experiment. This is an egg-adapted strain capable of killing 10-to 11-day-old chicken embryos within 36 hours after inoculation via the allantoic cavity. It has been through hundreds of serial passages in chicken embryos, but the exact number is unknown.

Hank's Balanced Salt Solution:

The balanced salt solution (BSS) was made with the following ingredients[37,19] on a basis of grams per liter of double distilled water: NaCl, 8.0; KCl, 0.4; $Na_2HPO_4.12H_2O$, 0.15; KH_2PO_4, 0.06; $MgSO_4.7H_2O$, 0.1; $MgCl_2.6H_2O$, 0.1; $NaHCO_3$, 0.35; glucose, 1.0; and phenol red 0.02gm. Calcium chloride, 0.14gm, was prepared separately to avoid flocculation, and added to the other ingredients after sterilization in an autoclave for ten minutes at ten pounds pressure. Penicillin, 10,000 units, and streptomycin, 10 mg per 100 ml were added. The BSS was at approximately pH 7.5

Preparation of Chorioallantoic Membranes and Propagation of virus:

Chorioallantoic membranes were collected from 10-to 12-day old embryonating chicken eggs from a commercial, disease-free flock. The eggs had been in a Jamesway Model 252 incubator at 99-99.5°F. The CAM was removed aseptically from the egg and washed in a sterile

petri dish containing about 20 ml of sterile physiological saline. The membrane was then transferred through three succesive washings in 200 ml of sterile saline contained in 500 ml Erlenmeyer flasks. The flasks were shaken vigorously by hand for thorough washing of the membranes. After the third washing, the CAM was removed to a sterile petri dish, and minced into small fragments of 2 to 5 mm with scissors. The fragments were then placed in an 8 ounce prescription bottle containing 19 ml of BSS. Unless specified otherwise, the cultures were incubated at 37°C. Minced CAM is the better medium for multiplication of IBV than whole CAM.[33]

It was necessary to adapt the embryo-cultivated virus to propagation in the isolated CAM in BSS by means of serial passage.[33] For the first passage virus-infected allantoic fluid was used, but virus-infected BSS from the CAM cultures was used for subsequent transfers. Virus from each passage was harvested 60 hours after inoculation and stored at -25°C until used. For the majority of the experiments virus from the 15th or higher CAM passage was used.

Infectivity Tests:

All titrations of viral infectivity were performed with 10- to 11-day old embryonating chicken eggs. In some instances, titrations were performed immediately after harvest of the virus-infected fluid, but in others it was necessary to store the samples at -25°C until they could be used.

Prior to titration, all samples of virus were centrifuged at 2,000 rpm for 5 minutes at 4°C with an International Centrifuge (Model PR-1) to sediment fragments of tissue which were present.

Serial ten-fold dilutions of the virus were made using 4.5 ml of sterile Difco nutrient broth and 0.5 ml of virus. Each of 5 embryos per dilution was inoculated with 0.1 ml via the allantoic cavity. After inoculation the eggs were re-incubated and candled once a day. Embryo mortality during the first 24 hours after inoculation was considered to be due to non-specific causes, and these embryos were not included in calculations of mortality rates. On the 9th day after inoculation, all living embryos were examined for gross pathological changes associated with infection with IBV.[44] Viral infectivity was calculated by the method of Reed and Muench[49] using embryo mortality and gross lesions as positive reponses. The results were expressed as the 50 per cent infectivity dose (ID_{50}) per 0.1 ml inoculum.

Isolation of Nucleic Acid:

The method described by Gierer and Schramm[36] for isolation of RNA from tobacco mosaic virus was used with a slight modification for attempts for isolation of RNA from IBV-infected fluid. Water saturated phenol was prepared and stored at 4°C. To each of two sterile 16 x 125 mm screw cap culture tubes in an ice bath, 3 ml of cold virus was added. An equal volume of water saturated phenol was added, the tube was closed tightly with a screw cap and the mixture was shaken vigorously by hand for 8 minutes at room temperature. After centrifugation for 4 minutes at 3,000 rpm at 4°C, the mixture had separated into the water and phenol phases. The water phase, 2.5 ml from each tube, was transferred to other tubes in ice bath each containing 2.5 ml of water saturated phenol. The mixture was again

shaken vigorously for 4 minutes, and then centrifuged. The water phase was transferred as before and shaken with an equal volume of water saturated phenol for 4 minutes and centrifuged. The total water phase, about 2 ml, was transferred to another culture tube and the phenol was removed by five successive extractions with an equal volume of peroxide-free ether. To remove the residual ether, dry nitrogen was bubbled through the solution at 0°C for about 10 minutes. The solution was adjusted to pH 6.8 to 7.0 with $NaHCO_3$ with phenol red as the indicator. This preparation will be referred as "undiluted" nucleic acid even though there was a slight dilution from adjusting the pH.

RESULTS

Adaptation of IBV to CAM Culture:

It was necessary to confirm a previous report[33] that the Beaudette strain of IBV had to be adapted by serial passage to propagation in CAM culture.

The result of the first and 6th passage of the virus is presented in Table I and Figure 1.

These data served as the basis for further investigations of the characteristics of the virus propagated in CAM culture, and emphasized that the maximum yield of virus was present 60 hours after infection. For all experiments, virus from the 15th passage in CAM culture or higher was used.

Growth of IBV in CAM Culture:

In order to ascertain the pattern of viral multiplication, twenty-seven cultures were prepared and inoculated with the 15th passage of the virus and incubated at 37°C. At certain periods, the infected fluids were collected from one culture and the virus was immediately titrated. The results are presented in Table II. The results for the first 60 hours after infection are shown in Figure 2.

The general pattern was a decline or lag phase from the initial titer of $10^{6.3}$ to a minimum of $10^{3.5}$ with a few fluctuations during the first 8 hours postinoculation. The lag phase was followed by a progressive increase or log phase to a maximum titer of $10^{6.5}$ at the 60th hour.

The results from the 8th hour through the 10th day are shown in Figure 3. From the maximum titer of $10^{6.5}$ at the 60th hour, there was a gradual decline during the next two and one half days to a titer of $10^{5.3}$ at the 5th day. Following this, there was a stationary phase during the next 3 days. After the 8th day there was marked decrease to a titer of $10^{4.4}$ at the 10th day.

Effect of pH on Growth of IBV:

Chorioallantoic membranes were suspended in BSS which previously had been adjusted to pH 4, 5, 6, 7, 8, and 9 by the appropriate addition of either 0.1 N HCl or 0.1 N NaOH. The pH of the BSS did not change appreciably after the addition of the CAM or the 17th passage of virus which was used as inoculum. At 8 and 60 hours after inoculation one ml of the infected fluid was collected and stored at -25°C prior to titration of viral infectivity and determination of the pH of the 60th hour samples. (Table III and Figure 4)

At the 8th hour, there was a direct relationship between pH of the BSS and increase in viral infectivity. One exception was noted where at pH 9 the viral infectivity was $10^{4.4}$ as compared to pH 8 where it was $10^{4.7}$.

At the 60th hour, maximum infectivity of $10^{6.8}$ occurred at pH 7.0 with lesser concentrations of virus at higher and lower pH values. The pH of the BSS collected at the 60th hour showed an increase of 1.5 and 0.5 units, respectively, for the initial values of pH 4 and 5. The BSS over the range of pH 6 to 9 showed a decrease of about 0.4 units for each initial value.

Effect of Temperature on Growth of IBV:

Chorioallantoic membrane cultures were incubated at 25, 30, 33, 35, 37, 39, 42, and 45°C for about two hours for thermal equilibrium prior to the inoculation with the 22nd passage of the virus. After 60 hours, the infected fluids were harvested and stored at -25°C until titrations were performed.

As shown in Table IV and Figure 5, maximum multiplication of the virus occurred at 37°C where the titer was $10^{6.2}$. From 25°C to 37°C there was a direct relationship between infectivity and increased temperature of incubation.

Growth of IBV in Different Amounts of CAM:

After the final washing, the CAM was blotted dry between layers of filter paper. The membrane was minced and 50, 100, 200, 300, 400, and 600 mg portions, respectively, were used for cultures. The 22nd passage of IBV was used as inoculum and the cultures were incubated at 37°C. At the 8th and 60th hours, respectively, one ml of BSS was removed from each culture and stored at -25°C until titrated.

The results of the infectivity tests are presented in Table V and in Figure 6. At the 8th hour, there was a decrease in titer of all cultures, but the least amount of virus was present in those containing 50 and 600 mg of CAM. The greatest amount of virus was present in the cultures containing 200 and 300 mg of CAM.

At the 60th hour, there was an increase of virus in all cultures, but the greatest amount was present in those containing 200 mg or more of CAM. The least amount of virus was present in the cultures containing 50 and 100 mg of CAM. Using the extremes of the concentration

of virus, the titer of the culture containing 400 mg of CAM was about $10^{2.6}$ times higher than the culture containing only 50 mg of CAM.

As shown in Table V, the pH of the BSS at the 60th hour decreased in direct proportion to increasing amounts of CAM.

The Effect of the Time of Incubation of CAM Culture After Preparation and Prior to Inoculation with IBV:

A series of CAM cultures was prepared. One culture was inoculated immediately after preparation and the others were incubated at 37°C for 24, 57, 81, 105, 144, and 216 hours before inoculation with the 27th passage of IBV. The control, which contained 19 ml of BSS only, was also inoculated with the virus. After 60 hours' incubation, samples were removed from each culture and titrated immediately.

As shown in Table VI and Figure 7, the cultures incubated for 24 to 81 hours prior to inoculation had the highest concentration of virus. The cultures inoculated immediately after preparation or after 105 hours' incubation had a slightly lower yield. The yield was lowest from the cultures which had been incubated for 144 and 216 hours. The control was innocuous after 60 hours' incubation.

As a further test of the validity of the decrease of infectivity of the control, the 36th passage of the virus was used. The bottle containing only BSS and virus was shaken vigorously and one ml was removed and titrated immediately. The mixture was incubated at 37°C, and samples were removed at 4, 8, and 12 hours after inoculation. As shown in Table VII and Figure 8, there was a rapid decline of viral infectivity, and the sample collected at the 12th hour was innocuous.

Thermostability:

The 24th passage of virus was dispensed in 1.5 ml portions in a series of 12 X 75 mm test tubes and incubated at 45°C in a water bath. After 2 minutes, the time required for the solution to reach thermal equilibrium, one sample was removed. This was considered to be at zero time. At subsequent intervals of 5, 10, 15, 25, 30, 40, 50, 60, 70, 80, 90, 100, and 120 minutes, other samples were removed. Immediately after removal, each sample was frozen in an alcohol bath at -30°C, and stored at -25°C until titrated. The results of the titrations are presented in Table VIII and in Figure 9.

It is evident that inactivation was bimodal consisting of two first order reactions. During the first 30 minutes, inactivation proceeded according to the linear regression of $Y = 6.19 - 0.154$ X (minutes). From 30 minutes through 120 minutes inactivation was $Y = 1.71 - 0.0053$ X (minutes).

At 56°C, the virus, which had an initial titer of $10^{6.2}$, was almost completely inactivated within 4 minutes where only 2 of 5 embryos showed positive responses to the inoculum.

The 28th passage of the virus was dispensed in 1.5 ml portions in 8 ml screw cap vials and stored at -25°C. After 1, 2, 3, 4, 6, 8, 10, 12, and 14 weeks the virus was titrated. The results are shown in Table IX and Figure 10. The virus decreased from $10^{5.8}$ at the beginning of the experiment to $10^{4.2}$ at the 14th week, or $Y = 5.76 - 0.113$ X (weeks).

Extraction of RNA from IBV:

The 34th passage of IBV was adjusted to pH 7.0 with 1.4 per cent

$NaHCO_3$. At the end of the extraction process, only about 1 ml of the fraction considered to contain RNA was obtained from 3 ml of the virus.

The undilited RNA fraction and dilutions of 10^{-1} through 10^{-4} were inoculated into two chicken embryos each, 0.1 ml per embryo, via the allantoic cavity. Two 7-week-old chickens received 0.2 ml of the fraction by the intranasal, intramuscular and intratracheal routes. A CAM culture was also inoculated with 0.2 ml of the sample. The fluid from the CAM culture was harvested at the 60th hour and inoculated into eggs.

No evidence of infection with IBV could be detected in any of the eggs or chickens.

Activation Energy for Inactivation of Infectivity:

The energy of activation, E, was calculated from the Arrhenius plot[58].

In calculating E, the following assumptions have to be made: the energy of activation for inactivation of the virus followed the rates of a monomolecular reaction; exponential inactivation was obtained at each temperature; slight differences in pH of each sample did not affect the rate of inactivation of each sample; and difference in the passage of the virus did not cause marked differences in the rates of inactivation.

The K values converted to day^{-1} were obtained from the slopes of the lines of the equations for thermostability of the virus at -25°C, 37°C, and 45°C.

The slope of the Arrhenius plot was -4.26 from which E was

calculated to be approximately 19.46 x 10^3 calories per mole.

TABLE I

GROWTH OF 1ST AND 6TH PASSAGES OF IBV

IN CAM CULTURES

	Time-hours								
	0	5 min.	12	24	36	60	72	170	288
	Log ID_{50}/0.1ml.								
1st passage	6.0	2.3	3.5	3.8	3.5	---	---	---	---
6th passage	6.2	5.3	5.2	5.8	6.5	6.7	4.8	5.8	4.8

TABLE II

GROWTH OF 16TH PASSAGE OF IBV

IN CAM CULTURE.

Time	Log ID_{50}/0.1 ml.	Time	Log ID_{50}/0.1 ml.
0	5.0	36 hrs.	6.0
5 min.	4.2	42 hrs.	6.3
15 min.	4.4	48 hrs(2days)	6.3
30 min.	4.5	60 hrs.	6.5
45 min.	4.6	72 hrs.(3days)	5.7
60 min.	3.9	84 hrs.	5.6
2 hrs.	4.3	96 hrs.(4days)	5.7
4 hrs.	3.8	108 hrs.	5.5
8 hrs.	3.5	5 days	5.3
12 hrs.	4.5	6 days	5.5
16 hrs.	4.6	7 days	5.5
20 hrs.	5.5	8 days	5.5
24 hrs.(1 day)	5.7	9 days	4.7
30 hrs.	5.8	10 days	4.4

TABLE III

EFFECT OF pH ON GROWTH OF 18TH PASSAGE OF IBV

IN CAM CULTURE.

Initial pH of CAM culture	pH of CAM culture at 60th hour	Log ID_{50}/0.1ml at 8th hour	Log ID_{50}/0.1ml at 60th hour
4.0	5.2	3.0	1.3
5.0	5.5	3.3	3.3
6.0	5.6	3.6	6.3
7.0	6.7	3.8	6.8
8.0	7.6	4.7	5.3
9.0	8.6	4.4	5.0

TABLE IV

GROWTH OF 23RD PASSAGE OF IBV IN CAM CULTURE AT VARIOUS TEMPERATURES OF INCUBATION

Incubation							
25°C	30°C	33°C	35°C	37°C	39°C	42°C	45°C
Log ID_{50}/0.1ml.							
1.8	2.6	4.0	5.6	6.2	3.8	2.7	0

TABLE V

GROWTH OF 23RD PASSAGE OF IBV

IN DIFFERENT AMOUNTS OF CAM IN CULTURE

Amount of CAM (mg.)	Log ID_{50}/0.1ml 8th hour	Log ID_{50}/0.1ml 60th hour	pH of CAM culture at 60th hour*
50	2.2	4.2	7.3
100	2.6	4.3	7.0
200	3.3	6.5	6.8
300	3.3	6.6	6.6
400	2.6	6.8	6.5
600	2.0	6.2	6.3

* CAM cultures at pH 7.5 at time of inoculation.

TABLE VI

EFFECT OF TIME OF INCUBATION OF CAM CULTURE AFTER PREPARATION AND PRIOR TO INOCULATION WITH 28TH PASSAGE OF IBV.

Time of incubation						
0	24	57	81	105	144	216
Log ID_{50}/0.1ml.						
5.7	6.2	6.0	6.2	5.7	5.5	5.3

TABLE VII

EFFECT OF TIME OF INCUBATION OF 36TH PASSAGE OF IBV IN HANK'S BSS WITHOUT CAM CULTURE.

Time-hours			
0	4	8	12
Log ID_{50}/0.1ml.			
5.3	2.8	0.5	0

TABLE VIII

THERMOSTABILITY OF 25TH PASSAGE OF CAM CULTURED IBV AT 45°C.

Time minutes	Log ID_{50}/0.1ml.	Time minutes	Log ID_{50}/0.1ml.
0	6.3	40	1.5
5	5.8	50	1.3
10	4.3	60	1.3
15	3.5	70	1.5
20	2.8	80	1.5
25	2.5	90	1.3
30	1.8	100	1.0
		120	1.1

TABLE IX

THERMOSTABILITY OF 28TH PASSAGE OF CAM CULTURED IBV AT -25°C.

Time-weeks									
0	1	2	3	4	6	8	10	12	14
Log ID_{50}/0.1ml.									
5.8	5.5	5.5	5.3	5.5	5.3	4.7	4.8	4.2	4.2

FIGURE 1

GROWTH OF 1ST AND 6TH PASSAGES OF IBV IN CAM CULTURE.

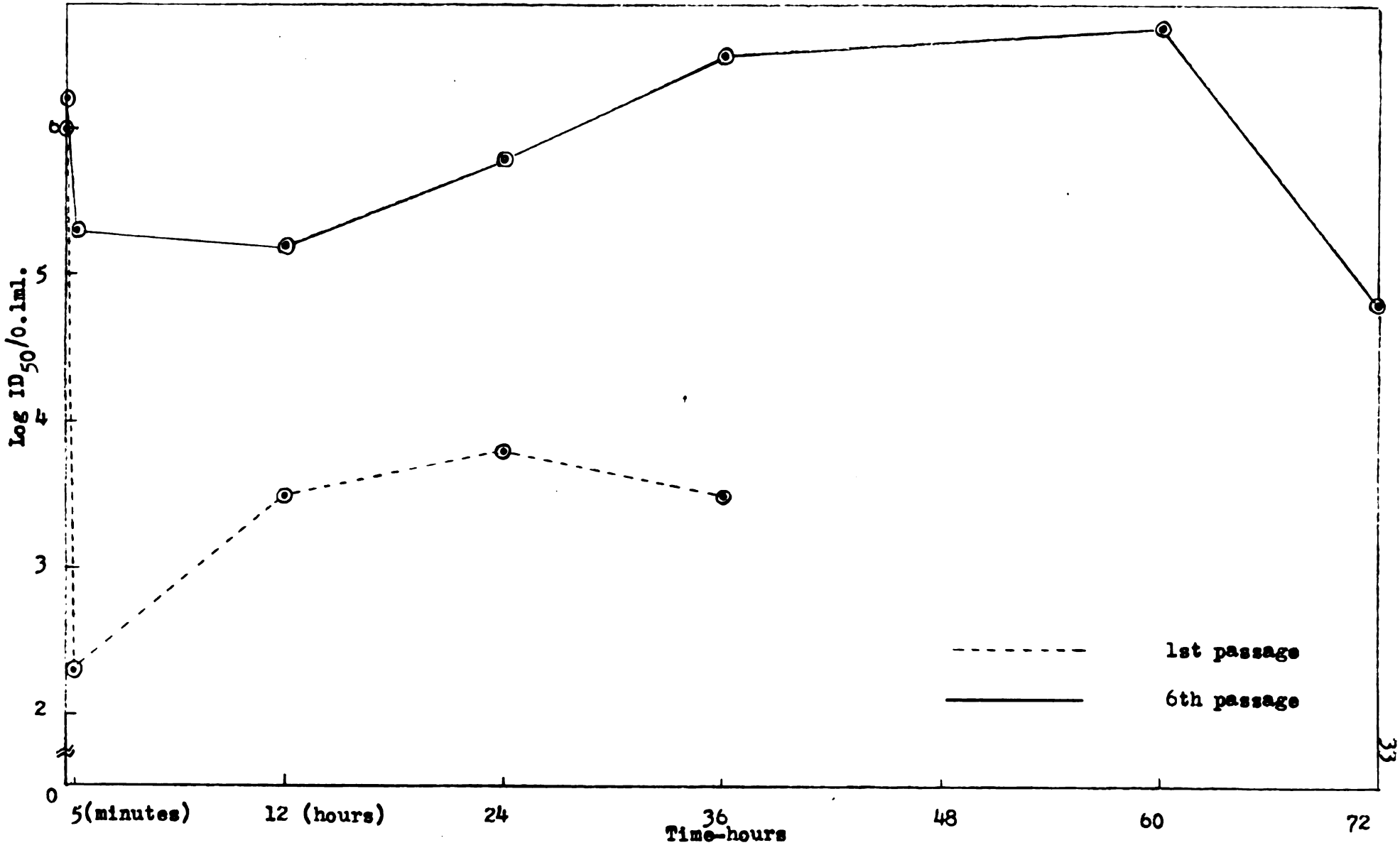


FIGURE 2

GROWTH OF 16TH PASSAGE OF IBV IN CAM CULTURE

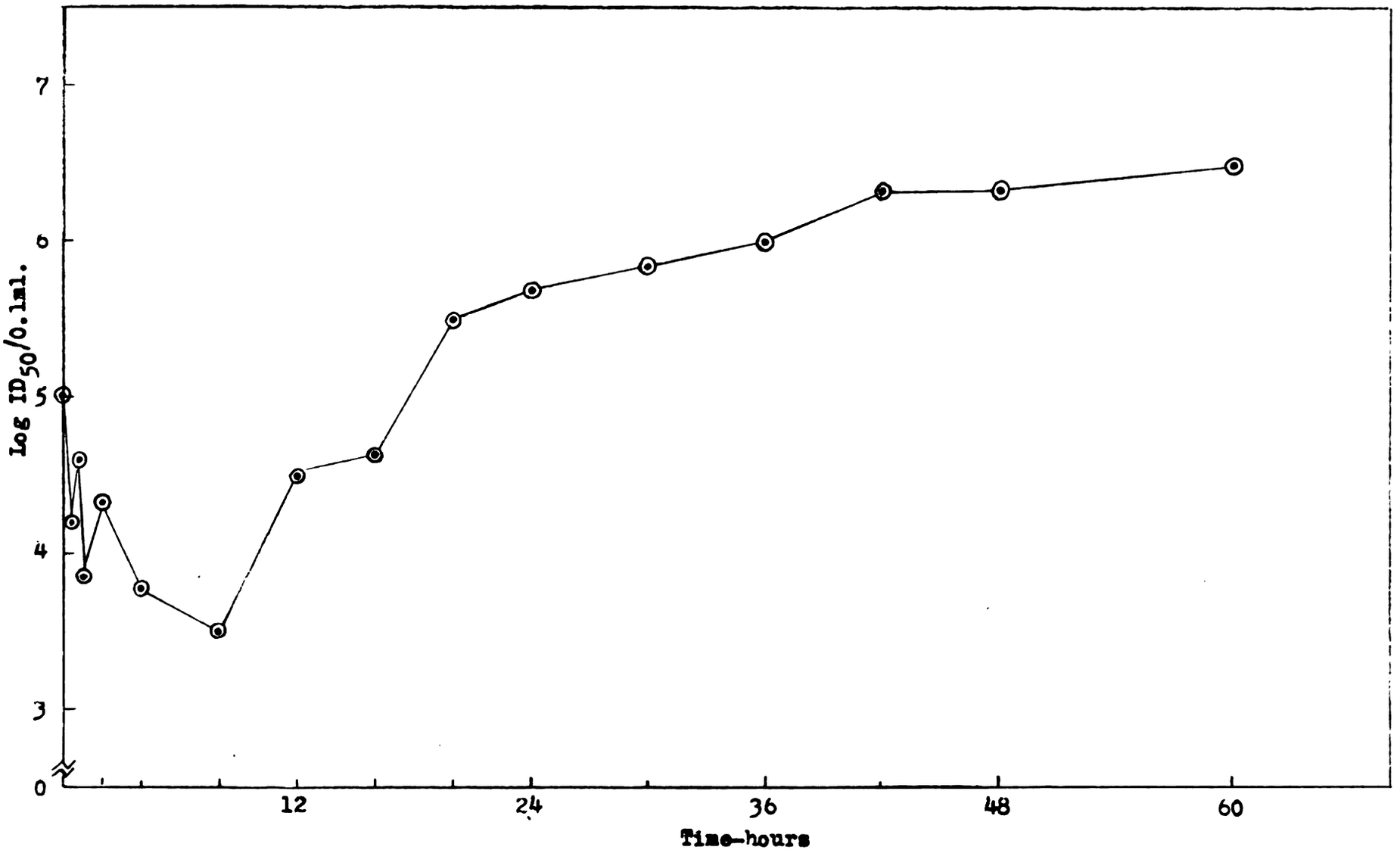


FIGURE 3

GROWTH OF 16TH PASSAGE OF IBV IN CAM CULTURE

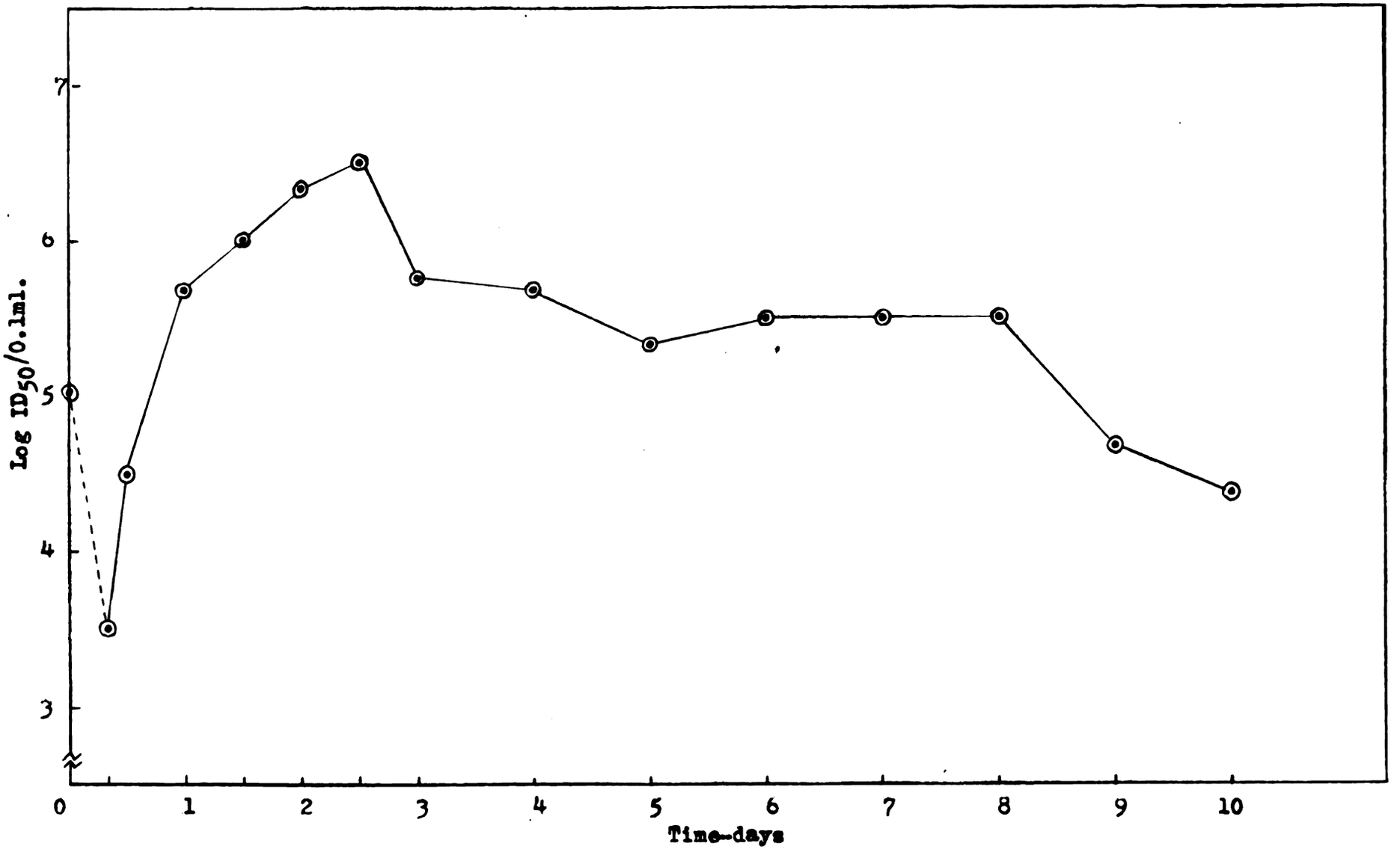


FIGURE 4

EFFECT OF pH ON GROWTH OF 18TH PASSAGE OF IBV IN CAM CULTURE

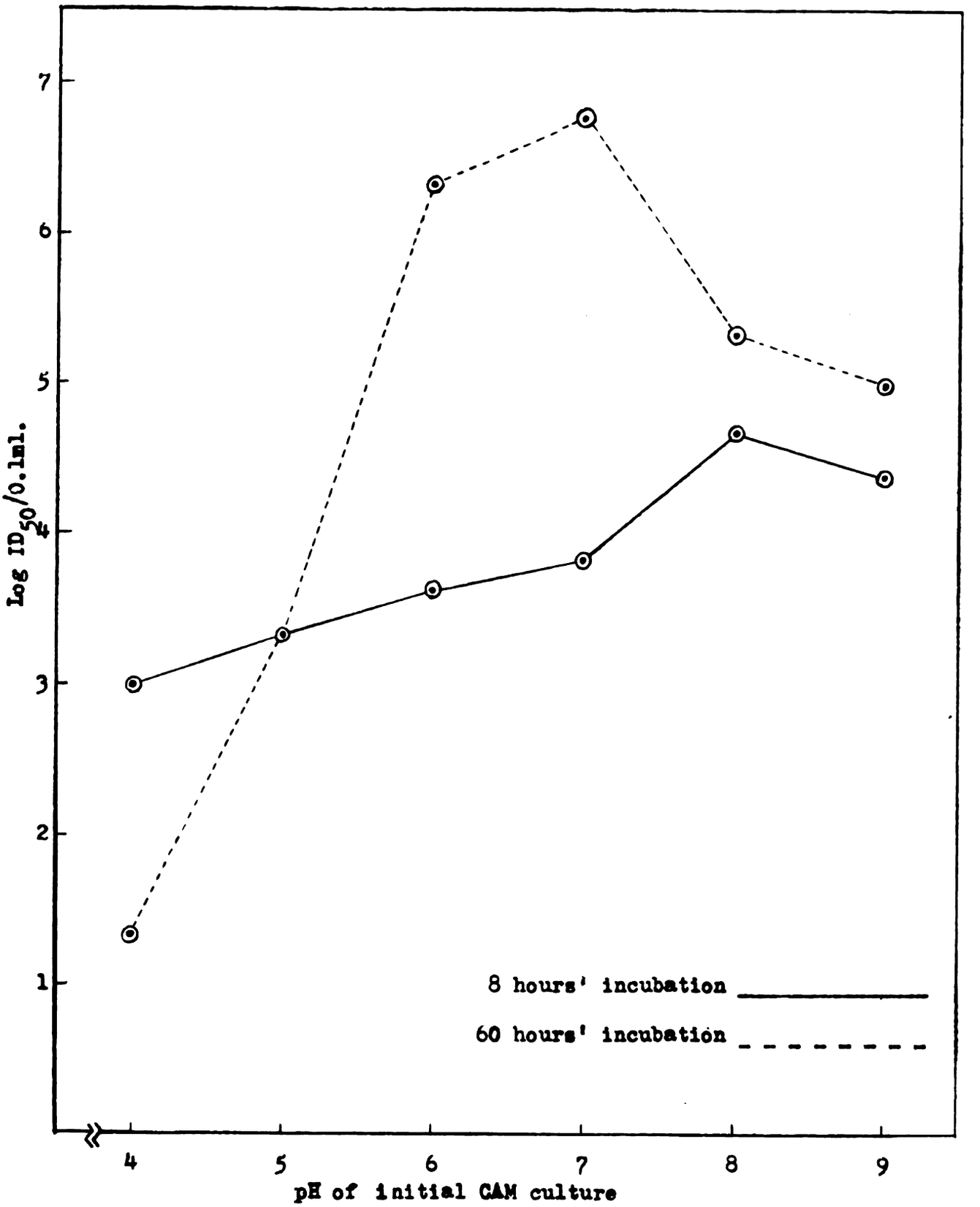


FIGURE 5

GROWTH OF 23RD PASSAGE OF IBV IN CAM CULTURE

AT VARIOUS TEMPERATURES OF INCUBATION.

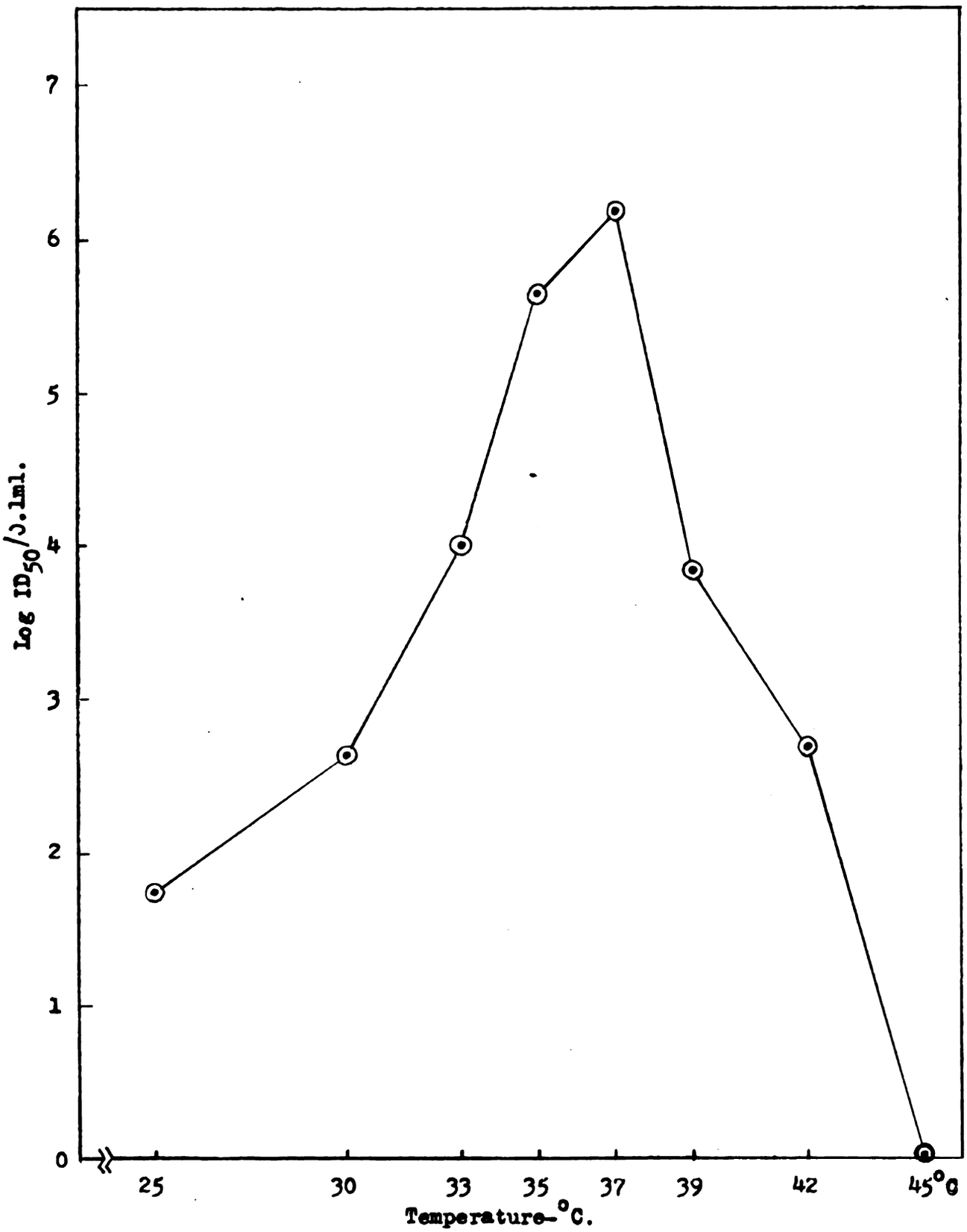


FIGURE 6

GROWTH OF 23RD PASSAGE OF IBV

IN DIFFERENT AMOUNTS OF CAM IN CULTURE

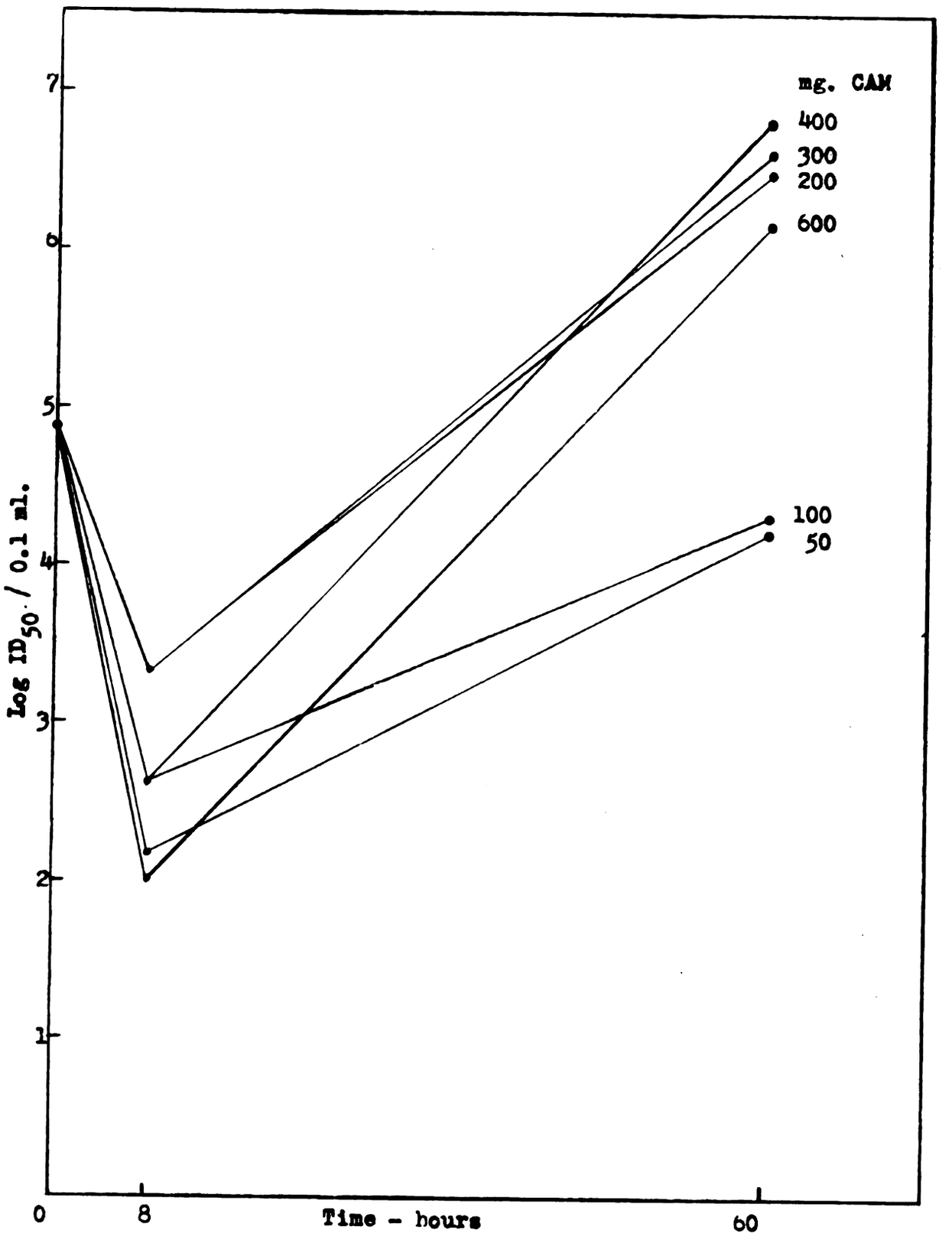


FIGURE 7

EFFECT OF TIME OF INCUBATION OF CAM CULTURE AFTER PREPARATION AND PRIOR TO INOCULATION WITH 28TH PASSAGE OF IBV.

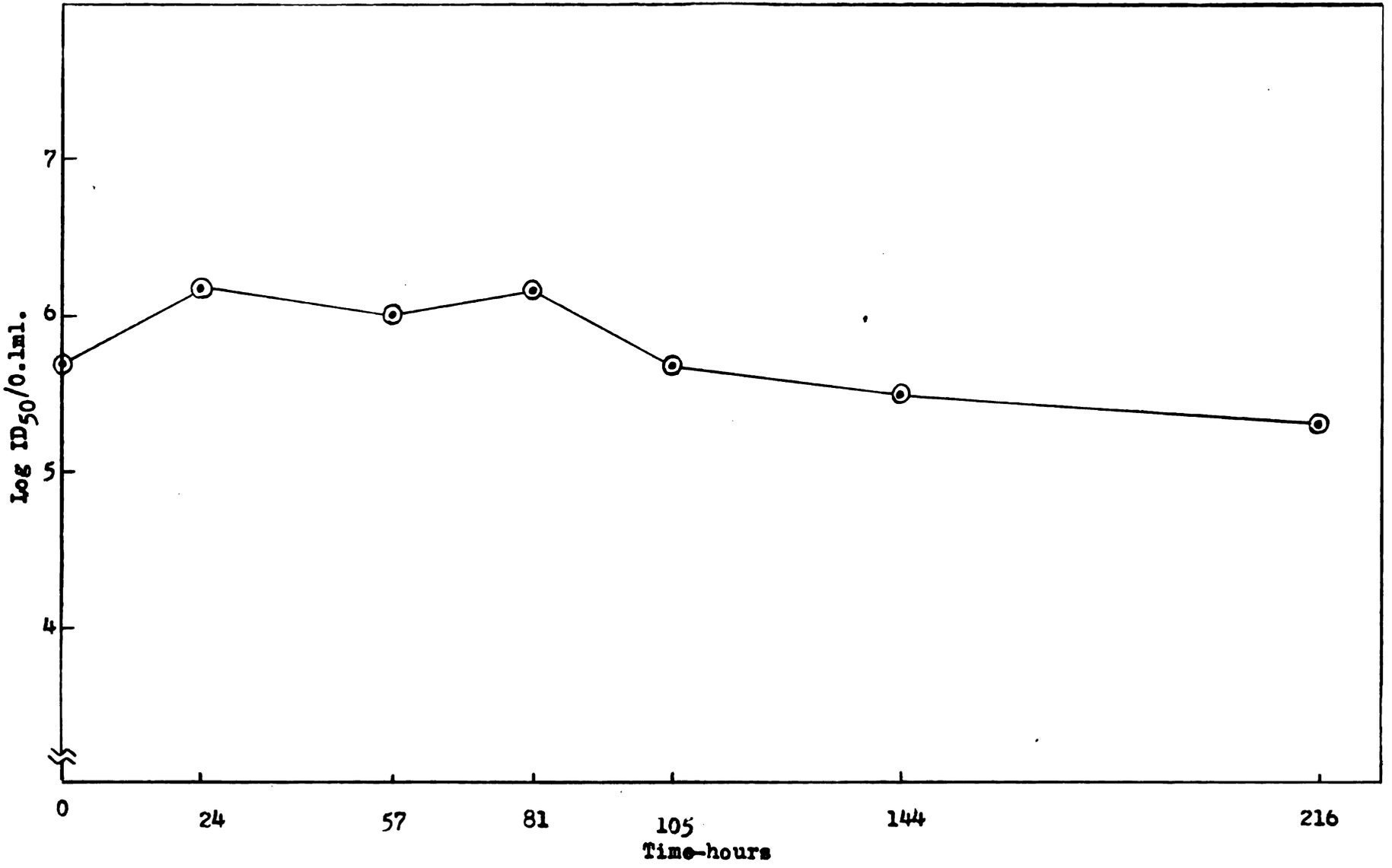


FIGURE 8

EFFECT OF TIME OF INCUBATION OF 36TH PASSAGE OF IBV IN HANK'S BSS WITHOUT CAM CULTURE

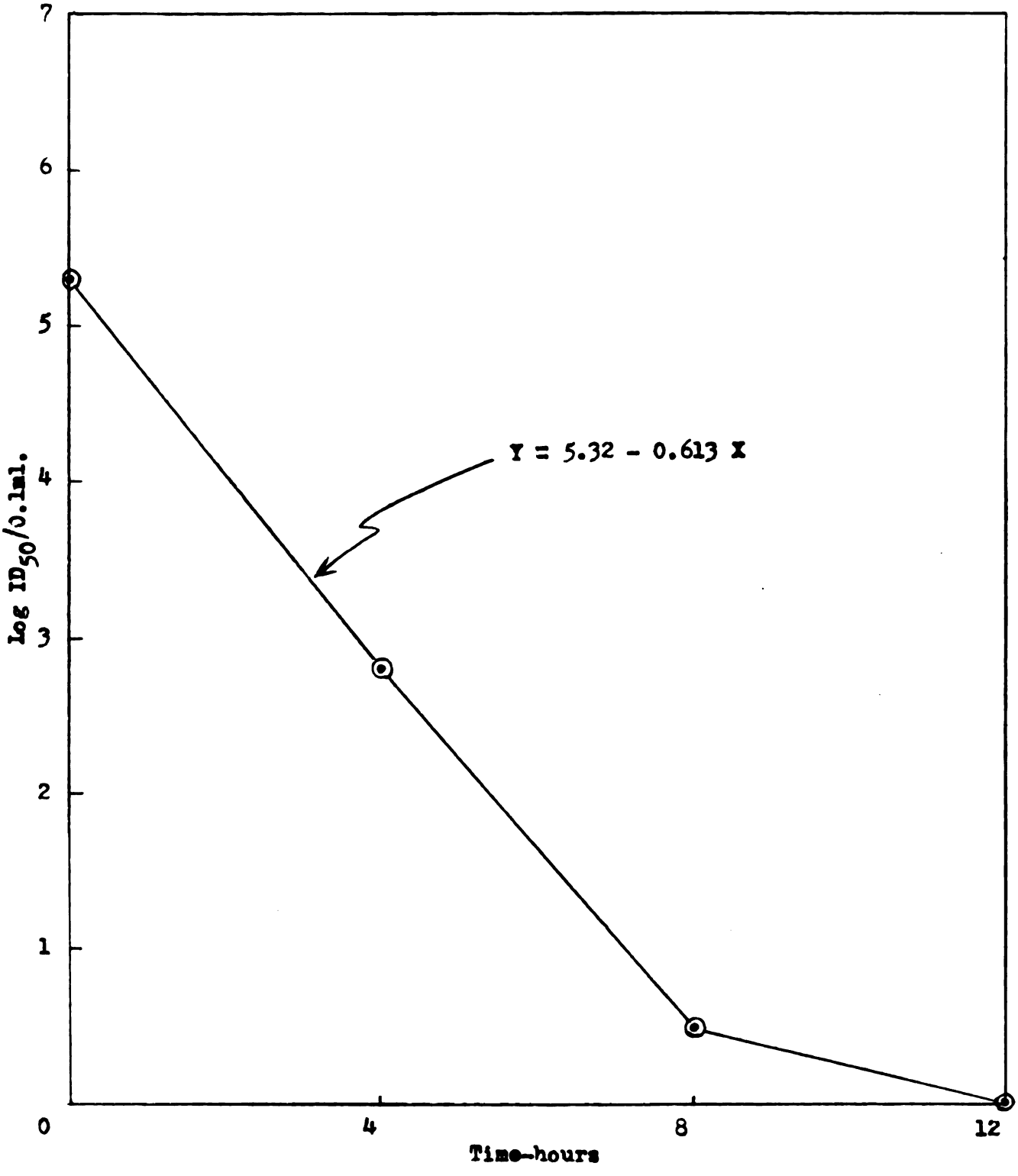


FIGURE 9

THERMOSTABILITY OF 25TH PASSAGE OF CAM CULTURED IBV AT 45°C.

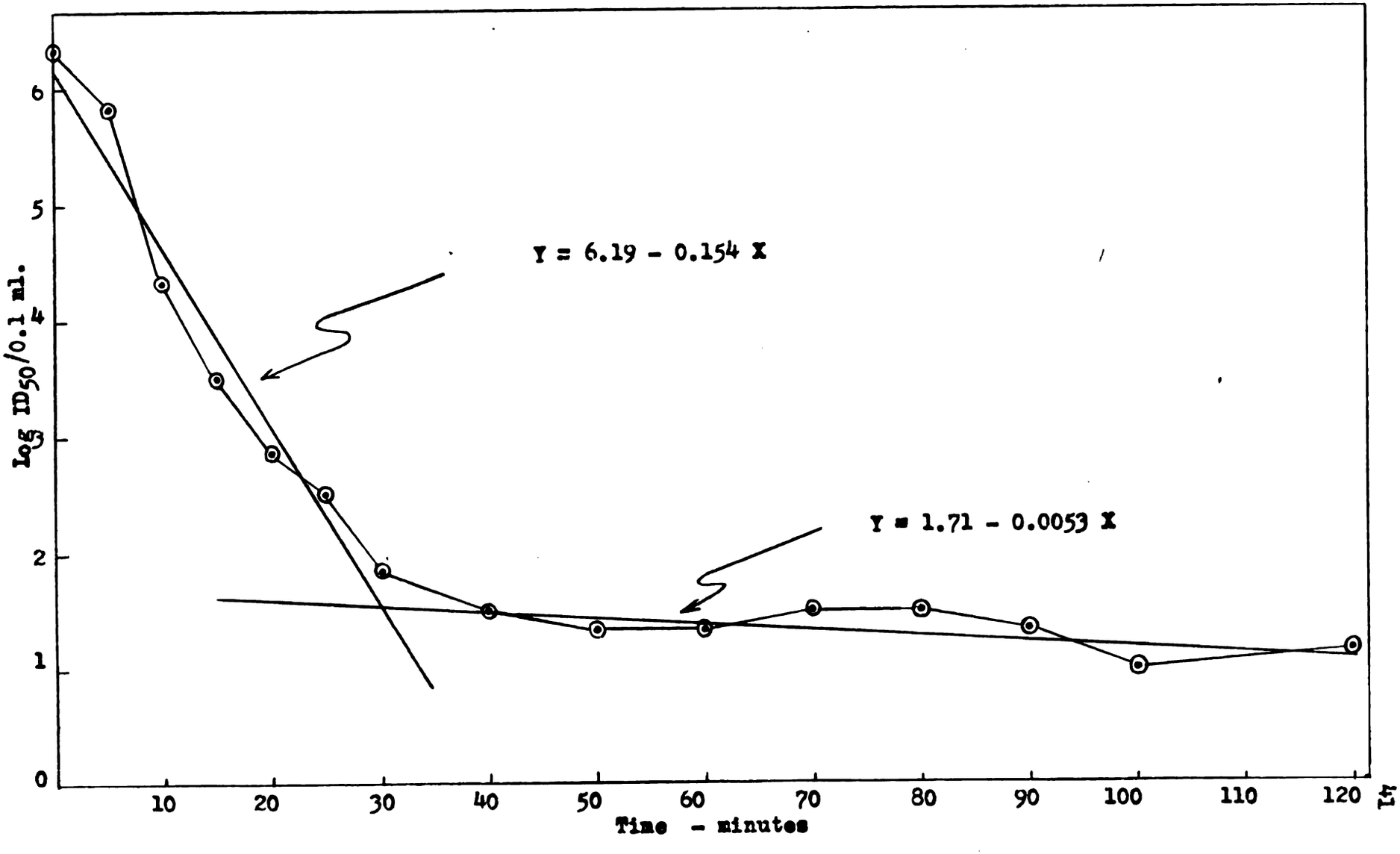


FIGURE 10

THERMOSTABILITY OF 28TH PASSAGE OF CAM CULTURED IBV AT -25°C.

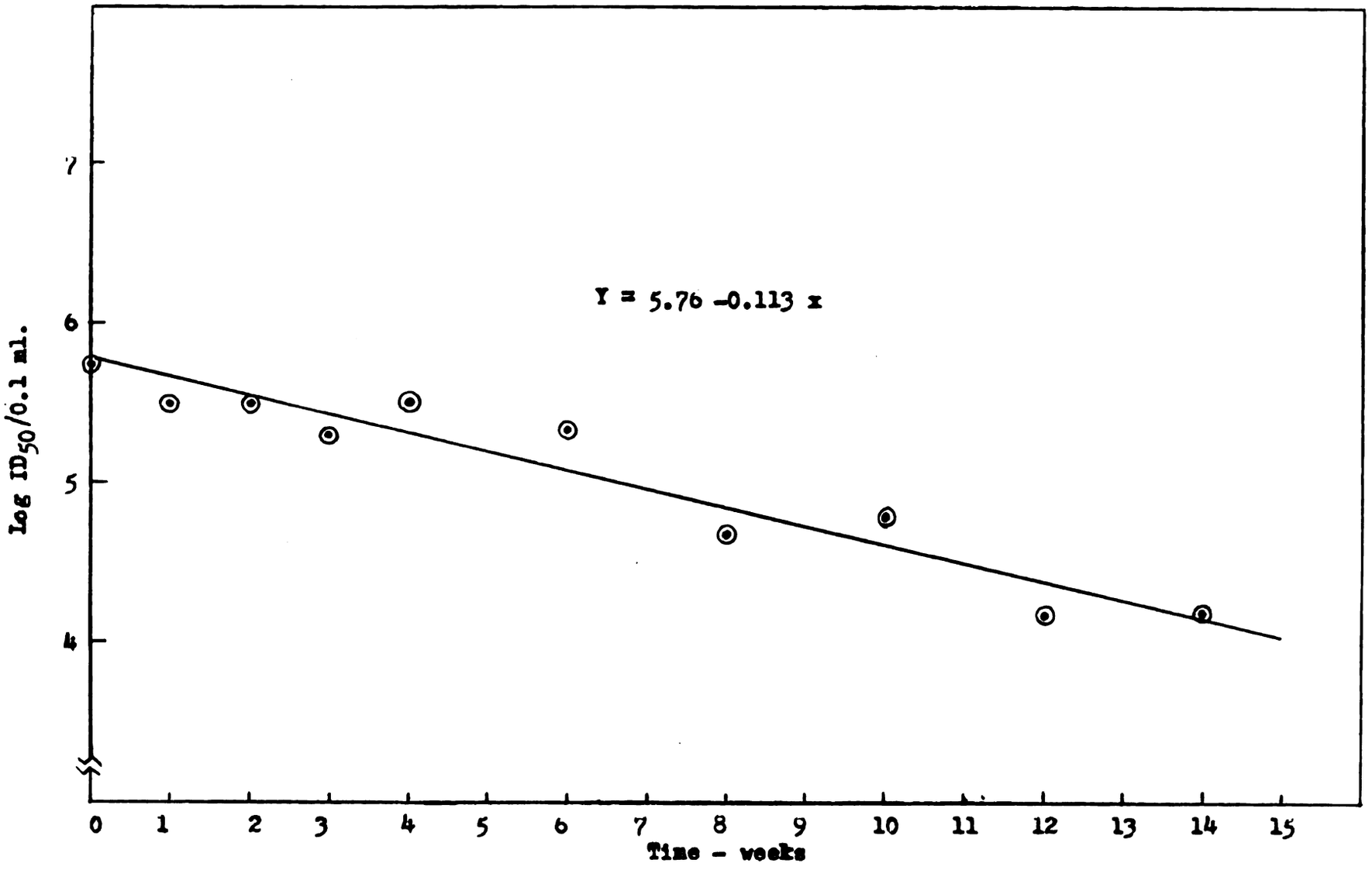
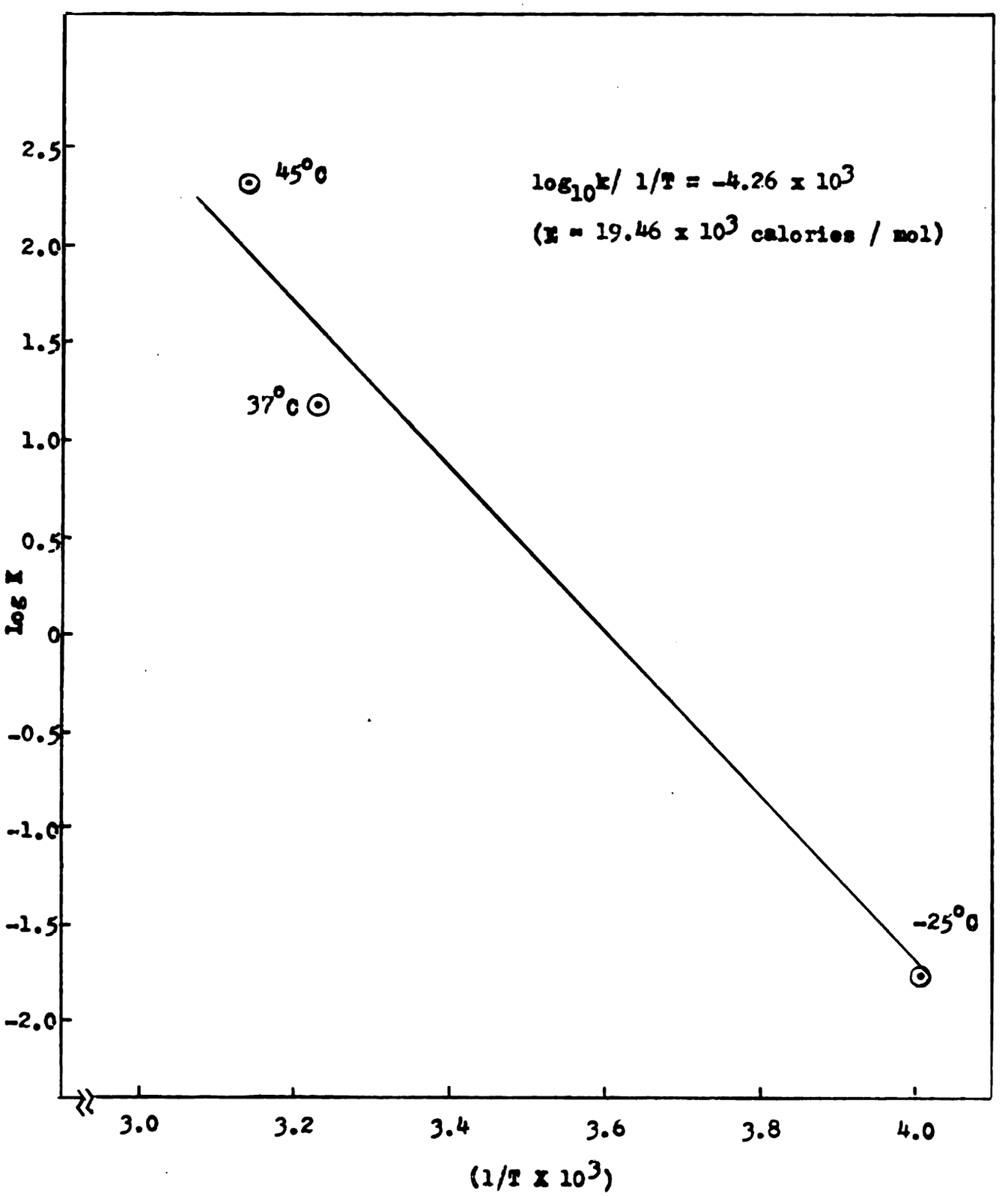


FIGURE 11

ACTIVATION ENERGY FOR INACTIVATION OF CAM CULTURED IBV.



DISCUSSION

A number of factors have been defined that profoundly influence the multiplication of viruses in tissue culture. Among these are the composition and pH of the medium, temperature of incubation, species and age of cells and the presence of naturally occurring inhibitors in the cells. Certain amino acids, carbohydrates, salt ions, vitamins or cofactors, and proteins are generally required for the growth of cells *in vitro*. Only glucose, glutamine, and salts[26] are required for the propagation of poliovirus. Glutamine is probably required as is any other amino acid for the synthesis of virus protein, and glucose may be necessary for the source of energy. In addition, glucose may serve as the primary source for alanine, serine, glycine, ribose and desoxyribose, lipid and carbohydrates, and enters into the biosynthesis of purines and pyrimidines.

The growth of IBV,[33] NDV,[65] and influenza virus[4] are deleteriously affected by the absence of either potassium, calcium, magnesium, or glucose in the extracellular medium in isolated CAM cultures.

The natural susceptibility or resistance of cells of different kinds and origin is of primary importance. The chorioallantoic membrane is an integral part of the developing embryo, and is composed of cells derived from the three primary germinal epithelial layers. When the CAM is grown *in vitro*, the nature and metabolic environment of the CAM can be considered to be different from that which is part of the embryo. Some of the fragments of the CAM culture adhered to the surface of the

bottle, somewhat as explants, around which narrow zones of new cells were formed. Microscopic examination of these new cells revealed the presence of a predominance of epithelial cells, although a few fibroblasts were present both under and at the periphery of the zone of epithelial cells.

Serial passage was necessary to adapt the egg-propagated virus to cultivation in the isolated CAM. In the first passage, viral infectivity was relatively low. The maximum titer was obtained at the 24th hour after infection which agreed with the results obtained by Ferguson[33] who had previously adapted the same strain of virus to CAM culture. In embryo culture, the maximum titer was also obtained at the 24th hour,[38,52] but this was accomplished after the virus had been completely adapted to the egg.

With the 6th passage, the titer at the 24th hour was 100 times greater than for the same period with the first passage. The maximum titer occurred at the 60th hour. The interval to obtain the maximum titer for the 6th and subsequent passages in CAM culture was more than twice as long as in eggs. This is considered to be a reflection on the cultivation of the virus in the same basic cells but in different environment.

There was a marked difference between the viral infectivity in the first and 6th passages 5 minutes after contact of the virus and the CAM. With the first passage, the infectivity was 1000 times less than that of the 6th passage. This may be considered to be another indication of a phase of the adaptation process of the virus to the CAM in a different environment. During the adaptation process in the new

cultural system, the molecular structure of IBV could have been modified so that the infectivity portion would become more stable after serial passage in the culture medium which had different osmotic pressure and hydrogen ion concentration.

Fluctuations in infectivity of the 16th passage of virus within the first 4 hours post inoculation were similar to the results obtained by Scott et al.[55] with Newcastle disease virus in the isolated CAM in Tyrode's solution. The fluctuations with IBV may have been due to a temporary adsorption of some of the virus to non-susceptible host cells and release of the virus without completion of its multiplication cycle. Some of the virus may also have been inactivated by cellular debris and extracellular substances[39] which is similar to experiences with poliovirus.[46]

The growth curve of the 16th passage of the virus during the first 60 hours may be divided into 2 phases; a lag phase of 8 hours and a log phase of the next 52 hours. The lag period indicated that about 8 hours were required for absorption of the virus to the cell, penetration into cell, and replication. Release of a new generation of the virus from the cell is manifested by the log phase. The 8 hour reproductive period for IBV corresponds to a 6- to 8-hour period for NDV.[55] This is in contrast to a 5- to 6-hour period for influenza virus[31] in the isolated CAM and 3 to 6 hours for poliovirus[45] in monkey kidney cells. The log phase of IBV was curvilinear as contrasted to the exponential log phase of NDV.

The log phase of IBV was followed by a primary decline phase of 2 and one half days, a stationary phase of 3 days, and a secondary

decline phase of 2 days.

It is considered that during the log phase the ability of the original CAM cells to produce virus was exhausted as a result of infection by IBV and few functioning cells remained in the culture.

The primary decline period may be a reflection of either inactivation of the virus or adsorption of the virus to the previously exhausted cells without replication. The virus may also have been absorbed into the newly formed cells of the CAM as the first phase of another multiplication cycle.

The stationary phase probably represents an equilibrium between production of virus by the new cells and inactivation of virus that had already been released from all cells. At the end of the stationary phase, it is considered that all cells had lost the ability to reproduce virus, and as a result the extracellular virus underwent inactivation as represented by the secondary decline phase.

With IBV, the maximum yield of the virus was obtained from the CAM culture at pH 7.0, whereas with the Roakin strain of Newcastle disease virus (NDV)[65], the maximum titer was obtained in the isolated CAM in Tyrode's solution at pH 9.5. These facts suggest that IBV and NDV require different enzymatic systems for multiplication, and that the system necessary for the reproduction of IBV is active at pH 7.0 whereas that for NDV requires a more basic environment.

As a result of cellular metabolism, the pH of the cultures after 60 hours' incubation decreased 0.3 to 0.4 units per pH value when the initial pH of the BSS was 6.0 or higher. When the initial pH was 5.0 or lower, there was an increase in pH during the

incubation period. This is considered to be a reflection of lack of metabolic activity of the cell. Degeneration and necrosis might have occurred. Release of the cellular contents, which in themselves have a buffering action, may have caused the shift of pH of the extracellular medium towards neutrality.

The titers obtained from the CAM cultures at pH 7.5 in the previous experiments were as high as $10^{6.5}$ or approximately the same as that obtained at pH 7.0 in the present experiment. From these data it may be concluded that the optimum pH for IBV in CAM culture is about 7.0, but that a pH range of 6.0 to 7.5 may be used for viral propagation.

The effect of temperature of incubation varies considerably with the virus-host system. The maximal growth of some viruses seems to be correlated with the optimal temperatures for growth for the host cells while in other systems there is no such correlation.

Colville et al.[18] demonstrated that in cultures of avian embryonic lung, increasing the temperature of incubation from 37 to 41°C did not prevent multiplication of influenza A virus.

Farnham and Newton[32] investigated the effect of variation of incubation temperature on the various stages in the growth cycle of herpes virus in HeLa cells. While the adsorption of the virus to the cells was largely temperature independent, the rates of cell penetration by virus and of intracellular viral growth were considerably decreased at temperatures lower than 37°C. This has been correlated with a rate of viral inactivation which is higher at 37°C than at 32°C.

Zuschek et al.[67] obtained the maximum growth rate of NDV in CAM suspended in Tyrode's solution and incubated at 42°C. The growth rate of NDV in the culture incubated at temperatures from 33 to 42°C followed a linear increasing response.

The chicken, the natural host for IBV, has a normal body temperature range from 40 to 41.6°C, but this does not necessarily mean that this is the optimum incubation temperature for cultivation of the virus in a tissue culture system.

The effect of temperature on cultivation of IBV showed two linear responses; a rising phase between 25°C and 37°C, and a declining phase between 37°C and 45°C. From 25 to 37°C, there was a progressive increase in viral production which might indicate a parallel increase of metabolic activity and activation of cellular enzymatic systems that were responsible for reproduction of the virus. The decrease of viral activity above 37°C represents the opposite reaction.

While there was no definite evidence that the virus multiplied in the culture at 25°C, some of the infectivity was maintained for 60 hours, whereas the infectivity was completely lost at 45°C.

The optimum temperature for the maximum yield of IBV is at 37°C.

The relation between the amount of virus present and the number of cells available for adsorption may offer an explanation as to the low concentration of virus present in the 50 and 600 mg CAM cultures at the 8th hour after inoculation.

A theory is offered that with the 50 mg culture only a

small amount of the virus was adsorbed and the virus remaining in the medium was inactivated. With the 600 mg culture a greater amount of the virus was adsorbed due to more cells being available for adsorption of the virus.

Although the 50 mg CAM culture was but a small portion of an entire CAM, average weight 200 mg, a sufficient number of cells was available for viral reproduction.

Had there been no CAM present, the virus would have been almost completely inactivated in the presence of BSS at the 8th hour sampling period as evidenced by this result in the experiment where only BSS and virus were present.

As a further test of the validity of this theory as to the number of cells available for viral reproduction, samplings made at the 60th hour showed two extremes: (1) the maximum yield of virus was obtained from the cultures containing 200 to 600 mg of CAM, (2) the least amount of virus was obtained from the culture containing 50 or 100 mg of CAM.

No significance is attached to the slight differences in the amount of virus obtained from the cultures containing 200 to 600 mg of CAM as only $10^{0.6}$ infective doses separated the extremes.

The low yields from the 50 and 100 mg of CAM are considered significant and support the theory that viral reproduction is dependent upon the relation of the number of cells available for infection and the amount of virus produced.

The effect of the time of incubation of the CAM culture prior to inoculation with IBV may perhaps be influenced by the multiplication

of the virus in the original cells as well as in the cells resulting from the explant zone of growth previously described. The latter might have been responsible for the slight increase of viral production in the cultures incubated from 24 to 81 hours prior to infection as contrasted to the results obtained with the culture inoculated immediately after preparation. It is considered that the progressive decrease of viral reproduction in the culture incubated for more than 105 hours represents a diminution due to exhaution of the virus-producing capability of the cell. The virus in the form of infected allantoic fluid was inactivated in 5 minutes at 56°C[48]. The 24th passage of the virus from CAM culture was inactivated in 4 minutes at 56°C. The slight difference in time of inactivation may be a reflection of the presence of less protective colloid in Hank's BSS than in allantoic fluid.

When subjected to 45°C, inactivation of the virus proceeded according to two successive first order reaction: one for the first 30 minutes and the other over a subsequent period of 90 minutes. During the first 30 minutes, the fastest acting, or the most thermolabile component was inactivated.

It is possible that the inactivated particles may have formed protective aggregates around the remaining active particles, thus accounting for the extremely low rate of inactivation during the subsequent 90 minutes. It is also possible that this bimodal type of overall reaction may be due to differential thermostability of original phase and derivative phase of viral particles in a mixed population of the egg adapted strain of IBV as presented by Singh[57].

Perhaps at 45°C, with virus in the form of infected BSS, selective separation occurred more readily than when the virus was subjected to 56°C in the form of infected allantoic fluid or BSS. This may be supported in part by the evidence that 2 of 5 embryos inoculated with a 10^{-1} dilution of virus in BSS and heated at 45°C for 70 minutes, and 1 out of 5 embryos inoculated with a 10^{-2} dilution of the virus heated for 80 minutes showed pathological alterations that were typical of early egg passage or original phase of the virus[44].

The activation energy for inactivation of IBV in CAM culture was 19,460 calories per mole below 45°C. Previous work by Page[48] with infected allantoic fluid showed an activation energy of 23,000 calories per mole. This difference in energy may also be attributed to the protective action of colloidal substances in the allantoic fluid.

All viruses on which chemical analysis have been made have been shown to contain at least nucleic acid in addition to proteins. The nucleic acids are considered to be the infective units, and are carriers of all the genetic properties of the viruses.

Lack of infectivity of the fraction of IBV extracted with phenol may be due to the following: (1) the extracted nucleic acids are relatively unstable, (2) the infective unit of IBV is not contained in the fraction, and (3) other components of the virus are necessary for initiation of infection.

Further investigations are necessary to establish the reason for the negative results obtained from chemical fraction of IBV.

SUMMARY

1. The Beaudette egg-adapted strain of infectious bronchitis virus was adapted to and propagated through 36 serial passages in minced chorioallantoic membrane culture suspended in Hank's balanced salt solution. Each culture contained 1.0 ml of virus and 19 ml of Hank's balanced salt solution.
2. The growth curve of the 16th passage of infectious bronchitis virus exhibited five phases: a variable lag phase of 8 hours, a logarithmic phase during the next 52 hours, a primary decline phase over the following 60 hours, a stationary phase of 72 hours, and a secondary decline phase of 48 hours.
3. The optimum incubation temperature was 37°C at pH 7.0.
4. The maximum yield of virus was obtained from cultures containing 200 - 600 mg of chorioallantoic membrane.
5. The chorioallantoic membrane cultures incubated at 37°C for 24 to 81 hours prior to inoculation yielded the highest concentration of virus. The cultures inoculated immediately after preparation or after 105 hours' incubation had a lower yield. The yield was lowest from the culture incubated more than 144 hours.
6. Viral infectivity decreased at the following rates: Log ID_{50} 0.113 per week at -25°C, and Log ID_{50} 0.63 per hour at 37°C. At 45°C, during the first 30 minutes the rate was Log ID_{50} 0.154 per minutes followed by Log ID_{50} 0.0053 per minutes for the subsequent

7.

8.

90 minutes.

7. The activation energy for inactivation of the virus was 19.46 X 10^3 calories per mole.

8. Fractionation of IBV for isolation of ribonucleic acid failed to reveal the infectivity portion of the virus.

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