SOME FACTORS AFFECTING PLAQUE SIZE OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS

by

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INTRODUCTION

Replication of viruses in monolayer cell cultures is often accompanied by the appearance of cytopathic effects. It is necessary to observe such effects microscopically when infected cell cultures are maintained in a fluid medium. However, incorporation of agar in the maintenance medium, as described by Dulbecco (14), limits the diffusion of infective virus particles. Cell destruction by the virus is thus restricted to discrete circular areas immediately surrounding the originally infected cells. These areas (plaques) can be rendered clearly visible to the unaided eye if surrounding normal cells are colored by a supravital stain such as neutral red. The character of plaques produced by a given strain of virus in a specific type of host cell culture is ordinarily quite constant.

Plaque diameter is a most readily measurable property, and one which makes the plaque technique a useful tool for strain separation, identification and study of viruses. When such a valuable measure of virus activity is observed to show marked or unexplained variation within the same virus strain, it becomes a matter of significance to the virologist. Such variation in plaque diameter was observed when western equine encephalomyelitis (WEE) virus was propagated in primary cell cultures obtained from 10-day old

chick embryos. Distinct plaques produced by WEE virus propagated in mouse brains or in chick embryo allantoic cavities averaged approximately 2 mm. in diameter when measured 48 hours after cell culture inoculation. Under identical conditions, WEE virus of the same strain, but having been passaged three times at terminal dilution in cell cultures, produced plaques with average diameter of 5 mm. or more. In short, the plaque size of a strain of WEE virus in chick embryo cell cultures was apparently affected by the method of propagation of the virus inoculum. Similar results were obtained in repeated trials.

Wagner (54) has reported that both eastern equine encephalomyelitis (EEE) and WEE viruses propagated in mouse brains produced small and indistinct plaques in chick embryo cell cultures. By comparison, plaques formed by cell-culture-propagated strains of these viruses were larger, more distinct and more uniform. However, no possible cause for this phenomenon was suggested. It was felt, therefore, that an investigation of some factors affecting the plaque size of WEE virus in chick embryo cell cultures would be of interest.

REVIEW OF LITERATURE

Introduction

WEE virus was originally isolated by Meyer et al. (42). These workers, who were investigating an outbreak of equine encephalomyelitis in the San Joaquin Valley of California in 1930, were the first to demonstrate propagation of this virus by intracranial injection of mice. A second method of laboratory cultivation of WEE virus came into common usage following the report in 1935, by Higbie and Howitt (23), of its growth on the chorio-allantoic membrane of developing chick embryos. Tissue culture methods of propagating WEE virus date from the work of Cox (10) who observed the growth of this virus in minced chick embryo fragments suspended in Tyrode's solution. The first reported use of the cell culture plaque technique for virus study was that of Dulbecco (14). His model system consisted of primary chick embryo fibroblast monolayers infected with WEE virus. The ease of propagation of this virus by several different laboratory methods has allowed researchers to collect data concerning the relationship of WEE virus to different host cells under a variety of conditions. However, only that portion of the available literature on WEE virus which pertains to the immediate problem will be reviewed.

Reported Properties of WEE Virus in Chick Embryo Cell Cultures

The plaque technique was first applied to a detailed study of the growth characteristics of WEE virus by Dulbecco and Vogt (15). They demonstrated the existence of a linear relationship between numbers of virus particles in the inoculum and the numbers of plaques observed on chick embryo fibroblast monolayer cell cultures. They therefore considered one WEE virus plaque-forming-unit (PFU) to be the approximate equivalent to one infectious virus particle. WEE virus inocula obtained from cell culture fluid media were reported to contain as high as 5×10^9 PFU/ml. Dulbecco and Vogt suggested a minimum incubation period of 30 minutes at 37° C. between inoculation of cell sheets and addition of agar overlay medium. This period allowed for adsorption to cells of 85 to 90% of potentially infective virus particles. Plaques were allowed to develop for 48 hours prior to the addition of neutral red to the cell cultures, and plaque counts were subsequently made 2 to 4 hours later. Depending on multiplicity of infection, the length of the latent period following inoculation and prior to release of second generation WEE virus from infected cells ranged from $2\frac{1}{2}$ to $3\frac{1}{2}$ hours.

Rubin *et al.* (47) reported the investigation of maturation and release of WEE virus from suspensions of chick

embryo cells, employing the technique of ultrasonic vibration. They discovered that release of virus from infected cells is a continuing and extended process. Their calculations indicated that the average WEE infective particle is released from the host cell less than 1 minute following its completed development within the cell. The observations of Dulbecco and Vogt were confirmed when the length of the true latent period was reported to be $2\frac{1}{2}$ hours. This was expressed as that time necessary for each infected cell to produce an average of one infective virus particle. The plaque technique was utilized by Rubin and his fellow workers as a means of detecting the appearance of the newly-released WEE virus.

Apparently neither of these research groups observed any significant variation in diameter of plaques produced by WEE virus in chick embryo monolayer cultures. At least, no mention of such a phenomenon was included in their reports.

Relation of Agar Overlay Constituents to Plaque Size

Plaques produced by viruses other than WEE virus, and in cell cultures other than those derived from chick embryos, have been noted to vary unexpectedly in diameter. Three strains of poliovirus were found to contain small-plaque-forming mutants, according to the report of Nomura

and Takemori (43). However, when fluid maintenance was used for HeLa or FL cell cultures, no differences in cytopathic effects or growth rates were observed between the mutants and wild type viruses. Takemori and Nomura (49) were later able to demonstrate an inhibitory effect on the rate of replication of the small-plaque mutants when an extract of Noble agar was added to cell culture maintenance media. Under identical conditions the growth rate of the large-plaque-producing wild types was not affected by the agar extract. These workers suggested that the presence of an inhibitory substance in the overlay medium (which contained Noble agar) was responsible for retarded development of plaques produced by the mutant virus strains. The mode of action of the inhibitor extracted from agar remained rather obscure. It was thought to act not directly on the virus, but possibly exerted its influence on the metabolism of the host cells.

The inhibitory agent in Noble agar is most likely a sulfated polysaccharide compound, according to Takemoto and Liebhaber (50). They reported that wild type mouse encephalomyocarditis (EMC) virus produced minute plaques, 1 mm. in diameter, when grown in mouse L cell monolayer cultures with Noble special agar overlay medium. As a result of further study, Takemoto and Liebhaber (51) indicated successful alteration of the plaque morphology of EMC virus.

Chemical inactivation of the polysaccharide-sulfate inhibitor by addition of small quantities of diethyl-amino-ethyl dextran (DEAE dextran) or protamine (salmine) to the overlay medium allowed more rapid replication of wild type EMC virus. Plaques produced by the wild type virus were 8 to 10 times greater in diameter than in identical cultures where the polysaccharide-sulfate inhibitor was unbound.

Replication of a large-plaque-forming mutant of EMC virus was not hindered by the presence of polysaccharide-sulfate in the overlay medium. But the growth rate of this mutant was actually demonstrated to be slower than that of the wild type in L cells maintained in fluid medium in the absence of agar. Another observation of note was that the large-plaque-forming mutant quickly reverted to the small-plaque-forming wild type after several passages in L cells in fluid medium. The reversion was more rapid when undiluted inoculum was used at each passage level.

According to Takemoto and Liebhaber (51), types 4, 5 and 6 ECHO viruses were observed to produce plaques in monkey kidney cell monolayers only after the addition of DEAE dextran to the overlay medium. It was suggested that the effect of the polysaccharide-sulfate inhibitor on other viruses should be investigated.

Ellem and Colter (18) have also noted the effect of the agar overlay medium on the size of EMC virus plaques

produced in L cells. They reported that the substitution of methylcellulose for agar in cell culture overlays greatly increased the size of EMC virus plaques.

Three variants of Mengo encephalitis virus have been observed by Ellem and Colter to produce plaques of significantly different size on L cell monolayers. In this case, however, all Mengo virus plaques were smaller under methylcellulose overlays than they had been in the presence of agar. Relative plaque size of the three variants with respect to one another remained unchanged, regardless of the change in overlay. One variant could be distinguished from the others on a basis other than plaque size, since it alone of the three possessed hemagglutinating activity.

Effect of Culture Oxygenation on Virus Plaque Size

Baron et al. (3) noted the effect of varied availability of oxygen on growth rate of several viruses, by observing changes in plaque size. Supplies of oxygen to host cell cultures were reduced by three methods. The rate of replication of many viruses was decreased significantly when agar overlays contained 0.01% to 0.03% sodium thioglycollate, when the amount of agar (and correspondingly the depth in the culture container) was increased from 3 to 24 ml., or when the agar concentration of overlay medium was

increased from 1.4% to 3%. Viruses known to be most susceptible to the action of interferon on their host cells were among those most inhibited by reduction in oxygen tension of cell cultures.

Interferon

The name 'interferon' was proposed by Isaacs and Lindenmann (35) in 1957 for a newly recognized substance possessing the property of interfering with influenza virus multiplication. These workers maintained viable pieces of chick embryo chorio-allantoic membranes in vitro by suspending them in tubes of Earle's balanced salt solution. These membrane bits were incubated for 24 hours in contact with heat-inactivated influenza virus before being thoroughly rinsed free of unadsorbed virus, and being placed in fresh maintenance medium. Isaacs and Lindenmann detected the subsequent appearance in the fresh maintenance medium of the substance they named interferon. The interferon content of the maintenance fluid could be titrated by noting its effect of suppressing influenza virus multiplication in fresh, otherwise unprotected pieces of chorio-allantoic membrane. The results were reported in terms of the difference in hemagglutinating units of influenza virus recovered from interferon-protected membrane pieces and from normal controls.

Further studies by Isaacs and his co-workers (6, 27, 28, 30, 31, 34, 35, 36, 39, 45) have yielded additional information concerning interferon and its probable mode of action. Interferon is apparently of cellular origin, being produced by the cell in response to exposure to heat-inactivated, ultra-violet-irradiated or live virus. It is thought to be protein in nature with a molecular weight approximating that of hemoglobin. Treatment with ether or trypsin will cause loss of its activity. Interferon is not sedimented by ultracentrifugation at 100,000 g for $\frac{1}{2}$ hour. No interfering activity can be removed from interferon preparations by adsorption with 1% fowl red cells for 2 hours at 2° C. Type-specific antisera, against the virus which stimulated its production, have no effect on interferon. It is non-dialyzable, and is stable after dialysis against buffers ranging in pH from 1 to 10. Heat stability of interferon apparently varies slightly with the pH of the preparation, but it may be stored indefinitely, when frozen or at refrigerator temperatures, with little decrease in activity.

Interferon inhibits replication of viruses other than the one which has stimulated its production. However, it has not been demonstrated to have direct antiviral activity, nor does it inhibit adsorption of virus to potential host cells. Interferon-treated cells, when exposed to live or

irradiated influenza virus, respond with additional interferon production.

The site of action of interferon is thought to be intracellular. Primary chick embryo fibroblast monolayer cell cultures seem to exhibit increased aerobic glycolysis after treatment with interferon. The volume of CO_2 produced by these cells is two to three times greater than the volume of CO_2 that is obtained from untreated control cells. Oxygen uptake by interferon treated cells is 30 to 50% greater than normal. Antiviral action of interferon, according to Isaacs and co-workers, is not due directly to stimulation of glycolysis, and the resulting unfavorable effect on pH of the cell system. Diminished oxidation of glucose by way of the pentose phosphate cycle would result in lower levels of available ribose for synthesis of viral ribonucleic acid. But this apparently is not a limiting factor. Substitution of 0.15% ribose and 0.1% pyruvate for glucose in the maintenance medium does not offset this effect of interferon.

In normal cells, the addition of uncoupling agents such as 2,4-dinitrophenol (DNP), dicumarol, sodium azide or Janus green will inhibit oxidative phosphorylation sufficiently to reduce levels of adenosine triphosphate (ATP) needed as an energy source for viral multiplication. Interferon has been demonstrated by Isaacs et al. (34) to have a similar effect

on oxidative phosphorylation.

In cancer cells, sufficient ATP is thought to be provided by glycolysis, and levels of ATP are not depleted by treatment with uncoupling agents that interrupt oxidative phosphorylation. As might be suspected, if interferon acts as an uncoupling agent, it would be difficult to demonstrate its antiviral activity in cell lines originally derived from malignant tumors. Isaacs and Baron (29) have also noticed lower than normal apparent activity of interferon preparations in early embryonic cells. The low sensitivity of these cells has been correlated with oxygen non-dependence and production of ATP by anaerobic processes.

Isaacs, Porterfield and Baron (37) showed an important parallel between availability of oxygen to cell cultures and activity of interferon. An increase in apparent interferon activity in these cultures accompanied reduction of available oxygen. Virus strains whose replication rates were most markedly reduced by the lowering of oxygen tension in cell cultures were those most susceptible to action of interferon.

According to Isaacs et al. (34), treatment of cells with DNP decreases their production of interferon. In contrast, the presence in cells of interferon does not inhibit further interferon production. This evidence has led Isaacs and co-workers to postulate a different site of

intracellular activity for these two agents. DNP is known to uncouple oxidative phosphorylation at both nuclear and mitochondrial sites. Interferon is thought to inhibit nuclear ATP formation, but not mitochondrial. Indirect evidence therefore would indicate that the production of interferon by the cell is dependent only on mitochondrial supplies of ATP.

Virus multiplication is apparently hindered unless ATP of both mitochondrial and nuclear origin are present as energy sources. DNP is known to be active in reducing available ATP at both sites, thereby inhibiting virus replication. The inhibitory effect of interferon on virus synthesis might be correlated with its property of uncoupling only nuclear phosphorylation.

Isaacs' research group (34) has suggested the following interferon-cell-virus relationships. Virus invasion of a cell apparently stimulates two activities. One is the multiplication of the virus. The other is production of interferon by the cell. The presence of interferon inhibits virus multiplication, and may act as a stimulant to hasten further interferon production by the invaded cell. Interferon has been likened to the cytoplasmic repressor substance which has been postulated to explain the occurrence of lysogenic bacterial strains. The presence of an interferon-like substance may explain the existence of

tissue culture cell lines which have been demonstrated to carry latent virus infection.

Titrations of interferon activity by Isaacs and his co-workers (35) were originally performed by measuring the effects of this substance on the cells of chick embryo chorio-allantoic membrane (CAM) pieces in vitro. Inhibition of influenza virus yield from the CAM pieces was titrated by measuring the hemagglutinating activity in these cultures. An inhibitor of hemagglutination produced by the CAM tissues and differences between batches of avian red cells prepared for the hemagglutinin assays introduced serious experimental error into the procedure unless numerous controls were utilized. These complications led to the development of a simpler interferon assay procedure by Porterfield (44), a colleague of Isaacs. The new method demonstrated inhibition of plaque formation in monolayer chick embryo cell cultures exposed to interferon preparations.

Effect of Interferon on EEE and WEE Virus Plaque Formation

The relationship between eastern equine encephalomyelitis (EEE) virus and WEE virus has been discussed by Ten Broeck and Merrill (52). Dulbecco and Vogt (14, 16) have shown the reliability and usefulness of the plaque technique for the study of the characteristics of animal

viruses. A sensitive plaque reduction test for EEE virus antibody has been reported by Daniels et al. (11). The susceptibility of both EEE and WEE viruses to the action of interferon on their host chick embryo cells was first noted by Wagner (54). He therefore proposed a simplified and sensitive technique for interferon titration with this test system. Interferon activity was measured as a function of observed reduction in size and number of EEE virus plaques on interferon treated chick embryo cell cultures as compared to interferon-free EEE-infected monolayers.

Wagner prepared interferon with the injection of an infecting dose of the WS (E) strain of type A influenza virus into the allantoic cavity of developing chick embryos. Centrifugation at 42,000 g for 1 hour freed the harvested allantoic fluid pools of a majority of infective influenza virus particles, with no apparent sedimentation of the interferon. Such preparations could be stored at -4° C. for as long as several months with little or no loss of biological activity.

Experiments led Wagner to believe that the inhibition of plaque formation in chick embryo cell cultures was largely a function of the concentration of the interferon, rather than being related to the concentration of the superinfecting EEE virus. This allowed him to inoculate cell monolayers simultaneously with virus and interferon

preparations. The failure of increased challenge doses of EEE virus to overcome interference suggested to him that the induction of interference by the allantoic fluid preparations could not be due entirely to competitive inhibition by remaining infective influenza virus particles.

The normal plaque size of EEE virus was diminished when chick embryo monolayer cell cultures were inoculated with EEE virus mixed with interferon preparations prior to inoculation. Wagner stated that neither EEE nor WEE virus which had been propagated in mouse brains could be utilized in preparation of standard dilutions of challenge virus in his interferon assay procedure. He reported that EEE and WEE viruses from this source produced small and indistinct plaques on primary passage in chick embryo cell cultures. A selected strain of EEE virus, which had been adapted to chick embryo propagation, was used instead as a challenge virus inoculum, since it was observed to produce plaques which were larger, more distinct, and more uniform in diameter. No probable cause for this phenomenon was suggested by Wagner.

The specificity of action of interferon with respect to host cell species was also observed by Wagner. Interferon prepared by inoculation of chick embryos with influenza virus showed activity at a dilution of 1:64 when titrated in chick embryo cell cultures. Undiluted interferon prepared

in an identical manner from duck embryos showed no titratable activity in chick embryo cell cultures. Interferon of duck origin was active at a 1:8 dilution however, when its effects were measured in duck embryo cell cultures--the homologous host cell.

The Probable Role of Interferon-like Substances in Chronic Cell Culture Infections

The discovery and investigation of an inhibitor of viral multiplication in primary human kidney and amnion cells was reported in 1959 by Ho and Enders (26). This inhibitor is apparently responsible in part for the initiation and maintenance of a chronic infection by type 2 poliovirus in these cells, and their resistance to infection by several other viruses. Ho and Enders' inhibitor, called 'virus-inhibitory-factor' (VIF), resembles interferon very closely.

Cooper and Bellett (9) also reported in 1959 that they could demonstrate a transmissible interfering factor present in vesicular stomatitis virus preparations. Further studies of the properties of the inhibitor by Bellett and Cooper (5) indicated a close resemblance to interferon, but did not rule out the possibility of its being incomplete or inactivated vesicular stomatitis virus. The search for the transmissible inhibitor was initiated after observation of an

interference phenomenon and low vesicular stomatitis virus yields from infected chick embryo cell cultures.

At a conference of virologists on latency and masking in viral and rickettsial infections in 1957, Deinhardt et al. (12) discussed the investigation of persistent infection of human (MCN) cells by Newcastle disease virus (NDV). In a later report by the same investigators, Henle et al. (22) confirmed the presence of an interferon-like substance which was apparently associated with maintenance of chronic NDV infection of these cells, and their resistance to superinfection by vesicular stomatitis virus.

HeLa cell cultures can become chronically infected with EEE virus, according to Bang et al. (2). Experimental evidence in this case led the observers to suggest that the chronic state of infection was achieved when all the cells in a culture were not uniformly susceptible to virus infection at a given time. Bang and his associates had observed phagocytosis of virus-killed HeLa cells by healthy HeLa cells which subsequently remained healthy. For this reason they intimated the nature of resistance to be intracellular in nature.

The reports by these workers have been reviewed in an attempt to establish the role of interferon-like substances as factors at least partially responsible for the existence of interference phenomena and observed chronic infections of

a variety of cell cultures.

Other Reported Sources of Interferon

Since 1957, numerous investigators have reported the detection of interferon elaborated by a wide variety of host cells, both *in vitro* and *in vivo*. Its production in the host cells is apparently capable of being triggered by the presence of any one of a rather wide variety of viruses. Table 1 is a list of some of the viruses which have been reported to stimulate the production of interferon.

Table 1. Some viruses reported to stimulate interferon production

	Name of virus	Virus treatment	Host cells	Reference
1.	fowl plague	UV irradiated[a]	CAM pieces[b]	6
2.	influenza A	heat inactivated	CAM pieces	35
3.	influenza A	UV irradiated	CAM pieces	39
4.	influenza A	UV irradiated	monkey kidney cell cultures	6

[a]Inactivated by ultraviolet light.

[b]Chick embryo chorio-allantoic membrane pieces *in vitro*.

Table 1. (Continued)

Name of virus	Virus treatment	Host cells	Reference
5. influenza A	UV irradiated	HeLa cell cultures	6
6. influenza A	virulent	CAM pieces	30
7. influenza A	virulent	chick embryo allantoic cavity	54
8. influenza A	virulent	mouse lung *in vivo*	33
9. influenza B	UV irradiated	CAM pieces	6
10. Newcastle disease[c]	virulent	MCN cell cultures	22
11. Newcastle disease	UV irradiated	CAM pieces	6
12. O'nyong-nyong virus	virulent	mouse brain *in vivo*	25
13. poliovirus[c]	virulent	human kidney cell cultures	26
14. polyoma	virulent	mouse embryo cell cultures	1
15. vesicular stomatitis[c]	virulent	chick embryo cell cultures	5, 9
16. West Nile	virulent	mouse brain *in vivo*	53

[c]Responsible for interferon-like substance.

Chronic Infection of Cell Cultures by WEE Virus

Results published by Chambers (7) indicate that WEE virus is capable of producing prolonged and persistent infections of mouse L cell cultures. Inoculation of mouse strain L cells with WEE virus resulted in virus multiplication, but varying severity of cytopathic effects. Inocula of higher virus concentrations frequently were noticed by Chambers to produce less cellular degeneration than were caused by more dilute inocula. A proportion of cells in infected cultures survived, proliferated, and new cell monolayers eventually grew. Production of WEE virus continued in these surviving cultures. Such chronically infected cultures were found to be resistant to superinfection at the time of subsequent challenge with WEE virus. Spontaneous loss of virus from persistently infected cultures was followed by increased susceptibility to challenge. Treatment of infected cultures with specific virus antiserum also caused cessation of virus replication and renewed susceptibility. To explain these phenomena, Chambers hypothesized a state of continuing autointerference, possibly resulting from the presence of incomplete or inactivated WEE virus particles. No attempt to assay culture fluids for an interferon-like inhibitor was reported.

Relation of Autoinhibition Phenomenon to WEE Plaque Size

Lockart, in a subsequent report (40), more conclusively demonstrated the relationship between autointerference and the existence of chronically infected mouse L cell cultures. The factor in the maintenance medium of carrier cultures responsible for inhibiting virus replication could be neutralized with specific immune serum. Also it appeared to be particulate in nature due to its sedimentation with intact virus in ultracentrifugation trials. For these reasons, Lockart suggested that it was heat inactivated WEE virus. Again, the possible presence of an interferon-like substance was not completely disproven.

That WEE virus is highly susceptible to the effects of moderate temperatures has been demonstrated by Lockart and Groman (41). WEE virus incubated for $2\frac{1}{2}$ hours at 37° C. lost 50% of its original infectivity while suspended in a serum-enriched Earle's balanced salt solution. The loss of infectivity was significantly less rapid when the suspending fluids had been previously conditioned by exposure to cell cultures. Some stabilizing factor of cellular origin could be shown to partially protect WEE virus from inactivation at 37° C.

Photographic evidence of exceptionally small WEE plaques on chick embryo fibroblast monolayers has been

offered by Lockart and Groman (41). According to these authors, the small plaques were the result of homologous interference with WEE virus multiplication due to previous exposure of the host cell cultures to heat-inactivated WEE virus preparations.

Apparent Genetic Changes in WEE and VEE Virus Populations

What is thought to be an interference phenomenon has been observed by Dunayevitch et al. (17) when titrating WEE virus obtained from naturally infected mosquitoes and birds. When young adult mice were inoculated with high concentrations of WEE virus from these sources, the mice survived infection. Mice of the same age were killed by an equal volume of more dilute suspensions of the same inoculum. However, this phenomenon was not observed when titrations were carried out in hamster kidney cell cultures or in infant mice. For this reason, Dunayevitch and his coworkers assumed that the interference observed was not the result of interference by inactivated virus nor the result of organic compounds liberated from the infected cells. They felt that the evidence favored the view that two viral genotypes were present in the brains of young adult mice. At high inoculum concentrations, a relatively large number of nonpathogenic virus particles might have multiplied

rapidly enough to suppress infection by the pathogenic genotype. The ability to select strains of WEE virus relatively avirulent for young adult mice from progeny of isolated plaques on chick embryo cell cultures was proposed by this research group as evidence in support of their assumption.

Selection of the relatively avirulent strains was not made on the basis of plaque size. Apparently, trial and error methods were used to select plaques for propagation. Isolated strains were then tested for pathogenicity by intracerebral titration in mice.

The causative agent of Venezuelan equine encephalomyelitis (VEE virus) and its relationship to other equine viruses have been investigated by Kubes and Rios (38) and by Beck and Wyckoff (4). An alteration in virulence of VEE virus after passage in mouse L cell cultures has been reported. Hearn, Brown and Hardy (20) suggested that the alteration in virulence was due to genetic changes in the virus itself, and that these changes were influenced by the host selected for VEE virus propagation. The plaque technique was not employed in their study, nor was there any reported attempt to demonstrate the presence of an interferon-like inhibitor in VEE virus inocula. Members of this research group, Hardy and Brown (19), had previously reported the occurrence of L cell cultures chronically

infected with VEE virus.

Other Reported Properties of WEE Virus

The hemagglutination of chick erythrocytes by WEE virus has been reported by Chanock and Sabin (8). However, carefully controlled conditions were required in order to achieve satisfactory hemagglutination. Red cells from chicks less than 24 hours old were most satisfactory. Hemagglutination occurred only over a very narrow pH range, and this pH was not the same as optimum pH for storage of viral hemagglutinin. Hemagglutinating properties were demonstrated only with recently isolated strains of WEE virus, since propagation for more than six passages intracerebrally in mice resulted in loss of activity. Even the size of inoculum given mice appeared to influence the production of WEE hemagglutinin in mouse brains.

Dozois et al. (13) demonstrated that addition of fresh rabbit serum containing complement significantly increased the virucidal action of heated specific immune serum against WEE virus. They called attention to the fact that the presence or absence of complement was an important consideration in analyzing results of WEE virus neutralization tests.

Summary and Statement of the Problem

Unexplained variations in the plaque size of WEE virus on chick embryo cell cultures have been observed by the author. A number of possible causes for the observed phenomenon have been considered in this review. Factors inherently associated with the plaque technique and known to affect the plaque-size of viruses in general were noted. Factors strictly related to known properties of WEE virus in particular were mentioned. The current research project was initiated in an attempt to establish which one, if any, of the factors indicated was the most likely cause of the observed variation in WEE plaque diameters.

EXPERIMENTAL PROCEDURE

The reader is here referred to Appendix A which contains information regarding such subjects as composition, preparation and storage of diluent and cell culture media; source of supply of special equipment items; and a brief outline description of glassware washing and sterilization procedures.

Source of Viruses

Western equine encephalomyelitis virus

A stock strain of WEE virus was obtained from Fort Dodge Laboratories[1] in 1951. It was maintained by the Department of Veterinary Hygiene, Iowa State University, for the succeeding ten-year period. Preservation was by freezing, with occasional chick embryo passages, usually at low dilution. This WEE virus strain was serially passaged five times at terminal dilution by intra-allantoic inoculation of 10-day-old chick embryos prior to its first inoculation in chick embryo cell cultures. The titer on initial cell culture passage exceeded 2×10^6 PFU/ml.

Swine influenza virus

The Shope 15 strain of swine influenza virus (SIV) was

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[1]Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

obtained as a chick embryo allantoic fluid pool through the courtesy of Dr. W. P. Switzer, Veterinary Medical Research Institute, Iowa State University. Eight serial passages at terminal dilution were made in the allantoic cavity of 7 to 11 day-old chick embryos prior to the use of this SIV strain for the preparation of interferon. The SIV pool utilized for interferon production contained $10^{8.6}$ chick embryo $L.D._{50}$ per ml. inoculum. Throughout this thesis, all 50% end points reported have been calculated from experimental data by the method of Reed and Muench (46).

Virus Propagation in Chick Embryos

Source of chick embryos

Fertilized eggs from a specific-pathogen-free flock of laying hens were obtained through the courtesy of Dr. M. S. Hofstad, Veterinary Medical Research Institute, Iowa State University. Eggs were routinely incubated for ten days at 37° C. in a humidified atmosphere, and then candled to determine fertility prior to virus inoculation.

Procedure of virus inoculation

A relatively avascular site immediately above the limiting border of the allantoic cavity in the air cell end of the egg was chosen by candling. The entire shell surface covering the air cell was disinfected by swabbing with

absorbent cotton saturated with 70% ethyl alcohol. A cotton-tipped applicator stick saturated with 2% tincture of iodine was used to further disinfect the inoculation site prior to drilling through the egg shell with a sterile stainless steel punch. A sterile 1 ml. syringe and a sterile 25 gauge, 5/8-inch needle were used to deliver 0.1 ml. of virus inoculum to each embryo allantoic cavity. At least five embryos were inoculated with each virus dilution. A small drop of fast-drying household cement closed each inoculation site.

Tubes of frozen virus to be used for inoculation were partially immersed in a 37° C. water bath to facilitate rapid thawing. Sterile 9 ml. dilution blanks were warmed to room temperature prior to use. Separate sterile 1 ml. serological pipettes were used for each transfer in the ten-fold dilution scheme. Inoculations were made immediately into previously prepared embryos. Erratic embryo death patterns resulted from a few attempts to inoculate embryos with fluids that were at lower than room temperature.

Procedure of harvesting virus

Inoculated embryos were incubated in a humidified 37° C. incubator, and were routinely candled at 4 hour intervals to determine the effects of inoculation. As soon as death could be determined by candling, each embryo was transferred

to a refrigerator where it was allowed to cool to 5° C. for a minimum of 1/2 hour prior to harvesting of allantoic and amnionic fluids containing the desired virus. The shell over each air cell was disinfected with 70% alcohol before its removal with a sterile forceps. Separate sterile forceps and a sterile 5 ml. pipette were used to aspirate the allantoic and amnionic fluids. Harvested fluids were pooled, tubed in 2 or 5 ml. aliquots, and immediately frozen at -40° C.

Virus Propagation in Mice

Source of mice

Mice were obtained from a colony maintained by the Department of Veterinary Hygiene, Iowa State University. No latent virus infection of this colony could be demonstrated by inoculation of normal mouse brain emulsions in chick embryo cell cultures. Mice were selected for virus inoculation approximately two weeks subsequent to weaning age.

Procedure of virus inoculation

Frozen brains which had been harvested from infected mice were rapidly thawed by partial immersion of their container in a 37° C. water bath. Each brain, together with the 2.5 ml. diluent in which it was suspended, was thoroughly emulsified in a sterile TenBroeck tissue grinder. The re-

sulting suspension was considered as the undiluted inoculum. Ten-fold dilutions of this suspension were made as described previously for allantoic and amnionic fluids. Prior to injection, the inoculation site on the cranium of each mouse was swabbed with a cotton-tipped applicator stick saturated with a 2% tincture of iodine. A sterile 1 ml. syringe and a sterile 27 gauge, 1/4-inch needle were used to inoculate each mouse intracranially with 0.03 ml. of the inoculum dilution. A minimum of five mice were inoculated with each virus dilution, and a separate cage was utilized for each group.

Procedure of harvesting virus

Inoculated mice were observed at frequent intervals so that the progress of infection could be determined. Infected mouse brains were harvested only from mice showing signs of acute encephalitis. In no case was the brain tissue of a mouse used for subsequent passage of virus if the mouse was found dead before harvesting could be performed. Mice in a moribund state, or mice in that stage of infection where severe convulsions could be elicited by slight stimulation, were quickly euthanized by using a small hemostat forceps to induce cerebral anoxia. The use of an inhaled anesthetic was contraindicated due to the possible adverse effects upon the virus. Each dead mouse was secured

to a mouse board, the anterior half of its body disinfected with 70% alcohol, and the skull aseptically opened. The entire brain was removed with sterile forceps and immersed in 2.5 ml. sterile diluent and immediately frozen at -40° C. until thawed for use.

Virus Propagation in Cell Cultures

Growth of cell cultures

Fertile hen's eggs were supplied by the Veterinary Medical Research Institute, Iowa State University. Beheaded 10-day-old chick embryos were aseptically transferred to GKN wash solution maintained at room temperature. After evisceration, the embryos were washed again in fresh GKN solution. Intact embryo bodies were fragmented by forcing them through a 20 ml. syringe containing a disc of wire mesh, and the resulting tissue fragments were again washed in GKN. The smaller particles and supernatant GKN were decanted after the larger particles were allowed to settle upon standing for a 15 minute period. The sedimented particles were resuspended in trypsin solution and gently agitated using a magnetic mixer. After 30 minutes of trypsinization at room temperature, the resulting cell suspension was freed of tissue debris by filtering through two layers of sterile gauze. Two cycles of centrifugation for 10 minutes at 1,000 r.p.m., with resuspension of packed cells in growth medium,

were used to wash cells free of residual trypsin. After the second centrifugation, packed cells were diluted to a final concentration of 1/500 by volume in growth medium. The resulting cell suspension was dispensed in 5 ml. amounts into 60 x 15 mm. disposable plastic petri dishes. Confluent monolayers of chick embryo fibroblasts were routinely grown by incubation of cells in these dishes for 48 hours at 37° C. Favorable environment for development of monolayers in the water-jacketed incubator was maintained by a humidified atmosphere of approximately 5% CO_2.

Preparation of virus inocula

WEE virus used for cell culture inoculations was prepared in the following ways, depending upon the methods which had been chosen for propagation of the virus. Allantoic and amnionic fluids from infected chick embryos were prepared exactly as previously described. Infected mouse brains used as a source of virus were emulsified in TenBroeck tissue grinders by a technique which has also been described previously. Frozen fluid maintenance medium containing virus harvested from infected cell cultures was thawed quickly, and mixed thoroughly with a sterile pipette before proceeding directly with serial dilutions. Squares of agar, containing virus harvested from previous plaque-technique passages in cell cultures, were another source of

virus. These bits of agar had each been frozen for storage in 5 ml. of diluent and were thawed rapidly immediately prior to use. A uniformly distributed virus suspension was prepared by dispersing the agar throughout the diluent using a sterile TenBroeck tissue grinder. The resulting suspension was regarded as the undiluted inoculum.

Regardless of the method of preparation of undiluted inocula described above, in each case serial ten-fold dilutions of the inoculum were made in 9 ml. sterile blanks containing Earle's balanced salt solution plus 0.5% lactalbumin enzymatic hydrolysate and buffered to a final pH of 7.4 with $NaHCO_3$.

Procedure of virus inoculation

After 48 hours incubation, individual culture plates were examined microscopically to determine that cell monolayers had achieved confluent growth and were free of contamination. Plates found to be satisfactory were grouped and labeled for inoculation. Growth medium was aspirated from the cell cultures using a sterile capillary pipette attached with tubing to a 2 liter flask which served as both vacuum source and receptacle for discarded medium. Five plates were immediately inoculated with each virus dilution, using 0.5 ml. inoculum per plate. The inoculum was evenly distributed over the cell layer by tilting and rotating the

plates, and the time of inoculation was noted. Since virus dilutions needed to be warmed to room temperature prior to inoculation of cell cultures, no more than four series of dilutions were prepared and inoculated in sequence, thus limiting the period of possible heat inactivation of virus particles to a maximum of 20 minutes, and also minimizing the effects of drying and change in pH which may occur when cell monolayers are kept for greater periods at atmospheric conditions when devoid of fluid medium. Culture plates were immediately returned to the incubator after completion of the inoculation procedures.

Virus adsorption

Procedures were standardized so that adsorption time allowed and environmental conditions during virus adsorption to the cells could be ruled out as the factors responsible for plaque size variation observed in this series of experiments. In all cases, inoculated cell sheets were maintained at 37° C. in a humidified 5% CO_2 atmosphere for 60 minutes, timed from the moment of exposure to inoculum until the addition of the agar overlay medium.

Addition of agar overlay

Well-mixed agar overlay medium, kept in a 42° C. water bath was added to the cell monolayers at the end of the 60 minute adsorption period. A sterile 25 ml. pipette was used

to accurately dispense 5 ml. of medium to each plate. As soon as the agar hardened, culture dishes were immediately returned to the incubator. Special attention was given to controlling the composition of the overlay medium and final depth of the agar overlay to minimize the effects that variation in these factors might have on plaque size of the virus inoculated.

Addition of second overlay

The second agar overlay (containing neutral red) was added to the infected cell cultures 32 hours subsequent to inoculation. The well-mixed agar and neutral red suspension, kept at 48° C. to avoid solidification, was spread on the surface of each plate in 1 ml. amounts. The cell cultures were returned to the incubator as soon as possible after the necessary interval at room temperature.

Observation of plaques

Distinct plaques could be routinely observed and counted any time on the second day following cell sheet inoculation. Accurate counts could be made on plates containing as high as 100 plaques. No attempt was made to count plates containing more than this number.

Measurement of plaque diameters (unless otherwise indicated) was made 48 hours subsequent to the recorded time of inoculation of the cell sheets. Beginning with plates

inoculated at terminal dilution, diameters of the first 100 clearly visible, individually occurring plaques were estimated to the nearest millimeter by observing them in comparison with a transparent plastic millimeter scale. Plaque diameters measured at 48 hours should not be interpreted as representing the maximum diameters obtainable, since plaques twice this diameter or greater could be observed when inoculated plates were allowed to incubate several additional days.

Photography

All photographs of infected cell cultures included in this thesis were taken as close to the time of plaque measurement as scheduling of the photographer's services would permit. In the few cases where more than two hours delay in photographing was anticipated, all plates were refrigerated at 5° C. for this time interval, with no measureable change in plaque size resulting. All prints are uniformly magnified to represent a 2x enlargement of actual size.

Harvest of plaques

Cell culture plates containing plaques to be harvested were first cooled for at least 30 minutes at 5° C. to insure extra firmness of the overlaying agar. A sterile, pointed scalpel was used to cut a square in the agar medium

circumscribing a single plaque. The scalpel was then used to transfer the excised agar square to 5 ml. of sterile diluent. The scalpel was immersed in 70% ethyl alcohol, briefly flamed, and cooled prior to each use. Plaques were at once frozen at -40° C. while suspended in diluent, and were stored in this manner.

Propagation of virus in fluid medium

The previous description concerns methods utilized in the plaque technique for virus propagation. In the few cases when infected cell cultures were maintained in fluid medium rather than a solid agar overlay, slightly different procedures were employed subsequent to virus inoculation. In these instances, 5 ml. of maintenance medium were added to each infected monolayer immediately after inoculation of virus, and without allowing the 60 minute period for virus adsorption. Cell sheets were then returned to the incubator.

Harvesting of propagated virus from infected monolayers was routinely performed when microscopically-observed cytopathic effects involved 50 to 75% of the cells present. This occurred often as early as 36 hours subsequent to virus inoculation. The maintenance fluid containing the virus was aseptically aspirated and pooled. Aliquots were dispensed in 2 or 5 ml. amounts and frozen at once for storage at -40° C.

Use of Special Protamine Overlay Medium

Purpose

Takemoto and Liebhaber (50, 51) have reported the presence of a polysaccharide-sulfate in Noble agar which exerted an inhibitory effect on plaque formation in chick embryo cell cultures by selected strains of EMC virus. The addition of 0.4 mg./ml. of protamine to the agar overlay medium was reported to chemically inactivate the inhibitor, and a significant increase in diameter of EMC virus plaques was observed.

In the present study, Noble agar was also used in preparation of the agar overlay medium. To indirectly determine the effect of the polysaccharide-sulfate compound on the diameter of plaques produced by WEE virus, the following procedure was used.

Procedure

WEE virus inocula were selected on the basis of predetermined plaque characteristics, and serial dilutions of each were prepared. Ten cell culture plates were inoculated at each dilution. One-half of the plates received regular agar overlays. Special protamine overlay medium was added to the other half. The standard plaque technique was otherwise followed with both groups. The effect of protamine addition was determined by comparison of treated plates with

controls after incubation for 48 hours.

Testing for Interferon-like Inhibitors

In the course of the experiments reported, attempts were made to demonstrate the presence or absence of an interferon-like inhibitor of virus multiplication in pools of WEE virus. Pools of swine influenza virus (SIV) were found to contain such an interferon-like substance as a result of their propagation in the allantoic cavities of developing chick embryos. Therefore, preparations of this inhibitor were used as positive controls in the test system to be described. Negative controls were obtained by applying identical techniques in making preparations from normal (non-infected) chick embryos and mice.

Source of fluids to be tested

WEE virus was propagated in chick embryos, in mice and in chick embryo cell cultures as previously described. Harvested virus pools were tested for the presence of soluble inhibiting factors.

SIV was propagated in the allantoic cavities of 10-day-old chick embryos. Allantoic and amnionic fluids were harvested from embryos killed by the virus. These fluids were used for the preparation of the interferon-like inhibitor.

Allantoic and amnionic fluids from normal embryos, suspensions of emulsified brains of normal mice, and maintenance medium recovered from uninoculated cell cultures were also prepared for testing as described below.

Ultracentrifugation

Aliquots obtained from frozen pools of each of the above materials were thawed, kept under refrigeration while being centrifuged, and the resulting preparations were immediately refrozen. Each aliquot was centrifuged for 5 hours at 100,000 g in the #40 head of a Spinco ultracentrifuge. Supernatant fluids were decanted and stored in convenient amounts. Sedimented pellets were resuspended and restored to the original fluid volume with sterile diluent before refreezing.

The purpose of centrifugation was to remove the majority of virus particles from the fluids to be tested for interferon-like activity. Fluids from normal sources were also centrifuged as control preparations. Although the supernatant fluids resulting from centrifugation were desired, the sedimented pellets were recovered as a source of additional information concerning efficiency of the centrifugation process.

Virus adsorption to chicken red blood cells

In the case of interferon-like preparations from SIV,

an alternative method could be used to reduce the concentration of virus particles. Chicken blood was aseptically collected in sterile sodium citrate solution. Red blood cells were washed three times in sterile saline (0.85% NaCl), and a 10% suspension in saline was prepared after the third centrifugation. Ten ml. of the 10% red cell suspension were mixed with a 30 ml. aliquot taken from the pool of allantoic and amnionic fluids harvested from SIV-infected embryos. After incubating 30 minutes at 5° C., the resulting mixture was centrifuged 10 minutes at 1,000 r.p.m. The packed red cells and adsorbed SIV were discarded, and the identical adsorption cycle was repeated with 30 ml. of the supernatant fluid. The supernatant fluid was recovered following the second adsorption cycle, and aliquots were again frozen. The resulting preparation represented a 9/16 dilution in saline of the original pool of allantoic and amnionic fluids.

Demonstration of interferon-like activity

Interferon can be titrated using the plaque-reduction method (44, 54). With this technique, the smaller size and reduced number of plaques appearing on an infected monolayer culture in the presence of interferon are contrasted to the size and number of plaques appearing on an infected culture devoid of interferon. This procedure was employed to determine the presence or absence of interferon-like activity

in the preparations under discussion.

A pool of WEE virus containing in excess of 10^8 PFU/ml. was prepared by propagation of the virus in chick embryo cell cultures using fluid maintenance medium. When aliquots from this pool were inoculated at appropriate dilution in chick embryo cell cultures, resulting plaques were of uniform size and routinely averaged more than 5 mm. in diameter. Chick embryo cell cultures to be used for the demonstration of interferon-like activity were inoculated with the dilution of an aliquot from this pool calculated to produce approximately 100 plaques per plate. Each plate was rotated and tilted immediately after inoculation to assure uniform distribution of infectious virus particles, and therefore uniform distribution of resulting plaques, over the entire surface of the cell monolayer. Routine procedure for cell culture inoculation was followed throughout, and the customary 60 minute period for virus adsorption was allowed.

The only deviation from the routine plaque technique occurred at the time of addition of the agar maintenance medium. Three ml. of agar maintenance medium (prepared in the regular fashion) were added to each infected monolayer rather than the usual 5 ml., and this partial overlay was allowed to harden. A short, sterile segment of aluminum tubing, 1 cm. in diameter, was placed upright in the center of each plate until the remaining 2 ml. of overlay medium

could be added and also allowed to harden. Thus a small cup was left in the center of the agar overlay above each cell monolayer when the piece of tubing was removed. This procedure was patterned after that described by Vainio et al. (53).

One drop of the fluid to be tested for interferon-like activity was deposited within the agar cup, using a 1 ml. serological pipette. All plates were prepared and all fluids to be tested were in readiness so that this process of adding the test fluids to a series of plates could be accomplished in the shortest period of time. Routinely, test fluids were added approximately 1 hour and 45 minutes after the original inoculation of cell monolayers with WEE virus. Culture plates prepared in this manner were placed in the incubator at once, using caution to avoid spilling of the test fluid beyond the confines of the center well.

Incubated cultures were subsequently stained and examined by procedures previously described. The interferon-like activity of the test preparation in the center well of the agar overlay was determined by observing its effect on the size and distribution of WEE plaques 48 hours after cell culture infection.

WEE Virus Heat-Inactivation Trials

Properties of test virus

WEE virus obtained from a single large plaque was passaged twice at terminal dilution in chick embryo cell cultures using fluid maintenance medium. Aliquots of 2 ml. were frozen from a large pool of virus propagated in fluid medium harvested after the second passage, and stored at -40° C. On repeated titrations, random samples from this pool yielded uniform large plaques whose mean size approximated 5 mm. in diameter. Virus titer was in excess of 10^8 PFU/ml.

Test procedure

At the start of the experiment, a tube selected at random from the WEE virus pool, was removed from the freezer and placed directly into a 37° C. water bath. Additional tubes were transferred from the freezer to the water bath at 12 hour intervals until a total of 48 hours had elapsed. At this time, the incubated samples and a freshly thawed sample were used to prepare serial ten-fold dilutions for cell culture inoculation. Five monolayers were inoculated with each dilution. Each group of cultures was handled identically by the routine plaque technique as described previously. After 48 hours incubation, plaques were counted and their diameters measured to determine if a reduction in

plaque size might possibly accompany a decrease in virus titer due to large amounts of heat-killed virus present in the inoculum.

Relation of Genetic Changes to WEE Virus Plaque Size

The presence of two WEE viral genotypes was proposed by Dunayevitch et al. (17) to explain the interference phenomenon observed when titrating this virus by intracerebral inoculation of mice. Alteration in virulence of VEE virus, apparently associated with genetic changes, has been reported by Hearn et al. (20). In the course of the experiment to be reported, it was possible to study the effect of several methods of WEE virus propagation on plaque size in chick embryo cell cultures. WEE virus was serially passaged four times both at high and low dilution in mouse brains, chick embryos, and chick embryo cell cultures. The plaque-forming properties of the resulting WEE virus preparations were compared with those of the parent populations.

Immunization of Laboratory Personnel

To minimize the risks involved in handling WEE virus preparations in the laboratory, workers were immunized prior to the start of the reported study. A bivalent EEE and WEE

vaccine of chick embryo origin was obtained.[1] Since this product was prepared for use in horses, it was diluted 1:1 with sterile saline before injection. In each case, 0.5 ml. doses of the diluted vaccine were administered intradermally at one week intervals, until three injections had been made. No adverse effects of this procedure were noted.

Blood samples were collected from one person before immunization, 14 days after completion of the injection series, and approximately one year later. The WEE virus antibody titer of these samples was compared using a serum neutralization test in chick embryo cell cultures.

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[1] Pitman-Moore Company, Indianapolis 6, Indiana

RESULTS AND DISCUSSION

Introduction

Original observations

On initial chick embryo cell culture passage, a strain of WEE virus, which had been serially propagated by allantoic inoculation of chick embryos, produced plaques varying greatly in diameter. Two types could be easily differentiated with respect to diameter, because those of the large type were at least twice the size of their smaller counterparts. Additional inoculations of WEE virus from this source resulted in recurrent observations of the same phenomenon.

Significance

The original purpose of WEE virus passage in chick embryo cell cultures by the plaque technique had been to prepare a pool of WEE virus suspended in cell culture maintenance medium. It was intended that the virus pool be the progeny of a single plaque and that it be homogeneous with respect to its observable properties. When difficulties were encountered in preparation of a WEE virus pool consisting of only one plaque type, an investigation of the factors affecting the plaque size of WEE virus in chick embryo cell cultures was initiated.

Code designations used throughout the experiments to identify pools of virus have also been used in reporting and discussing results. Table 2 is a short explanation of those codes appearing in this section and in Appendix B.

Table 2. Explanation of code letters and numbers used for identification of virus pools

Code	Explanation
WEE	western equine encephalomyelitis virus
SIV	swine influenza virus
E (after number)	refers to intra-allantoic cavity passage in chick embryos
M (after number)	refers to intracranial passage in young mice
TCF (before number)	refers to passage in chick embryo cell cultures using fluid maintenance
TCP (before number)	refers to passage in chick embryo cell cultures with agar overlay
Series 5766E - 5783E	denotes passage level in chick embryos of WEE virus
Series #1A - #15A	denotes lot number of cell cultures
Series #1M - #12M	denotes passage level of WEE virus in mice
Series #1S - #8S	denotes passage level of SIV in chick embryos

Relation of Experimental Procedure to Plaque Size

Factors controlled

In order to establish that WEE virus plaque size variation was attributable to some property of the inoculum, factors associated with handling of the virus inocula and experimental procedure of cell culture inoculation, incubation and observation were necessarily standardized. Careful attention was devoted to controlling those conditions reported by previous workers to affect plaque development. The time of harvesting virus-laden tissues and fluids was regulated with respect to the time of death of infected embryos, mice or cells in cultures. Storage temperature of the virus pools was kept constant. Limits were placed on the intervals of exposure of virus inocula to room temperature. Time, temperature and pH were carefully controlled during the period of virus adsorption to cell cultures. The composition and amounts of media used, the composition of diluent and the dilution technique, cell culture pH and oxygenation were among the factors regulated. All plaque size comparisons were made within a series of cell cultures grown from the same lot. During most of the experiments, an aliquot from a pool of virus having known plaque-producing properties (WEE TCF #4A) was included as a control inoculum. Differences in plaque diameters (if they occurred) were found

to be most striking when cultures were stained at 32 hours after virus inoculation, and when plaques were counted, measured and photographed at 48 hours subsequent to inoculation. Adherence to these time intervals was as strict as possible in all experiments.

Results

Figures 1 and 2 show plaques produced by inoculation of WEE 5766E virus, an aliquot from a pool harvested from passage in chick embryos at 10^{-5} dilution. Plaques produced by WEE 5766E virus after chick embryo passage at 10^{-2} dilution are depicted in Figures 4 and 5. Under identical conditions, the mean diameter of plaques resulting from low dilution passage was notably less than the mean diameter of plaques produced by the same WEE virus strain following passage in chick embryos at higher dilution. The WEE 5766E virus pool represents allantoic and amnionic fluids harvested after the second passage in chick embryos of WEE virus isolated from a single large plaque in a chick embryo cell culture. The first passage of the plaqued virus in embryos was at terminal dilution.

When they were allowed to develop in the same lot of cell cultures, and under identically controlled conditions, even smaller plaques resulted from inoculation with WEE #2M virus. Figures 7 and 8 are photographed results of this

passage. The original chick embryo propagated laboratory strain of WEE virus had been passed twice at terminal dilution in mice to obtain the WEE #2M virus inoculum used in this passage. Results presented are those from the first cell culture passage of this virus.

Figures 3, 6 and 9 accompanying the photographs are graphical representations of data recorded from these specific WEE virus passages. Appendix B contains data from these and other experiments reported, tabulated in the form originally recorded. Succeeding illustrations and graphs presented throughout this thesis were similarly obtained.

Significance

Unexplained variation in diameters of plaques produced by WEE virus in chick embryo cell cultures continued to occur, despite rigidly controlled experiments. Differences in plaque size observed seemed to be related to the passage history of the WEE virus inocula. Both the host selected and the dilution used for propagation of the inoculum apparently exerted some unexplained effect on the diameters of plaques produced. At this point in the investigation, it remained to be shown whether the indicated differences among WEE inocula were the result of genetic changes in the virus itself, or were due to some sort of inhibitory agent carried with the inocula.

Figure 1. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-5} dilution of WEE 5766E virus. Inoculum was propagated by passage at 10^{-5} dilution in chick embryos

Figure 2. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-6} dilution of the same inoculum as in Figure 1

Mean plaque diameter of plaques represented in both figures = 3.9 mm.

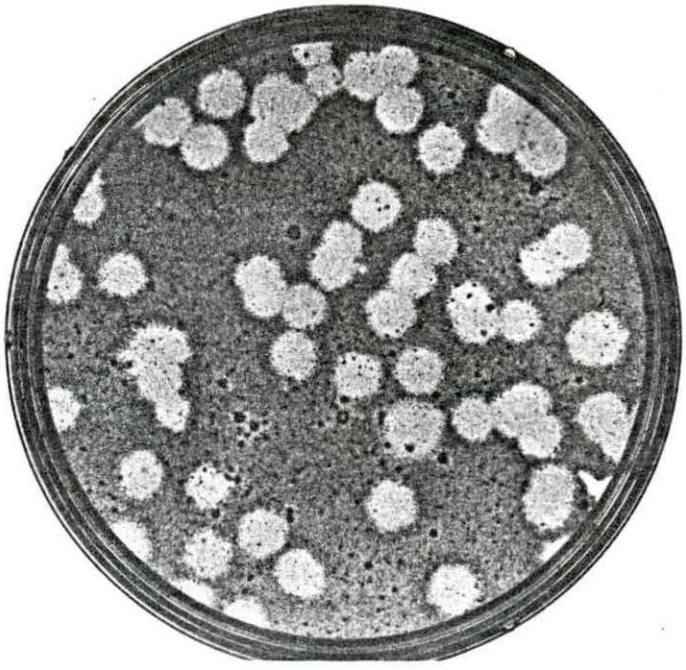


Figure 3. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figures 1 and 2

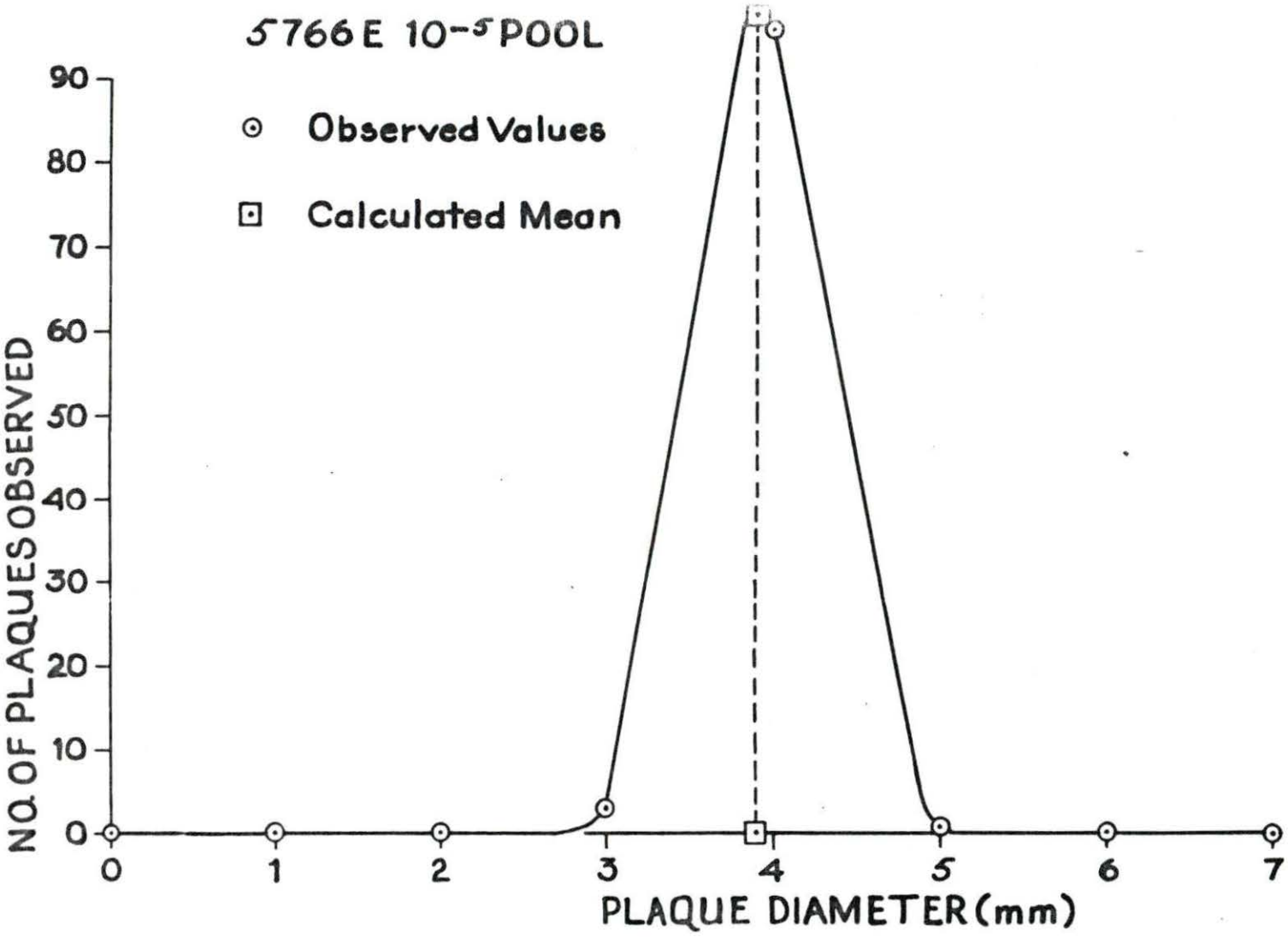
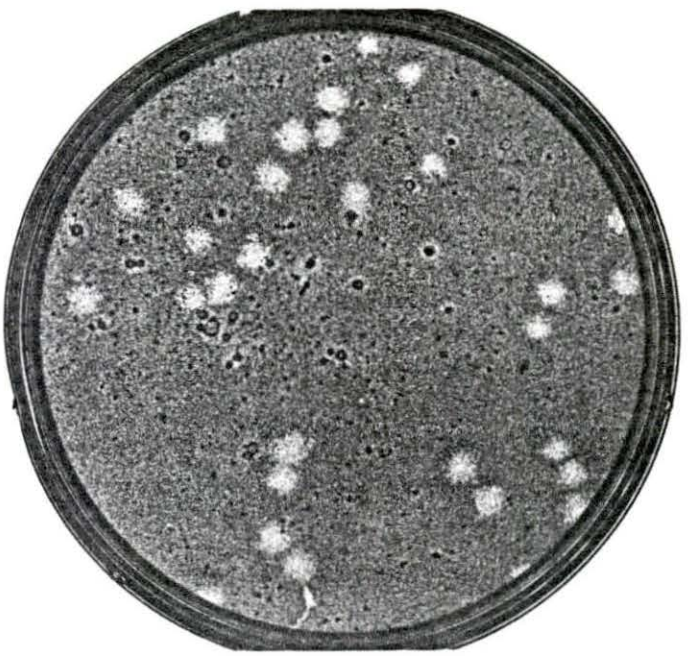

5766 E 10⁻⁵ POOL
Observed Values
Calculated Mean
NO. OF PLAQUES OBSERVED
0
10
20
30
40
50
60
70
80
90
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

Figure 4. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of WEE 5766E virus. Inoculum was propagated by passage at 10^{-2} dilution in chick embryos

Figure 5. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-8} dilution of the same inoculum as in Figure 4

Mean plaque diameter of plaques represented in both figures = 2.2 mm.



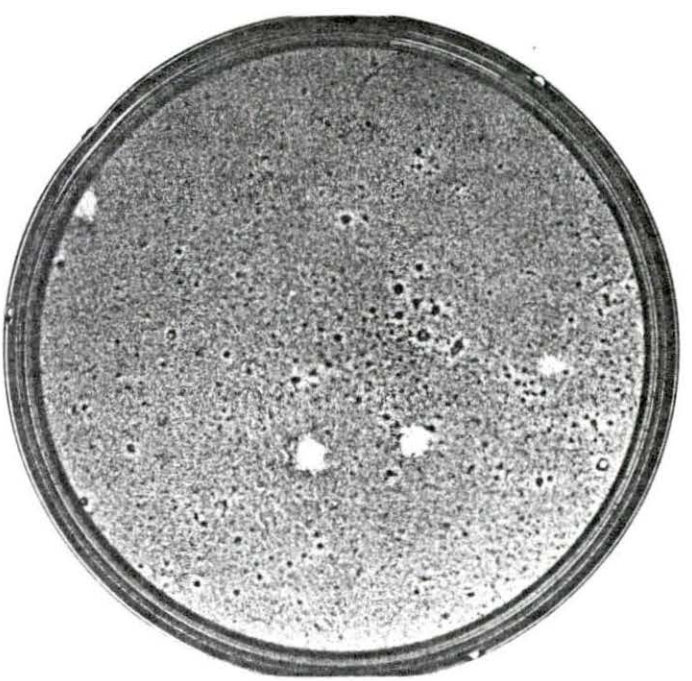


Figure 6. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figures 4 and 5

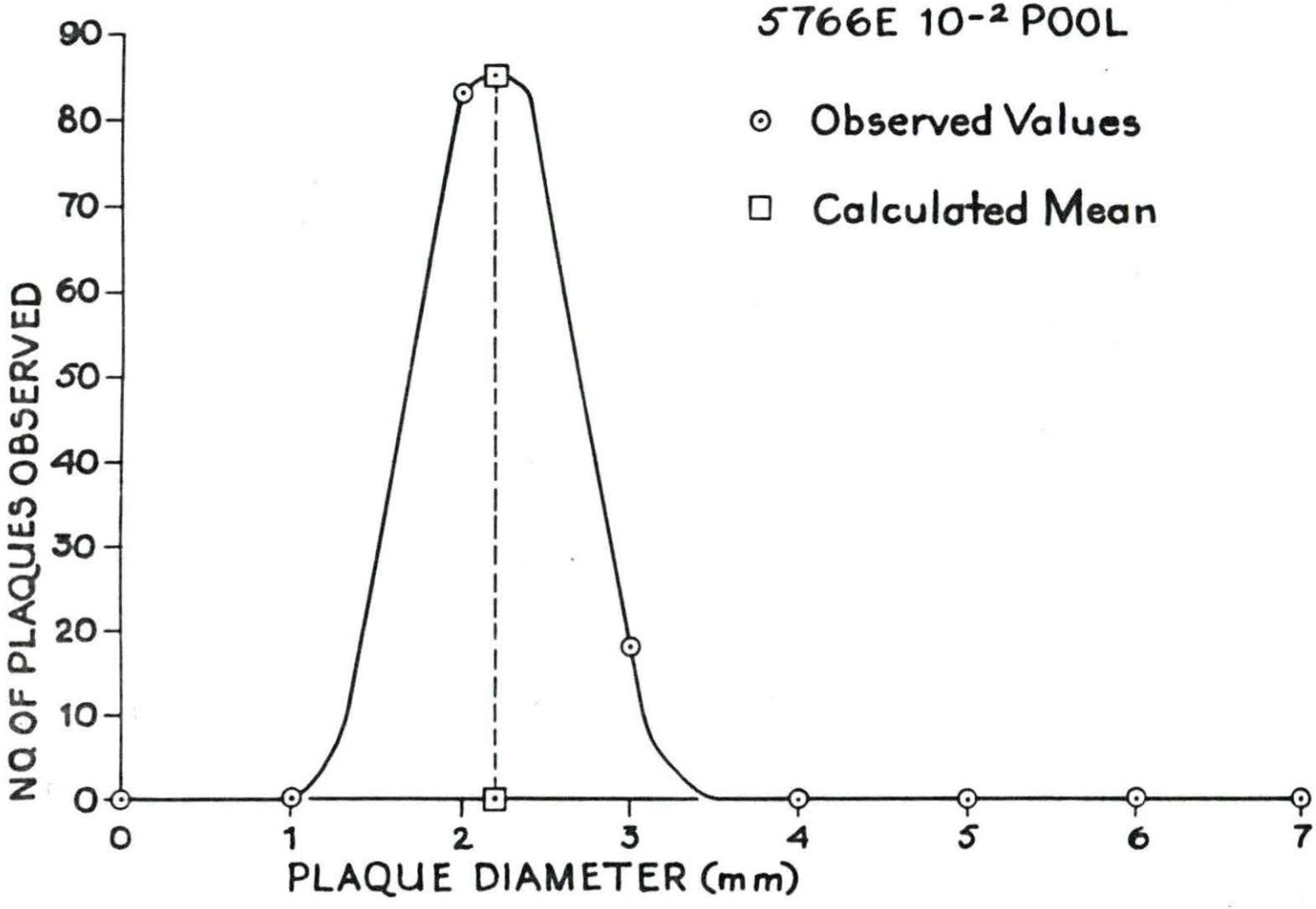
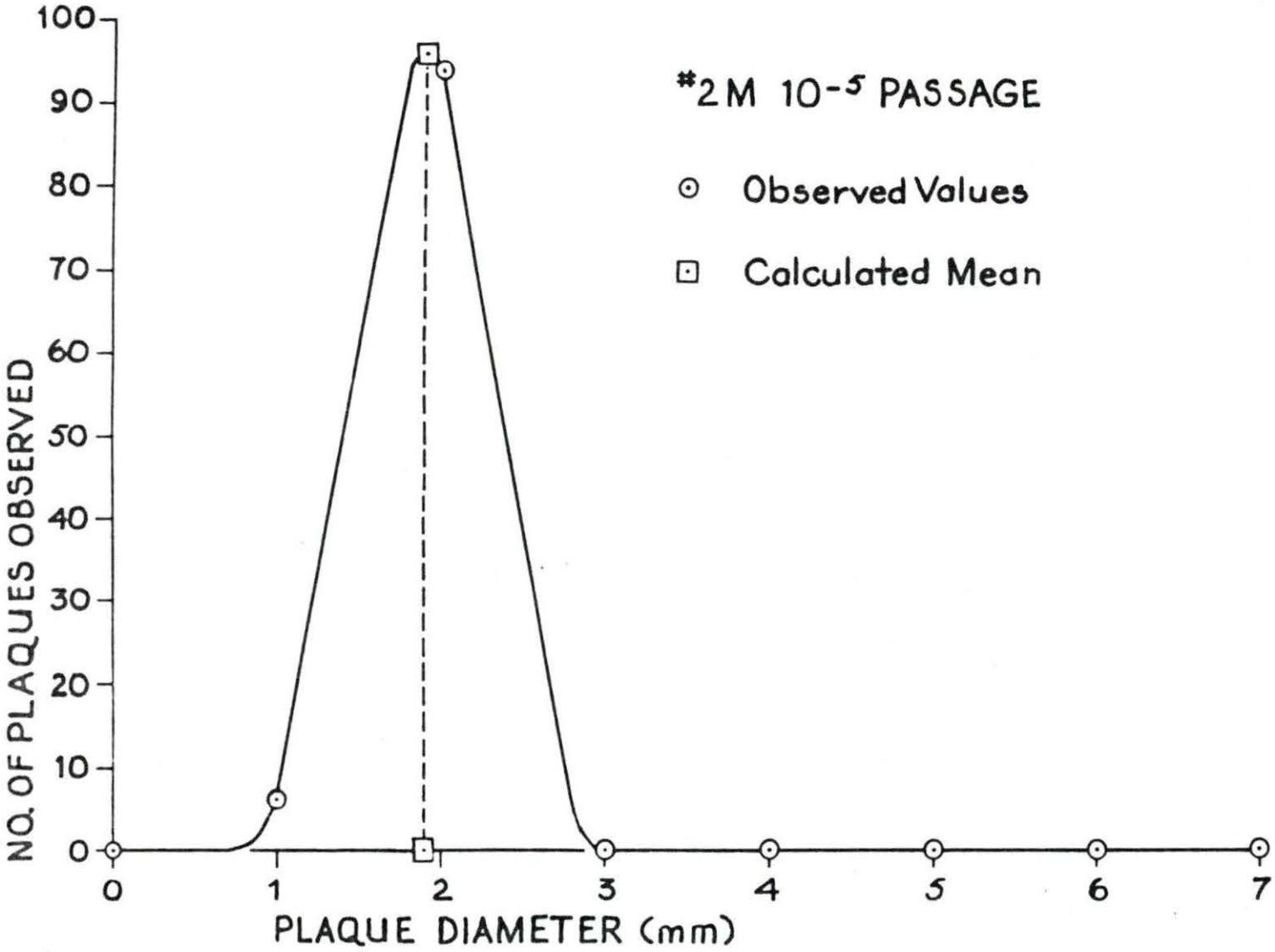

5766E 10-2 POOL
Observed Values
Calculated Mean
NO OF PLAQUES OBSERVED
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

Figure 7. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-6} dilution of WEE #2M virus. The inoculum was propagated by passage at 10^{-5} dilution intracranially in mice

Figure 8. Plaques produced 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of the same inoculum as in Figure 7

Mean plaque diameter of plaques represented in both figures = 1.9 mm.

Figure 9. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figures 7 and 8


100
90
80
70
60
50
40
30
20
10
0
NO. OF PLAQUES OBSERVED
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)
*2 M 10-5 PASSAGE
Observed Values
Calculated Mean

Although plaque diameter variations were observed regardless of strict control of the factors already known to affect plaque diameter, it was necessary to keep these factors standardized during the course of further studies. Significance of the results of investigation of other variables depended on the reliability of the plaque technique as an experimental method.

Effect of the Addition of Protamine to the Overlay Medium

Results

Aliquots from three pools of WEE virus were selected for passage in chick embryo cell cultures in the protamine experiments, since their plaque-forming properties had been determined from earlier experimental data. Inocula were chosen deliberately which would produce large, medium and small plaques respectively under regular agar overlay medium.

WEE TCF #4A virus was used to inoculate one series of plates. This inoculum was an aliquot of a pool propagated at 10^{-3} dilution in chick embryo cell cultures and was the progeny of a single large plaque isolate. The mean diameter of 100 plaques was increased by the presence of protamine sulfate from 5.1 to 6.0 mm.

WEE M_3TCP #6A virus, originally the same strain as TCF #4A, had been passed at terminal dilution three times in

mice, and plaqued once on chick embryo cell cultures prior to its passage in this experiment. The mean diameter of 100 plaques produced by WEE M_3TCP #6A virus was increased from 3.9 to 5.4 mm. in the presence of protamine sulfate.

WEE 5769E TCP #7A was also derived from TCF #4A parent virus, but had been passed twice at terminal dilution in chick embryos, once in embryos at 10^{-2} dilution, plaqued once on cell culture, returned to chick embryos at 10^{-2} dilution, and finally plaqued one additional time on chick embryo cell cultures. The WEE 5769E TCP #7A inoculum for this trial consisted of an emulsified small plaque isolated from the last cell culture passage. The control plaques on this trial averaged 1.5 mm. in diameter, whereas those on identically prepared plates in the presence of protamine sulfate achieved a mean plaque diameter of 6.0 mm. in the same growth period.

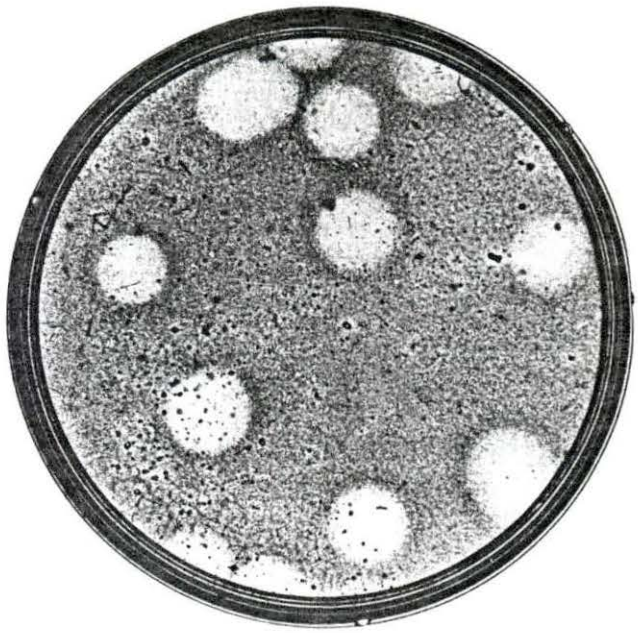
Photographs of plaques appearing on the control plates under regular overlay medium are shown in Figures 10, 12 and 14. Photographs of the larger plaques resulting from multiplication of the three WEE virus inocula under overlays containing an added 0.4 mg./ml. of protamine sulfate are pictured in Figures 11, 13 and 15. A summary of the results of this particular experiment are graphed in Figure 16.

Figure 10. Plaques observed 48 hours after inoculation of WEE TCF #4A virus on chick embryo cell cultures with regular overlay medium

Mean plaque diameter = 5.1 mm.

Figure 11. Plaques observed 48 hours after inoculation of WEE TCF #4A virus on chick embryo cell cultures with special protamine overlay medium

Mean plaque diameter = 6.0 mm.



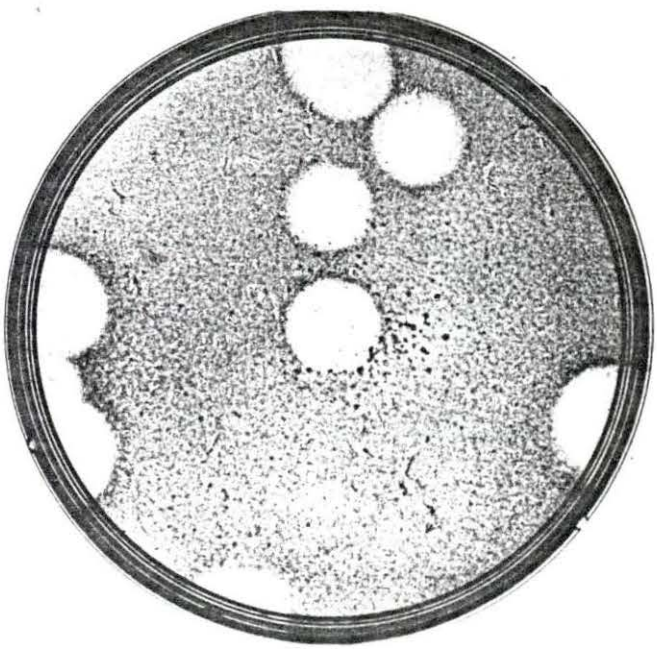
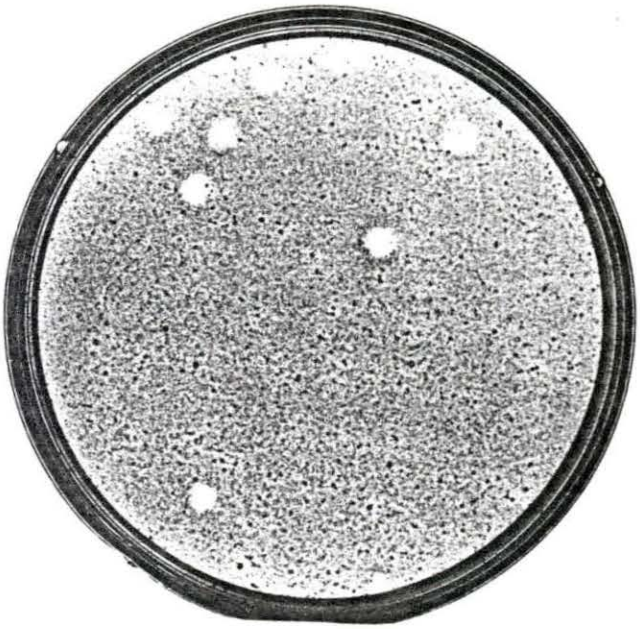


Figure 12. Plaques observed 48 hours after inoculation of WEE M_3TCP #6A virus on chick embryo cell cultures with regular overlay medium

Mean plaque diameter = 3.9 mm.

Figure 13. Plaques observed 48 hours after inoculation of WEE M_3TCP #6A virus on chick embryo cell cultures with special protamine overlay medium

Mean plaque diameter = 5.4 mm.



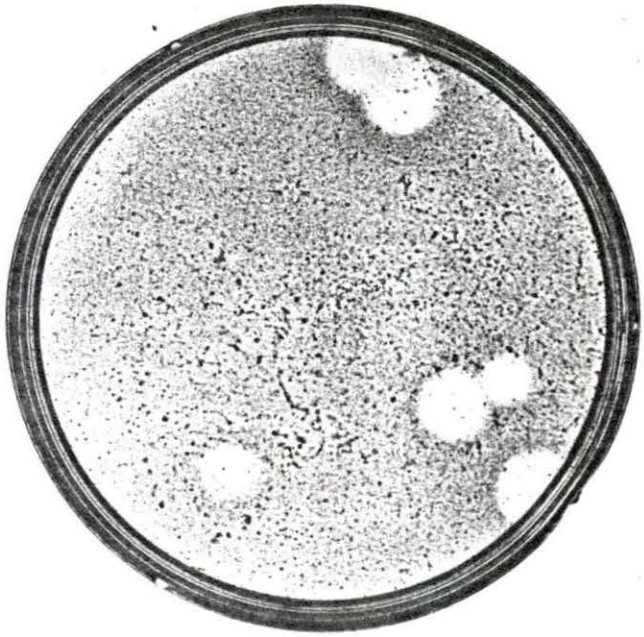


Figure 14. Plaques observed 48 hours after inoculation of WEE 5769E TCP #7A virus on chick embryo cell cultures with regular overlay medium

Mean plaque diameter = 1.5 mm.

Figure 15. Plaques observed 48 hours after inoculation of WEE 5769E TCP #7A virus on chick embryo cell cultures with special protamine overlay medium

Mean plaque diameter = 6.0 mm.

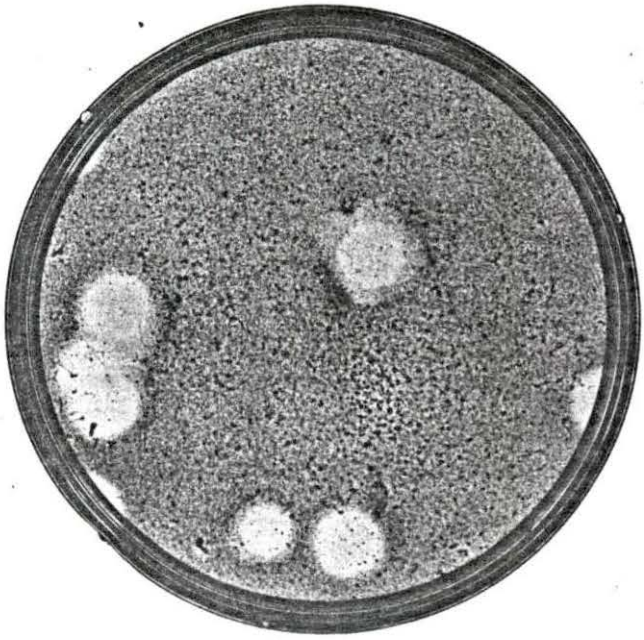
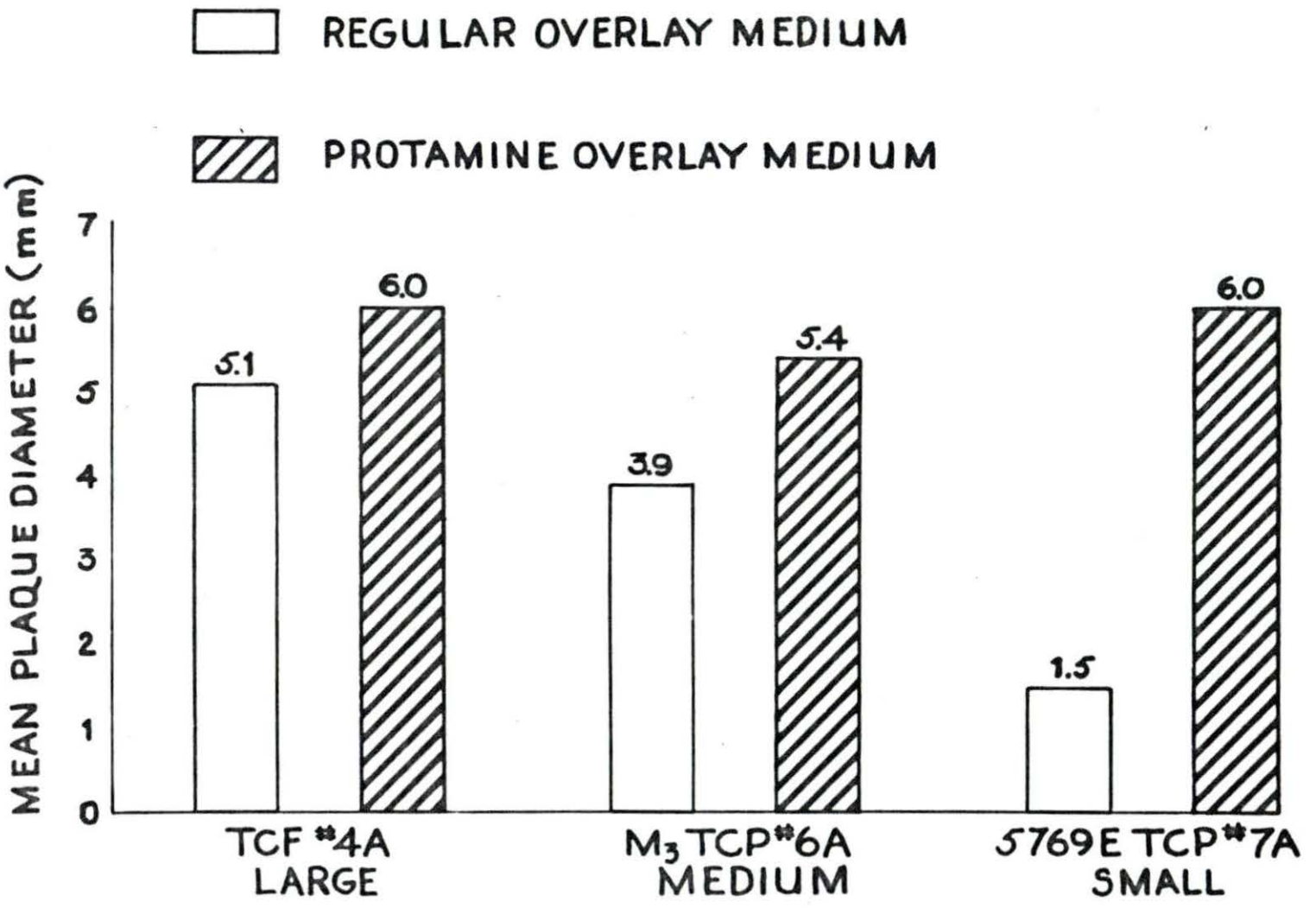


Figure 16. The effect of addition of protamine sulfate to the overlay medium on mean plaque diameters of three strains of WEE virus in chick embryo cell cultures (All plaques measured 48 hours following inoculation)


REGULAR OVERLAY MEDIUM
PROTAMINE OVERLAY MEDIUM
MEAN PLAQUE DIAMETER (mm)
0
1
2
3
4
5
6
7
5.1
6.0
3.9
5.4
1.5
6.0
TCF #4A
LARGE
M3 TCP#6A
MEDIUM
5769E TCP#7A
SMALL

Significance

Evidence seems to indicate that the addition of protamine sulfate, in the concentration used, markedly increased the diameter of plaques produced by WEE virus in chick embryo cell cultures. The presence of protamine sulfate apparently exerted a comparatively greater influence in cultures inoculated with small-plaque-producing types of WEE virus. The differences in plaque diameters routinely observed in cultures overlayed with regular medium were virtually eliminated in the presence of protamine sulfate.

Results of this experiment parallel the reported observations of Takemoto and Liebhaber (51), who were able to alter the plaque morphology of EMC virus by the addition of protamine sulfate to the overlay medium. Until additional experimental evidence should prove otherwise, and in light of the preliminary results reported here, one might be tempted to assume that the mode of action of protamine sulfate in this case is similar to its action in the slightly different culture system described by Takemoto and Liebhaber. These workers performed more extensive experimentation with EMC virus than time factors would allow in the present study. Their evidence supported the theory that the presence of protamine sulfate in cell culture overlays indirectly increased the size of EMC virus plaques by chemically inactivating a polysaccharide sulfate inhibitor of plaque

development present in Noble agar.

A similar assumption, if made in this case, would agree well with experimental evidence to be presented later in this thesis. That assumption is that certain strains of WEE virus are highly susceptible to the inhibitory effects of a polysaccharide sulfate compound in Noble agar, and as a result are capable of producing only relatively small plaques under overlay media in which Noble agar is utilized. The comparatively larger size of plaques produced by other WEE virus strains may be a result of decreased susceptibility of virus inocula in this category to the effects of the inhibitor.

Assay for Interferon-like Activity

WEE virus multiplication is reportedly susceptible to the inhibitory properties of interferon when both are present in chick embryo cell cultures (54). From a review of the literature on the subject of interferon, one might be led to conclude that propagation of WEE virus in chick embryos or mice could be responsible for stimulating the production of interferon by the cells of these hosts. If present in WEE virus inocula, an interferon-like inhibitor might well be partly responsible for the small plaques resulting when such inocula are introduced and propagated in chick embryo cell cultures. This possibility was considered

in attempts to experimentally determine the cause of observed variation in WEE virus plaque diameters.

Results

A series of chick embryo cell cultures was inoculated with WEE TCF #4A virus of known titer and known large-plaque-producing properties. The cup in the center of the agar overlay of each culture plate received one drop of one of a variety of fluids prepared for assay. Table 3 contains a complete list of fluids assayed, a description of the source and preparation in each case, and the results of the assay procedure. A positive result is indicated in cases where the assayed fluid demonstrated interferon-like activity. In these instances, diffusion of the inhibitor through the agar surrounding the cup apparently protected the chick embryo cells from the effects of the WEE TCF #4A virus inoculum. Plaques which would have been observed near the cup were absent due to the action of the inhibitory substance. At the outer limits of the zone into which the inhibitor had diffused, smaller than normal plaques appeared. This would suggest only partial protection of the cells from the cytopathic effects of the WEE virus present. Normal plaques typical for WEE TCF #4A virus appeared at the outer edges of the culture plate. Figure 17 is a photograph of a cell culture used for assay of interferon-like activity, and

Table 3. Source, methods of preparation, and results of assay for interferon-like activity in WEE TCF #4A virus infected chick embryo cell cultures

Preparation				Assay by plaque inhibition[d]
Inoculum[a]	Time elapsed[b]	Material harvested	Treatment[c]	
Diluent	48 hr.	allantoic fluid from normal, live chick embryos	uncentrifuged	negative
			supernatant	negative
			pellet[e]	negative
WEE 5782E passed at 10^{-1} dilution	18 hr.	allantoic fluid from WEE-killed chick embryos	uncentrifuged	negative
			supernatant	negative
			pellet	negative
WEE 5783E passed at 10^{-4} dilution	24 hr.	allantoic fluid from WEE-killed chick embryos	uncentrifuged	negative
			supernatant	negative
			pellet	negative

[a]Refer to identification code (Table 2). See plaque properties in Appendix B.

[b]Time elapsed after injection of host and before harvest of material tested.

[c]Ultracentrifugation at 100,000 g for 5 hours at 5° C.

[d]Refer to Figures 17 and 18. Negative = no inhibition. Positive = inhibition.

[e]Pellet restored to original fluid volume in diluent.

Table 3. (Continued)

Preparation				Assay by plaque inhibition[d]
Inoculum[a]	Time elapsed[b]	Material harvested	Treatment[c]	
WEE 5783E passed at 10^{-5} dilution	48 hr.	allantoic fluid from surviving chick embryos	uncentrifuged supernatant pellet	negative negative negative
SIV #8 passed at 10^{-1} dilution	60 hr.	allantoic fluid from SIV-killed chick embryos	uncentrifuged supernatant pellet	positive positive negative
SIV #8 passed at 10^{-7} dilution	60 hr.	allantoic fluid from SIV-killed chick embryos	uncentrifuged supernatant pellet	positive positive negative
Diluent	7 da.	brain emulsion from normal, live, mouse	uncentrifuged supernatant pellet	negative negative negative
WEE #11M passed at 10^{-1} dilution	3 da.	brain emulsion from WEE-infected moribund mouse	uncentrifuged supernatant pellet	negative negative negative
WEE #12M passed at 10^{-6} dilution	5 da.	brain emulsion from WEE-infected moribund mouse	uncentrifuged supernatant pellet	negative negative negative
WEE #12M passed at 10^{-8} dilution	7 da.	brain emulsion from surviving mouse	uncentrifuged supernatant pellet	negative negative negative

a positive result is demonstrated.

When fluids devoid of interferon-like activity were deposited in the center well of such cultures, no inhibitory effects could be detected when plates were observed after 48 hours. Figure 18 indicates the appearance of such a culture in which negative results were obtained.

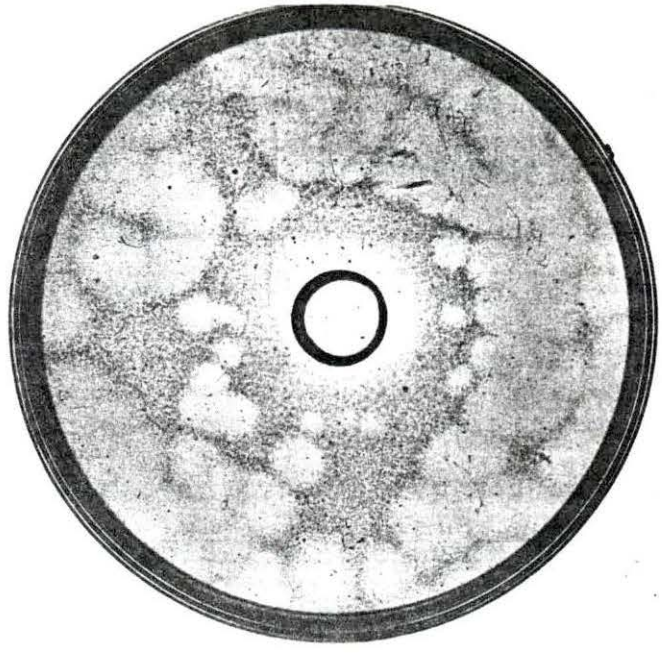
No interfering substances were detected by this assay procedure in test fluids prepared from normal chick embryo allantoic and amnionic fluids. Supernatant fluids prepared from emulsions of normal mouse brains also yielded negative results when tested.

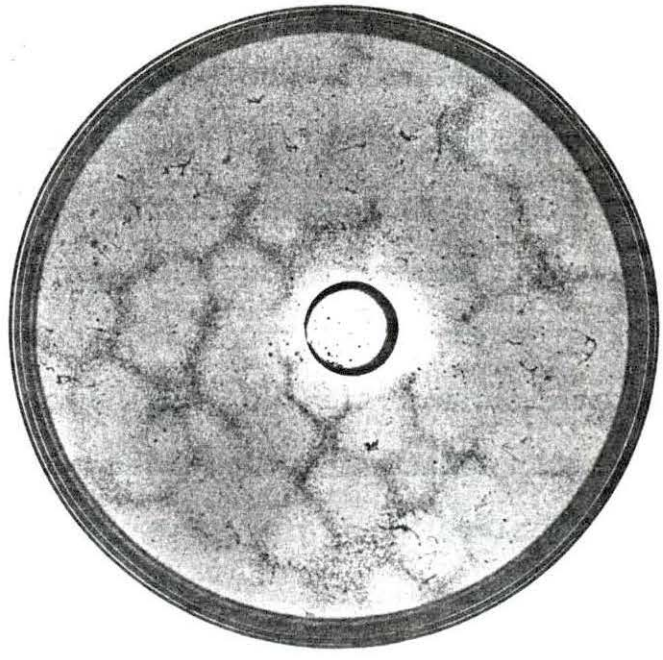
Fluids prepared from WEE virus infected chick embryos at varying times after infection were negative at the time of assay. Test materials obtained from the brains of WEE-virus-infected mice were also devoid of demonstrable interfering components.

When allantoic and amnionic fluids from chick embryos infected with either high or low dilution passaged #8 SIV were prepared and tested by identical methods, these fluids were observed to elicit good inhibition of plaque formation by WEE TCF #4A virus in chick embryo cell cultures. The fact that identical methods of preparation were successful with SIV containing materials, and unsuccessful with WEE virus preparations, tends to establish the validity of the negative results.

Figure 17. WEE TCF #4A plaques 48 hours after inoculation on chick embryo cell culture plate for assay of interferon. Limits of diffusion of fluid from the center well are marked by total absence or decreased size of WEE virus plaques. The allantoic fluid contains an interferon-like substance

Figure 18. WEE TCF #4A plaques 48 hours after inoculation on chick embryo cell culture plate for assay of interferon. Presence of allantoic fluid diffusing from center well did not inhibit WEE virus plaque formation, therefore was thought not to possess interferon-like activity





With all preparations, an attempt was made to remove a large percentage of infective virus particles from the fluids assayed for presence of interferon-like activity. This step in the procedure was necessary to establish that any inhibitory activity observed was not related to competitive inhibition of WEE plaque development related to the presence in the test fluids of either infective or incomplete virus particles. Ultracentrifugation of preparations at 100,000 g for 5 hours was employed for this reason. In the case of SIV preparations, an alternative method was feasible. Hemadsorption of infective SIV particles to chicken red blood cells and removal by centrifugation was employed. This method also assured more likely removal of any incomplete virus hemagglutinins originally present. Hemagglutination techniques were not employed with WEE virus preparations since hemagglutination of chicken red cells by this virus necessitates such critically controlled conditions. This rather laborious procedure was not justified, since no inhibiting activity of crude preparations from WEE virus infected hosts was observed.

Figure 19 demonstrates the relative success of the two different methods of preparation of #8 SIV allantoic and amnionic fluids for assay. The hemadsorption technique more efficiently removed infective SIV from the interferon-like preparations, as evidenced by results of titrations of these

Figure 19. Titers of infective swine influenza virus (SIV), expressed as chick embryo LD_{50}/ml., in fluids prepared by different methods for assay of interferon-like activity

SIV inoculum used: #8S 10^{-1} passage

Letters designate the following:

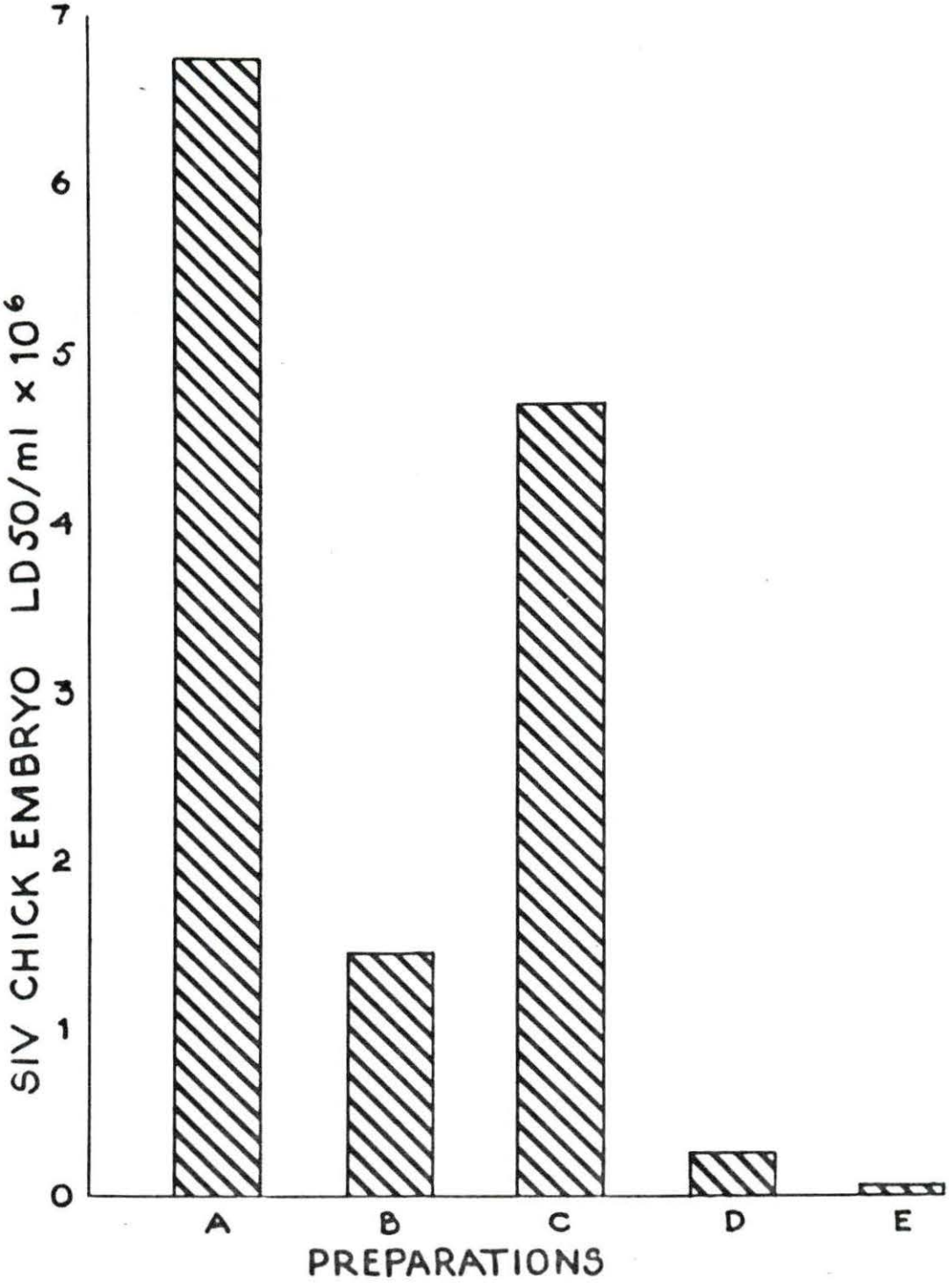
A. An aliquot of a pool of allantoic and amnionic fluids from SIV-infected embryos harvested 60 hours following allantoic cavity inoculation

B. Supernatant fluid recovered after an aliquot from the same pool was centrifuged at 100,000 g for 5 hours

C. Pellet sedimented during ultracentrifugation of aliquot B (above) and restored to its original fluid volume in diluent

D. Supernatant fluid recovered after adsorption to chicken erythrocytes of SIV particles in another aliquot of the same pool. A period of 30 minutes at 5° C. was allowed for virus adsorption followed by centrifugation for 10 minutes at 1,000 r.p.m.

E. Supernatant fluid recovered from aliquot D (above) after a second identical red cell adsorption cycle


SIV CHICK EMBRYO LD50/ml x 10⁶
0
1
2
3
4
5
6
7
A
B
C
D
E
PREPARATIONS

fluids in 10-day-old chick embryos by allantoic cavity inoculation.

Titration of chick embryo LD_{50}/ml. contained in materials prepared by ultracentrifugation, on first observation, might suggest a possible discrepancy in results. When summed, the number of chick embryo LD_{50}/ml. remaining in the supernatant fluid (B, Figure 19), plus the number of chick embryo LD_{50}/ml. in the resuspended pellet (C, Figure 19), do not quite equal the number of chick embryo LD_{50}/ml. calculated from titrations of the original SIV suspension (A, Figure 19). This small difference might indicate that a slight drop in numbers of infective SIV particles occurred during ultracentrifugation for 5 hours at 5° C. Incomplete dispersal of the sedimented pellet throughout the resuspending diluent may also have been a factor which lowered the titrated value obtained for preparation C, Figure 19. A sterile tissue grinder was used to facilitate resuspension of the pellet however, and this latter effect mentioned may have been rather slight.

Titrations to determine the level of interferon-like activity were performed on preparations derived only from SIV-infected embryos, since only these fluids demonstrated inhibitory effects in tests with undiluted materials. Test cultures each received one drop of the particular 2-fold dilution of inhibiting fluid. Triplicate cultures used at

each dilution yielded the results shown in Table 4, when observed 48 hours after inoculation. Apparently, removal from the fluids of a large majority of infective SIV particles did not reduce the inhibitory activity of the preparations tested. More conclusive evidence that the inhibition of WEE virus plaque development demonstrated by these substances is not directly related to their content of infective SIV, is demonstrated by the lack of interfering activity of the test material prepared from the resuspended SIV pellet (C, Figure 19), and (C, Table 4).

Significance

The properties of the interfering substance prepared from SIV-infected chick embryos very closely resemble the described properties of interferon. Neither are reduced in activity following incubation with chicken red cells, a procedure known to remove most of the infective SIV particles and also incomplete virus hemagglutinins. Neither can be effectively sedimented by high speed ultracentrifugation. Both exhibit the same WEE virus plaque inhibiting properties in chick embryo cell cultures. The inhibitor prepared in this experiment has been referred to as an interferon-like substance, and all available evidence concerning its properties point to the probability that it is, in fact, interferon.

Table 4. Titration of allantoic and amnionic fluids from SIV-infected embryos for interferon-like activity inhibiting plaque formation of WEE virus in chick embryo cell cultures

Fluid titrated[a]	Results in triplicate at each dilution indicated[b]				
	1/2	1/4	1/8	1/16	1/32
A	+	+	+	+	-
	+	+	+	+	-
	+	+	+	+	-
B	+	+	+	+	-
	+	+	+	+	-
	+	+	+	+	-
C	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
D	+	+	+	+	-
	+	+	+	+	-
	+	+	+	+	-
E	+	+	+	+	-
	+	+	+	+	-
	+	+	+	+	-

[a] Refer to Figure 19 for descriptions.

[b] Inhibition observed = (+)
No inhibition = (-).

Experimental results confirm a previous report (54) of the susceptibility of WEE virus in chick embryo cell cultures to the action of interferon. Interferon, when present in chick embryo cell cultures, does effectively reduce the

diameters of these WEE virus plaques produced at the border of zones of inhibition. However, assay of a large variety of WEE virus preparations for similar inhibitory effects on plaque development of WEE TCF #4A challenge virus yielded negative results in each case. Therefore, the occurrence of small plaques on chick embryo cell cultures, inoculated with WEE virus strains propagated at low dilution in mouse brains or chick embryos, is apparently not due to the presence of an interferon-like inhibitor of plaque formation in these inocula.

Another observation worthy of mention is the fact that the plaque size of WEE TCF #4A virus was not decreased in the presence of normal mouse tissue emulsions, nor in the presence of allantoic and amnionic fluids from uninfected chick embryos. This might also serve to indicate that the small plaques which result from WEE virus inocula suspended in these fluids are not associated with the presence of inhibitory substances in these materials, but must be related to some property of the viruses themselves.

Relationship of Autoinhibition by Heat Inactivated WEE Virus Particles to WEE Virus Plaque Diameter

A report by Lockart and Groman (41) has been reviewed, which indicates the sensitivity of WEE virus to the effects of moderate temperatures. This report also includes mention

of smaller than normal plaques produced by WEE virus inocula in chick embryo cell cultures previously treated with suspensions of heat inactivated WEE virus particles. In light of these reported observations, an experiment was designed to determine if similar autoinhibitory effects were related to phenomena observed in the present study. The effects of prolonged incubation at 37° C. on the titer and mean plaque diameter of WEE TCF #4A were investigated. Five aliquots were selected at random from this pool of WEE virus known to produce plaques of large and uniform diameter, and each aliquot was transferred from the freezer to the 37° C. water bath at specified intervals. Time intervals were arranged so that results of heat inactivation could all be observed on one lot of cell cultures.

Results

Plaques produced in chick embryo cell cultures were counted, measured and photographed 48 hours after inoculation of the cultures with the aliquots which had been maintained at 37° C. for 0, 12, 24, 36 or 48 hours. Results are illustrated in Figures 20 through 24. As can be seen, no significant decrease in plaque size occurred as a result of heat exposure. In fact, as illustrated in Figure 25, although titer of infective WEE virus dropped markedly during 48 hours incubation at 37° C., mean diameter of plaques

Figure 20. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-6} dilution of WEE TCF #4A virus previously incubated 0 hours at 37° C.

Mean plaque diameter of 100 plaques from this passage = 5.2 mm.

Virus titer = 6.0×10^{7} PFU/ml.

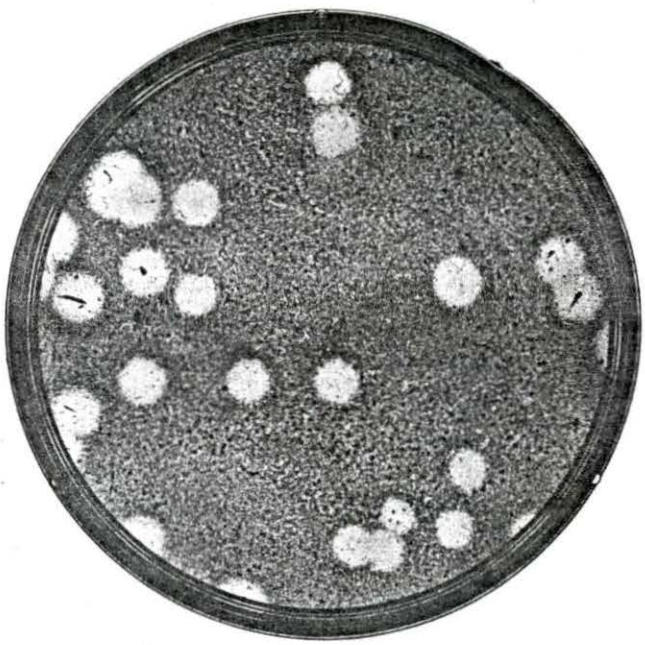


Figure 21. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-6} dilution of WEE TCF #4A virus previously incubated 12 hours at 37° C.

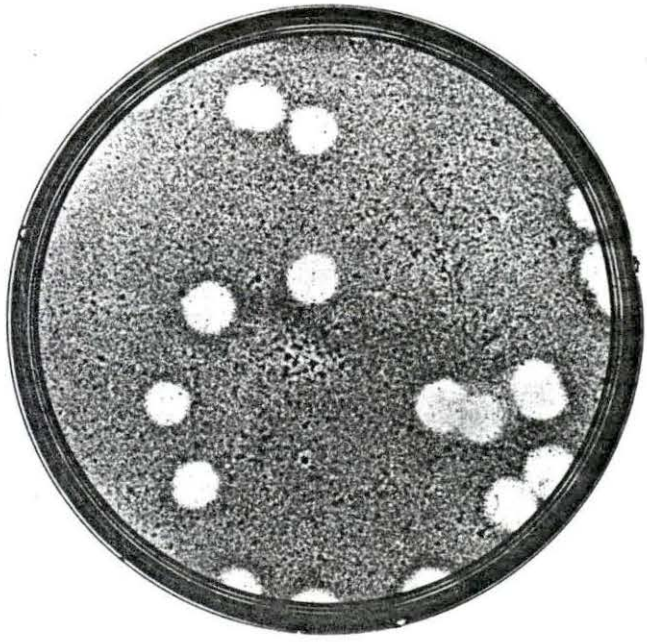
Mean plaque diameter of 100 plaques from this passage = 5.2 mm.

Virus titer = 3.2×10^{7} PFU/ml.

Figure 22. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-6} dilution of WEE TCF #4A virus previously incubated for 24 hours at 37° C.

Mean plaque diameter of 100 plaques from this passage = 5.0 mm.

Virus titer = 1.6×10^{7} PFU/ml.



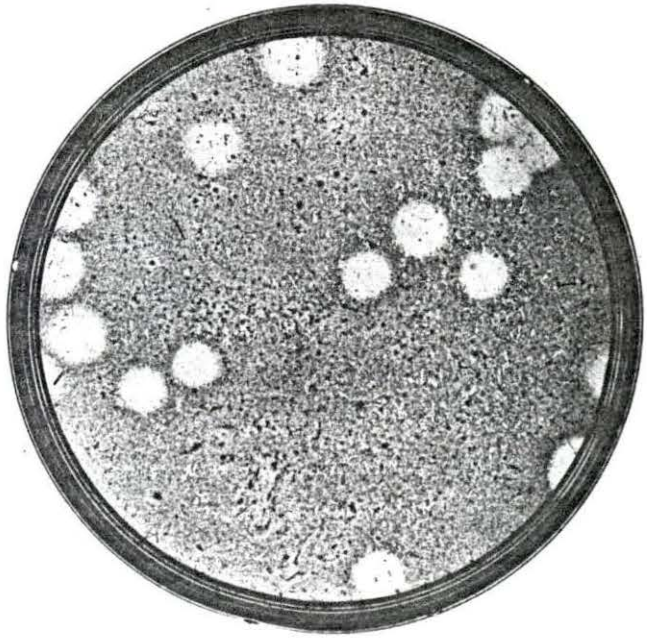


Figure 23. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-5} dilution of WEE TCF #4A virus previously incubated 36 hours at 37° C.

Mean plaque diameter of 100 plaques from this passage = 5.8 mm.

Virus titer = 4.5×10^{6} PFU/ml.

Figure 24. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-4} dilution of WEE TCF #4A virus previously incubated 48 hours at 37° C.

Mean plaque diameter of 100 plaques from this passage = 5.4 mm.

Virus titer = 1.3×10^{6} PFU/ml.

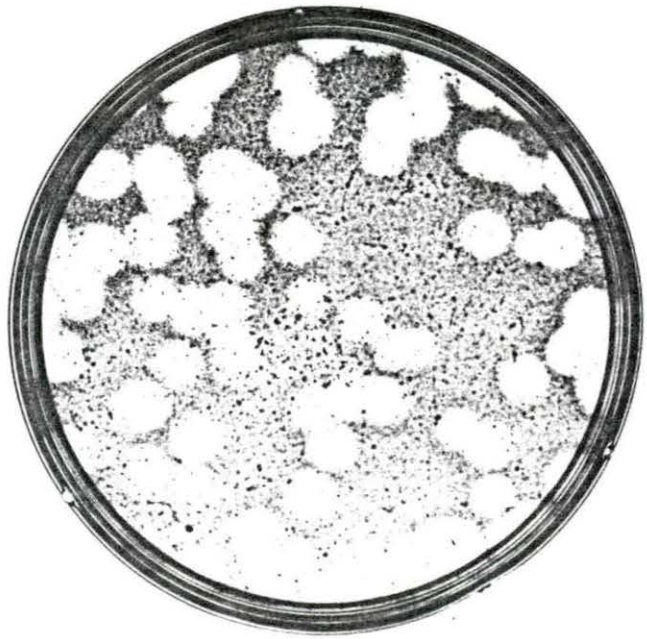
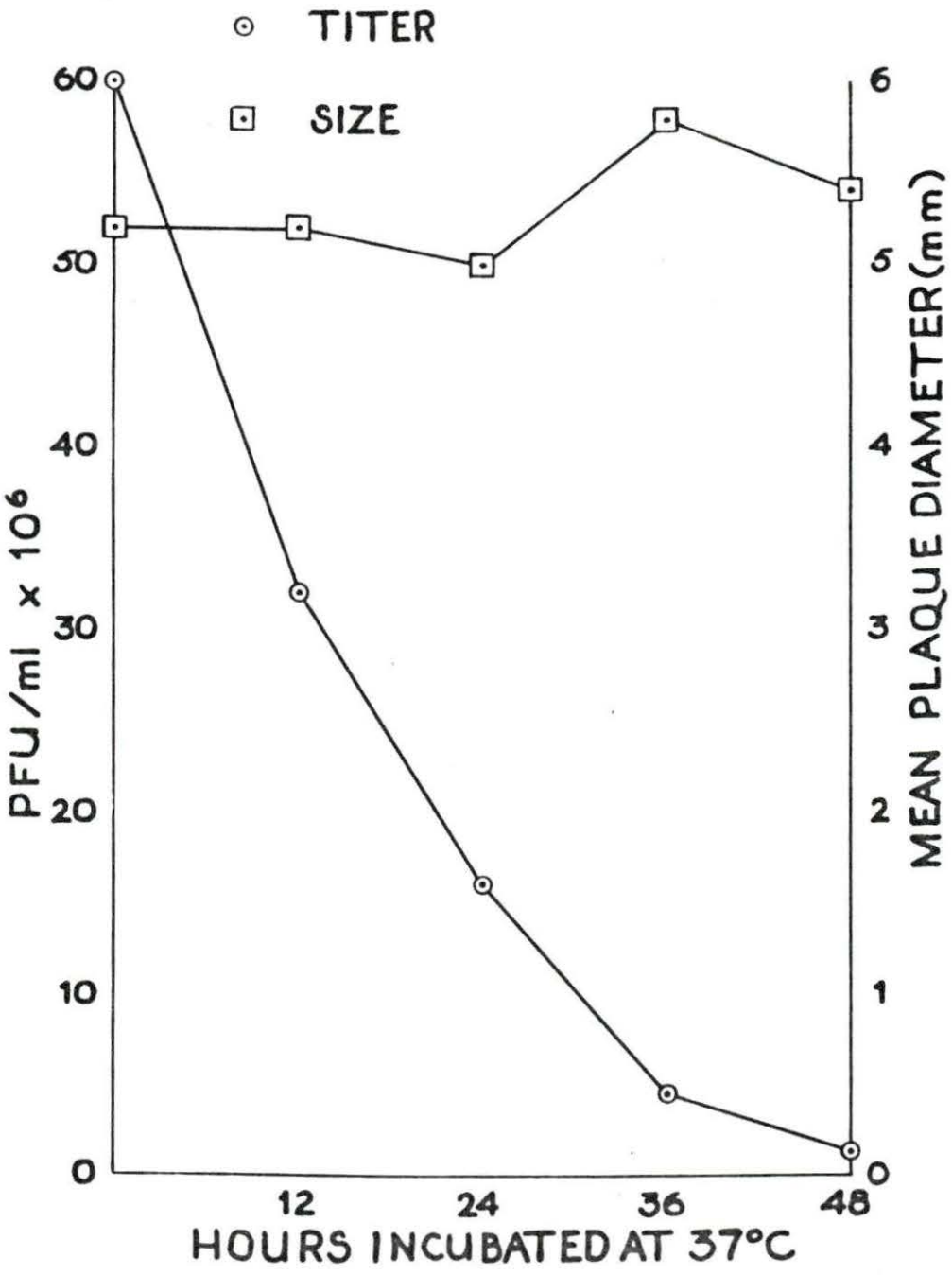


Figure 25. Mean plaque diameter of WEE TCF #4A virus on chick embryo cell cultures compared to titer expressed in PFU/ml. of inoculum after incubation of aliquots from WEE TCF #4A pool for 0, 12, 24, 36 or 48 hours at 37° C.


TITER
SIZE
60
50
40
30
20
10
0
PFU/ml x 10⁶
6
5
4
3
2
1
0
MEAN PLAQUE DIAMETER(mm)
12
24
36
48
HOURS INCUBATED AT 37°C

produced by inocula containing a high percentage of inactive WEE virus particles was not changed, or if anything, tended to increase.

Significance

Experimental results would seem to indicate that autoinhibition by heat inactivated WEE virus particles is of very little significance as a factor responsible for the variations in WEE virus plaque diameter observed in the study being reported. Results did confirm the rather susceptible nature of WEE virus with regard to exposure for extended periods to moderate temperature. For this reason, during the course of further experiments, precautions were still taken to minimize exposure times of WEE virus inocula to temperatures above those of storage at -40° C.

Genetic Changes Related to WEE Virus Plaque Size

Preliminary investigations in this study revealed WEE virus plaque diameter variations which could be related to the passage history of the inoculum employed. The host chosen for propagation of a strain of WEE virus apparently was a factor affecting plaque size. Passage of the same inoculum at high or low dilution often resulted in different mean plaque diameters when the harvested virus was plaqued. The literature contains reports of several phenomena

attributed to the existence of genetically mixed populations of WEE (20) or related VEE viruses (17).

For these reasons, an experiment was devised which would elucidate the effects of passage of aliquots from a selected WEE virus pool at high and low dilutions in several different hosts. Aliquots chosen at random from the WEE TCF #4A virus pool repeatedly had been observed to produce relatively large plaques of uniform diameter in chick embryo cell cultures, and thus were a natural choice for use as a parent virus control. Effects on resulting plaque size were observed after passage of WEE TCF #4A virus at high and low dilutions in chick embryo cell cultures with fluid maintenance and in mice (Table 5). A later experiment investigated the same effects of chick embryo passage on plaque size.

Results in cell cultures and in mice

An aliquot from the WEE TCF #4A virus pool served as a control inoculum on chick embryo cell culture Lot #14A. Figure 26 is a photograph of resulting plaques whose mean diameter was 5.2 mm. In this particular experiment, distribution with respect to diameter of the first 100 plaques measured, as graphed in Figure 27, assumes a great deal of significance in interpretation of the results obtained. It can be seen that the control virus inoculum produced

Table 5. Scheme for determining effects of passage in cell cultures and mice on the plaque size of WEE TCF #4A virus

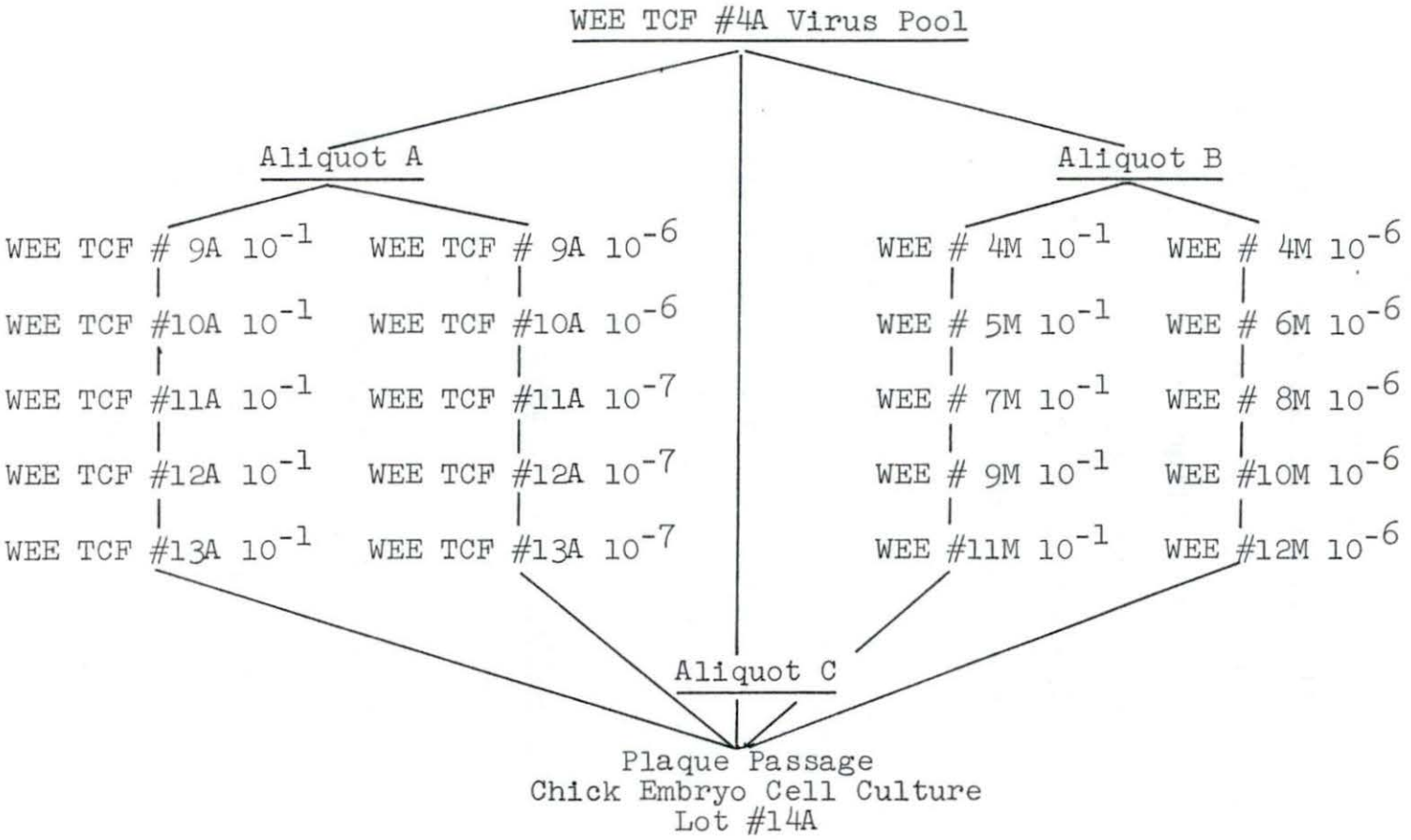


Figure 26. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of WEE TCF #4A virus (Parent virus control passage)

Mean plaque diameter = 5.2 mm.

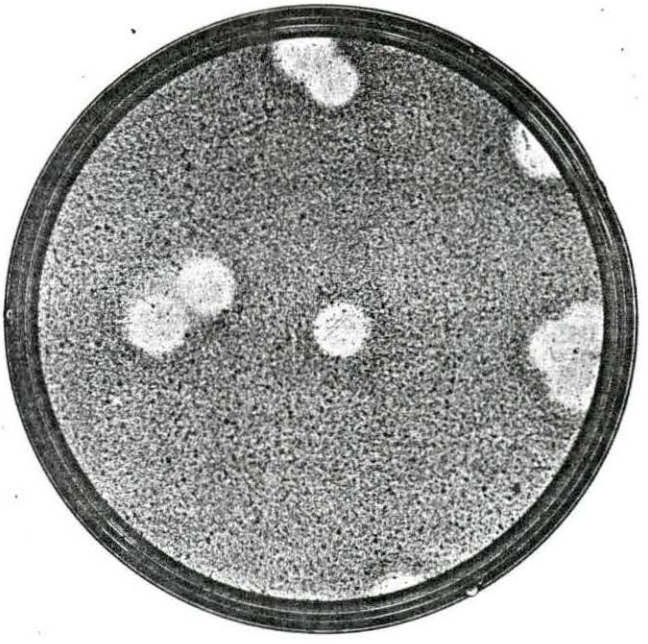
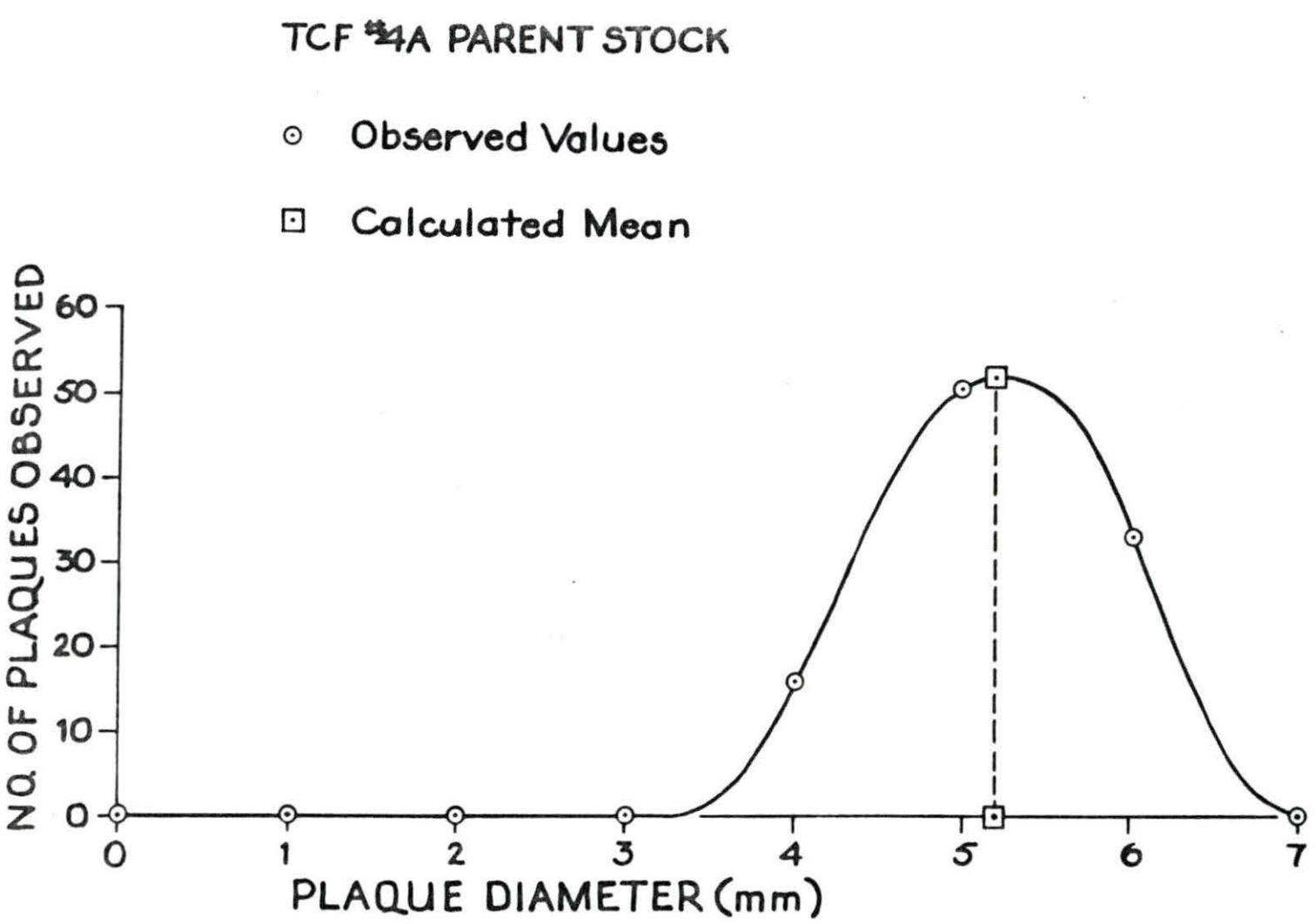


Figure 27. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figure 26


TCF #4A PARENT STOCK
Observed Values
Calculated Mean
NO. OF PLAQUES OBSERVED
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

entirely large plaques of fairly uniform diameter.

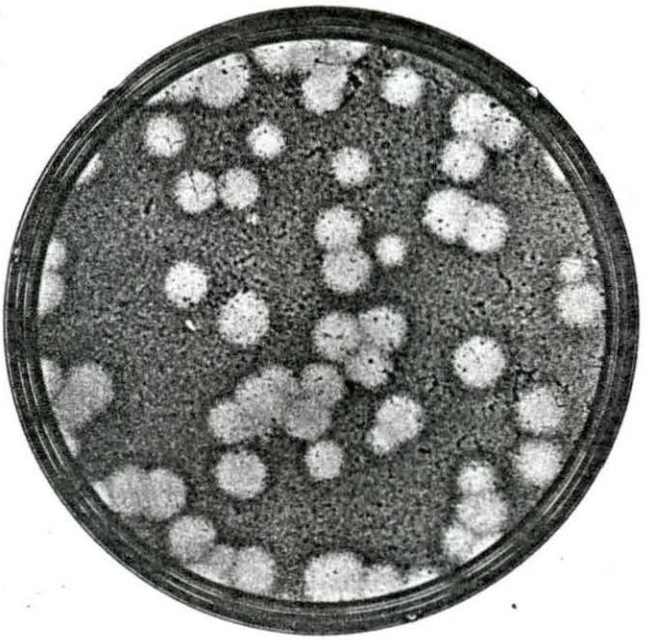
An aliquot from the WEE TCF #4A pool was passed serially five times at relatively high dilution (10^{-6} or 10^{-7}) in chick embryo cell cultures maintained in fluid medium. WEE TCF #13A 10^{-7} virus was the code designation for the virus pool harvested from the fifth passage. When plaques from this pool on Lot #14A cells were photographed at 48 hours, they appeared as shown in Figures 28 and 29. Figure 30 indicates that the first 100 plaques measured fell into two categories, large and small. They were distributed about two diameter means calculated to be 1.0 mm. for the smaller plaques, and 5.0 mm. for the larger plaques. The large plaques predominated.

The WEE TCF #13A 10^{-1} pool was derived by concurrent passage of the WEE TCF #4A parent inoculum in the same lots of cell cultures, and with identical procedure as that used for propagation of WEE TCF #13A 10^{-7} just discussed, except for one significant difference. Serial passage in this case was at comparatively low dilution, 10^{-1}. Figures 31 and 32 are photographs taken 48 hours after inoculation of WEE TCF #13A 10^{-1} virus on Lot #14A cell cultures. Distribution with respect to plaque diameters is presented in Figure 33. Again, mean plaque diameters could be calculated for both large and small plaques measured. Significantly, in this case, the smaller of the two plaque types represented the

Figure 28. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-6} dilution of WEE TCF #13A virus, after five serial passages at 10^{-7} dilution, of the parent TCF #4A virus

Figure 29. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of the same inoculum as in Figure 28

Mean plaque diameters of plaques represented in both figures = 1.0 mm. (small), 5.0 mm. (large)



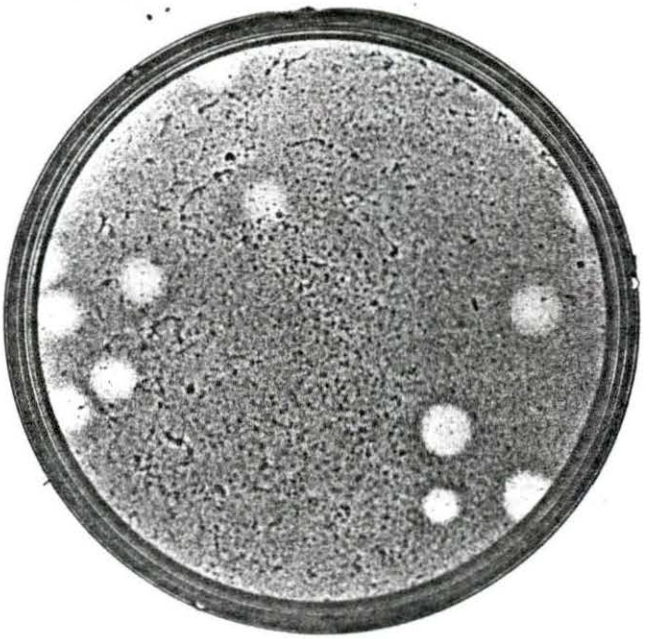


Figure 30. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figures 28 and 29

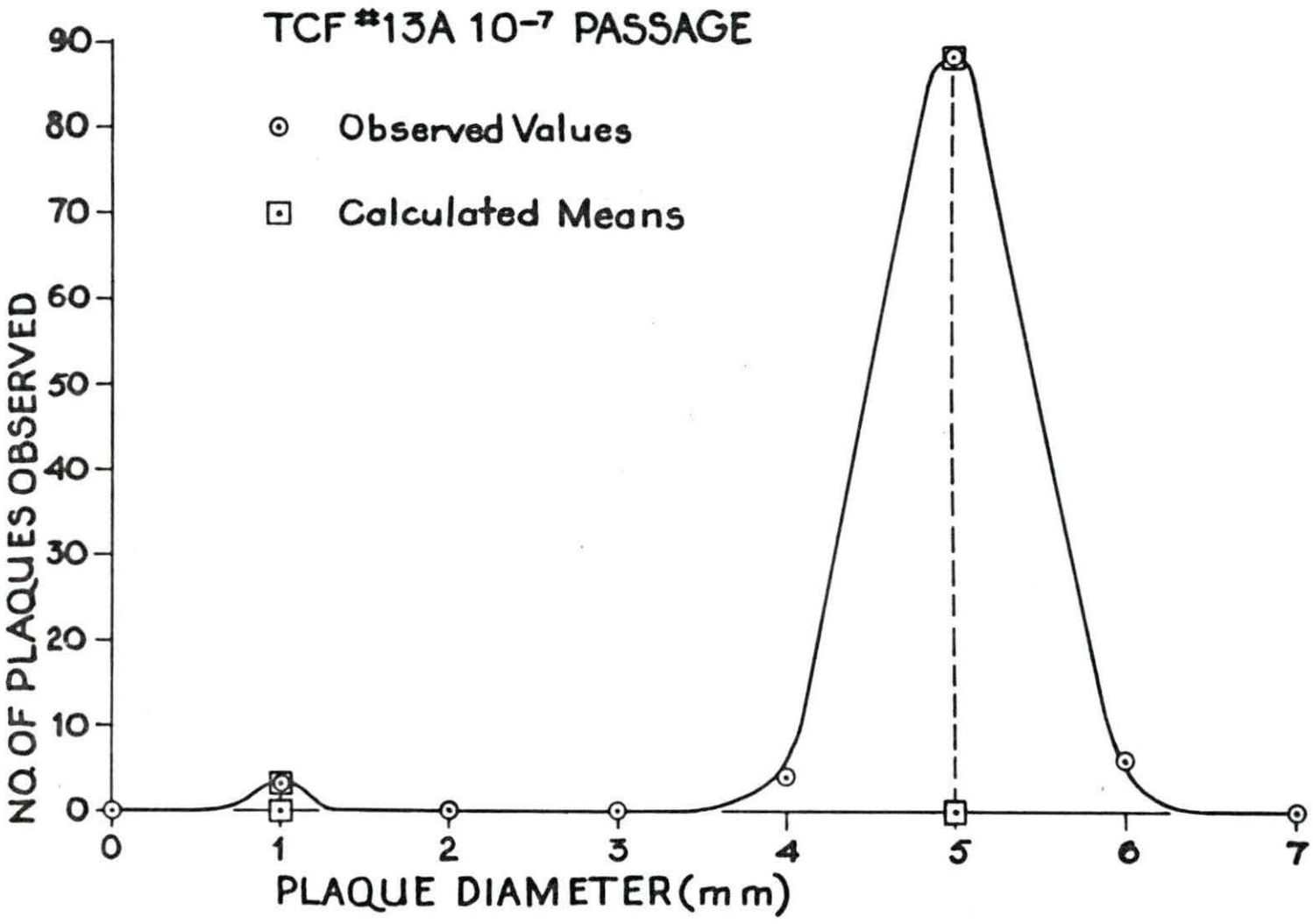

TCF #13A 10⁻⁷ PASSAGE
Observed Values
Calculated Means
NO OF PLAQUES OBSERVED
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

Figure 31. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-6} dilution of WEE TCF #13A virus, after five serial passages at 10^{-1} dilution, of the parent TCF #4A virus

Figure 32. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of the same inoculum as in Figure 31

Mean plaque diameters of plaques represented in both figures = 1.2 mm. (small), 4.7 mm. (large)

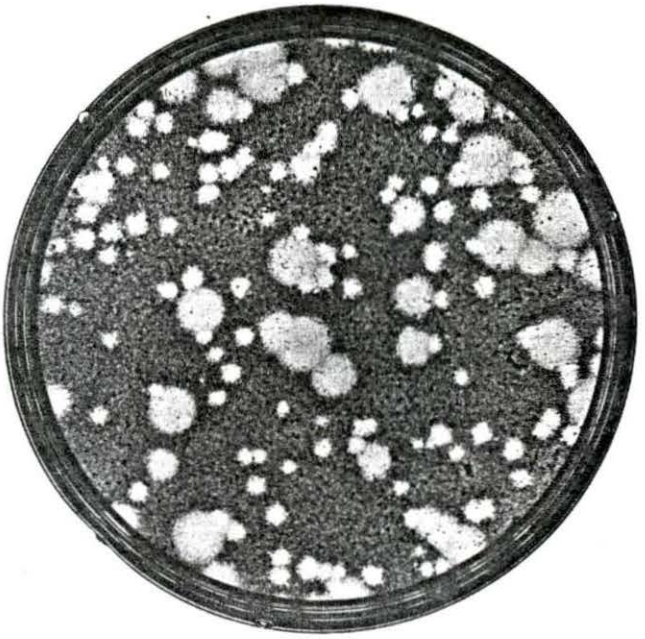
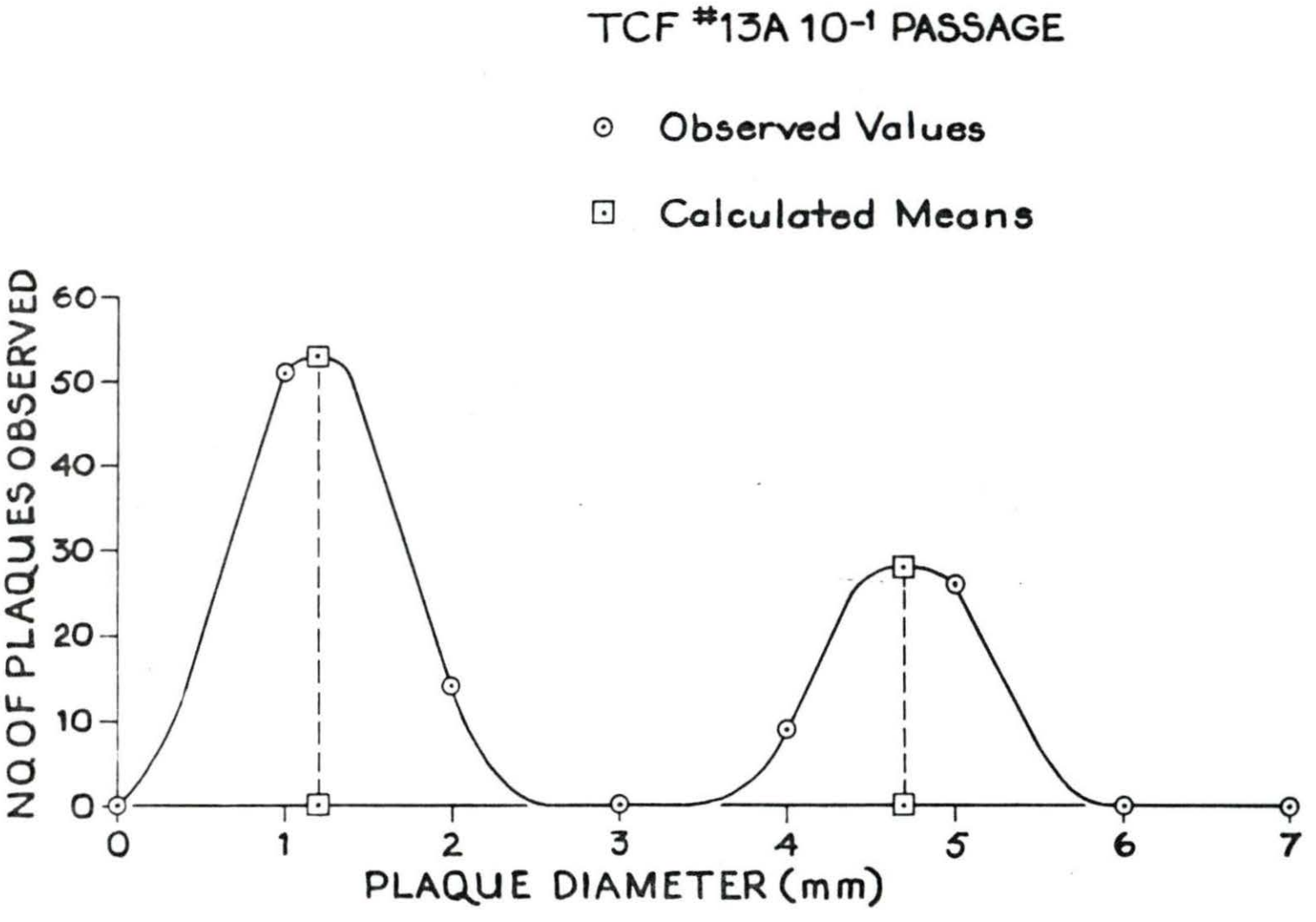


Figure 33. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figures 31 and 32


TCF #13A 10-1 PASSAGE
Observed Values
Calculated Means
NO OF PLAQUES OBSERVED
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

majority observed on these plates. A comparison of Figures 30 and 33 indicates a phenomenon of interest. A distinct difference in the diameters of plaques produced by the two virus populations is indicated by the graphs.

Figures 34 and 36 present results obtained in a similar fashion on Lot #14A cell cultures 48 hours following inoculation of WEE #12M virus. The WEE #12M virus inoculum had been propagated by serial passage of WEE TCF #4A parent virus five times at 10^{-6} dilution intracerebrally in mice. The WEE #11M pool was prepared identically, except it was the result of propagation of the same WEE TCF #4A virus through five passages in mice at 10^{-1} dilution. Plaques produced by WEE #11M on Lot #14A cell cultures appeared as in Figure 35, and their diameter distribution was as graphed in Figure 37. Again, results shown in Figures 36 and 37 suggested that the populations resulting from low dilution mouse passage had undergone more significant changes in relation to the control.

Significance of observations after passage in cell cultures and in mice

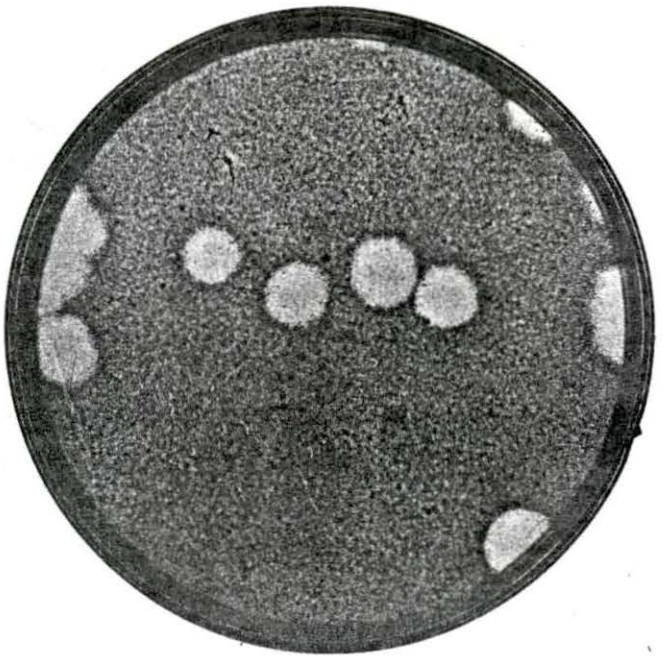
The dilution chosen for serial passage of a population of WEE virus (consisting of predominantly large-plaque-producers) was a factor definitely influencing plaque diameters when progeny were again plaqued in chick embryo cell cultures. Differences in plaque diameters resulting from

Figure 34. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of WEE #12M virus, after five serial passages intracranially in mice at 10^{-6} dilution, of the parent TCF #4A virus

Mean plaque diameters of plaques observed in the cell culture passage illustrated = 1.0 mm. (small), 3.7 mm. (large)
(No small plaques appear in this photograph)

Figure 35. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of WEE #11M virus, after five serial passages intracranially in mice at 10^{-1} dilution, of the parent TCF #4A virus

Mean plaque diameters of plaques observed in the cell culture passage illustrated = 1.0 mm. (small), 3.0 mm. (large)



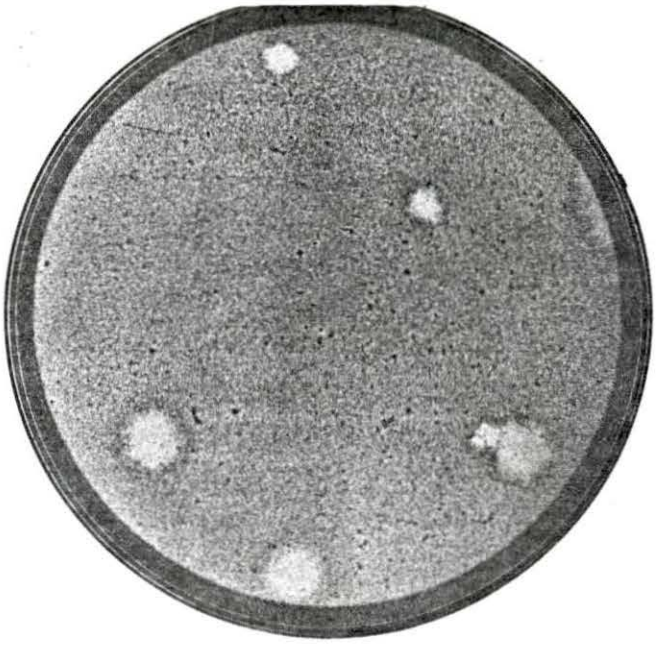


Figure 36. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figure 34

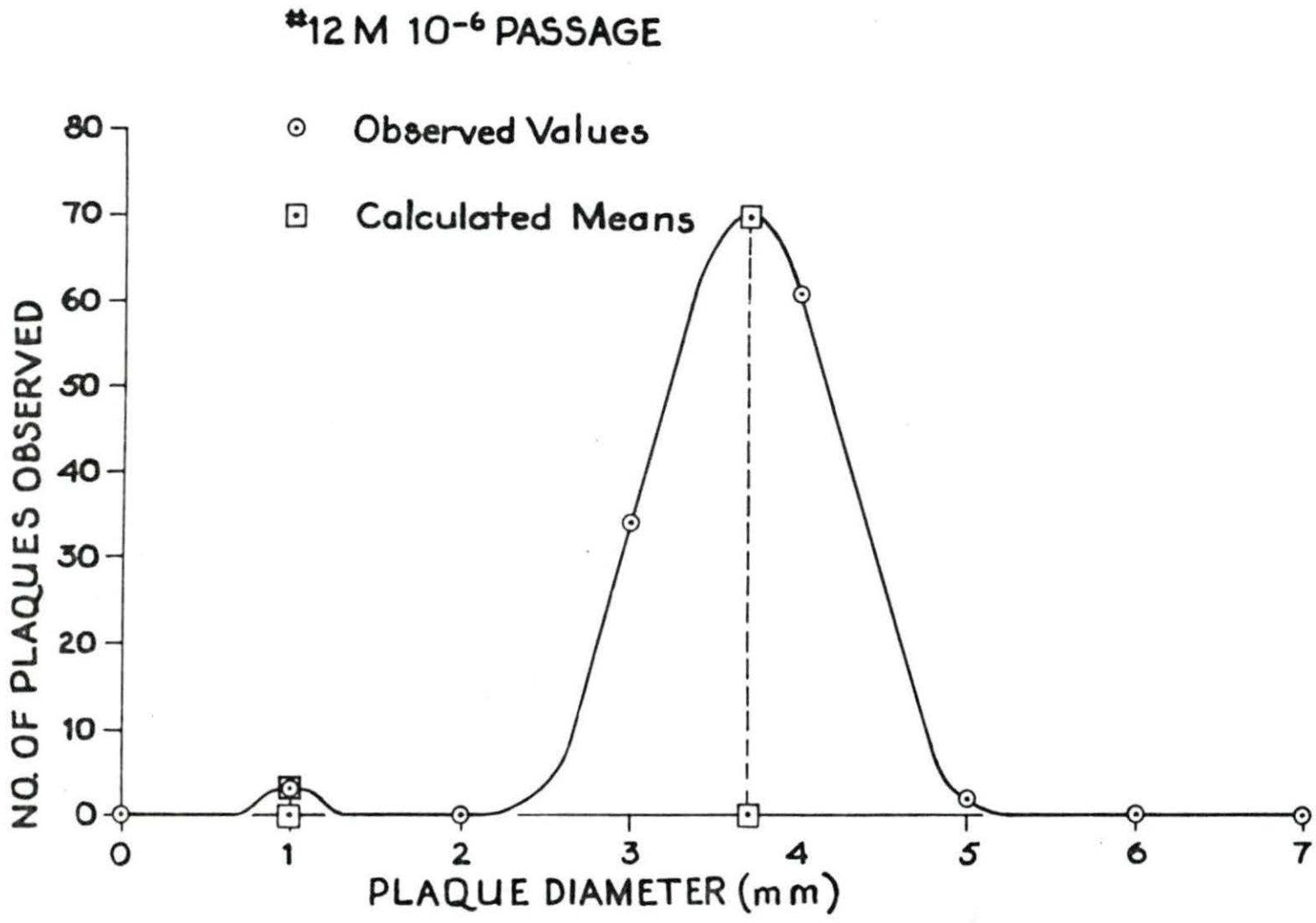
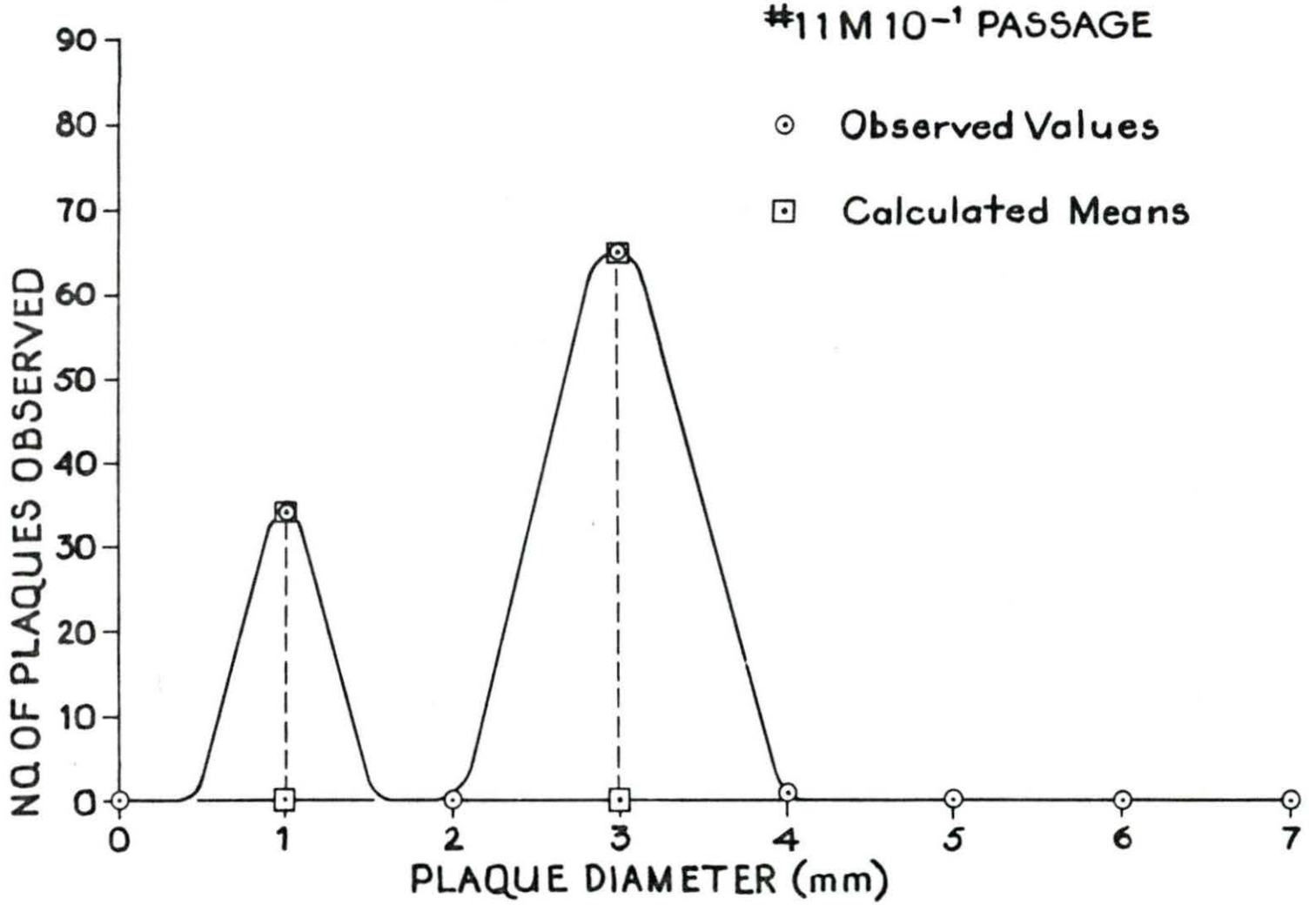

#12 M 10-6 PASSAGE
Observed Values
Calculated Means
NO. OF PLAQUES OBSERVED
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

Figure 37. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figure 35


#11 M 10⁻¹ PASSAGE
Observed Values
Calculated Means
NO OF PLAQUES OBSERVED
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

alternative choice of hosts were only slight, and comparatively of less significance. Data obtained might be interpreted to support the claims of other workers (17, 20) who have suggested the occurrence of genetically mixed populations of WEE virus.

The existence of two types of WEE virus in the population originally plaqued on chick embryo cell cultures could explain the occurrence of two sizes of plaques. Although large plaques were carefully selected by three clonings, apparently the WEE TCF #4A virus population resulting still contained a slight minority of the small-plaque-producing type.

Small plaques usually failed to appear when aliquots from the WEE TCF #4A virus pool were plaqued directly, and at terminal dilution, on cell cultures. This evidence indicates that they represented less than 1% of the total number of WEE virus particles present.

After five serial passages at high dilution in cell cultures (with fluid maintenance), or in mice, small plaques accounted for approximately 3% of the total plaques produced when the resulting populations were titrated on cell cultures.

After five serial passages in these hosts at 10^{-1} dilution, the type producing small plaques represented a majority of the virus population derived. The small-plaque-

producer apparently multiplies more rapidly than the type responsible for larger plaques when they are propagated in cell cultures with fluid maintenance or in mice. Serial passage at high dilution offsets this advantage, since at each passage level, terminal dilution tends to select a greater proportion of the large type which originally were the majority. It is only with serial passage at low dilution that the difference in replication rates is readily discernible.

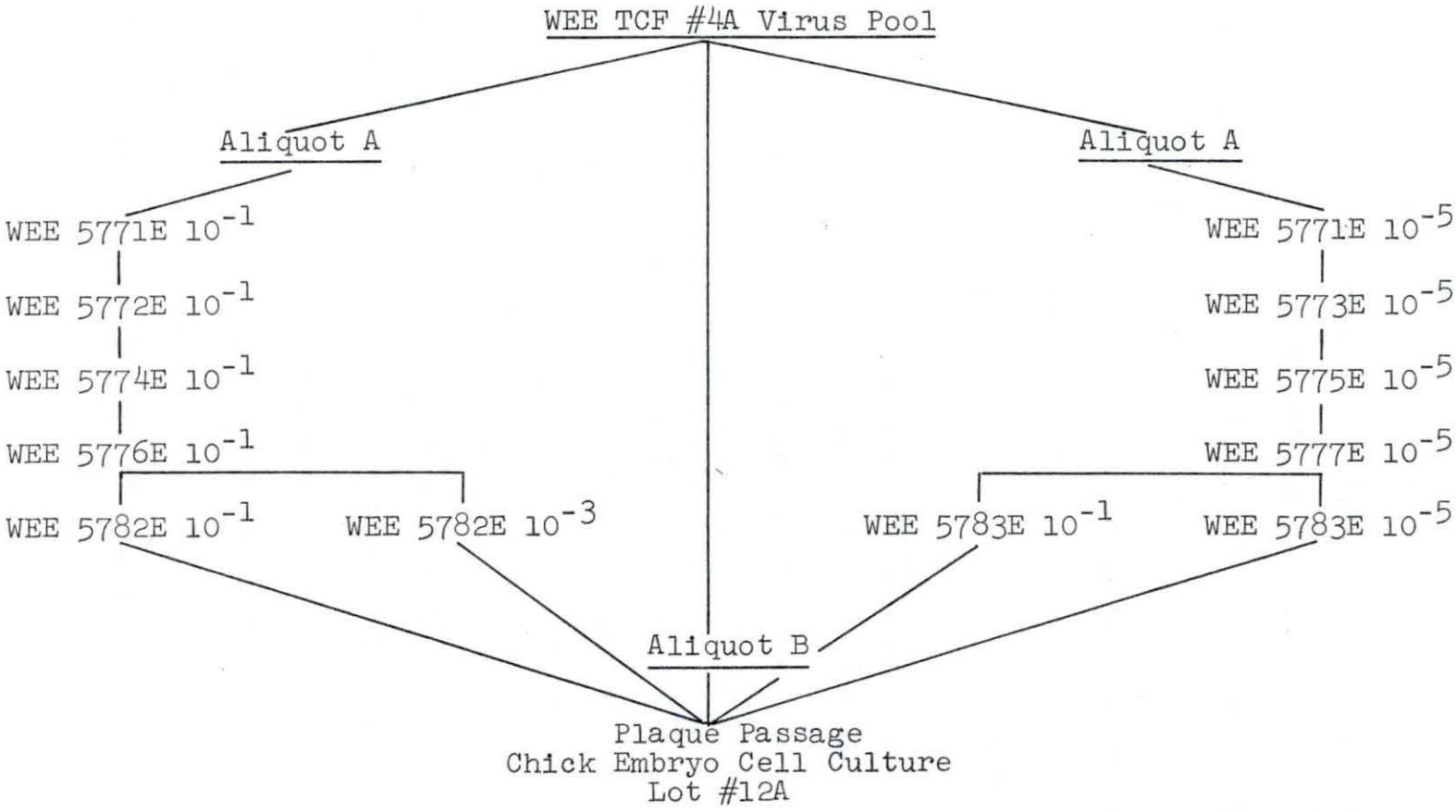
Results of chick embryo passage

An aliquot from the WEE TCF #4A pool was randomly selected for passage in chick embryos at high and low dilution, using the scheme shown in Table 6.

The WEE 5782E 10^{-1} virus pool resulted from harvest of allantoic and amnionic fluids from chick embryos infected during the fifth passage of the parent virus at 10^{-1} dilution. When the fifth passage of the parent virus was made instead at 10^{-3} dilution, the virus population obtained was identified as WEE 5782E 10^{-3}.

The WEE 5783E 10^{-5} virus pool was obtained by serial passage of the original WEE TCF #4A aliquot at 10^{-5} dilution. After four passages at 10^{-5} dilution, the WEE 5783E 10^{-1} virus pool was produced by one final passage at 10^{-1} dilution.

Table 6. Scheme for determining effects of passage in chick embryos on the plaque size of WEE TCF #4A virus



Following chick embryo passages, the diameters of plaques produced by the different WEE virus populations were compared with the range in plaque size of an aliquot of the original parent pool. All five titrations were performed in plates prepared from chick embryo cell culture Lot #12A, and typical cultures from each were photographed at 48 hours. These photographs showing plaques produced after inoculation of WEE TCF #4A control virus, and virus pools WEE 5783E 10^{-1}, WEE 5783E 10^{-5}, WEE 5782E 10^{-1} and WEE 5782E 10^{-3} are reproduced in Figures 38, 40, 41, 43 and 44 respectively. Data obtained from measuring the diameters of 100 plaques in each population are graphed in Figures 39, 42 and 45. By comparison of the photographs, it may be observed that plaques produced by the chick-embryo-propagated WEE virus pools tended to be decidedly smaller than those which resulted from inoculation of the parent virus control in the same set of cells. Calculated mean plaque diameters, graphically represented, indicated that these small plaques averaged less than half the size of the larger variety produced by WEE TCF #4A virus.

As shown in Figure 45, the mean diameter of plaques produced by WEE 5782E 10^{-1} inoculum was only 2.6 mm., in contrast to the 7.0 mm. mean plaque size of the control graphed in Figure 39. Superimposed on the plaque population curve for WEE 5782E 10^{-1} in Figure 45 is the curve for WEE

Figure 38. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of WEE TCF #4A virus (Parent virus control passage)

Mean plaque diameter = 7.0 mm.

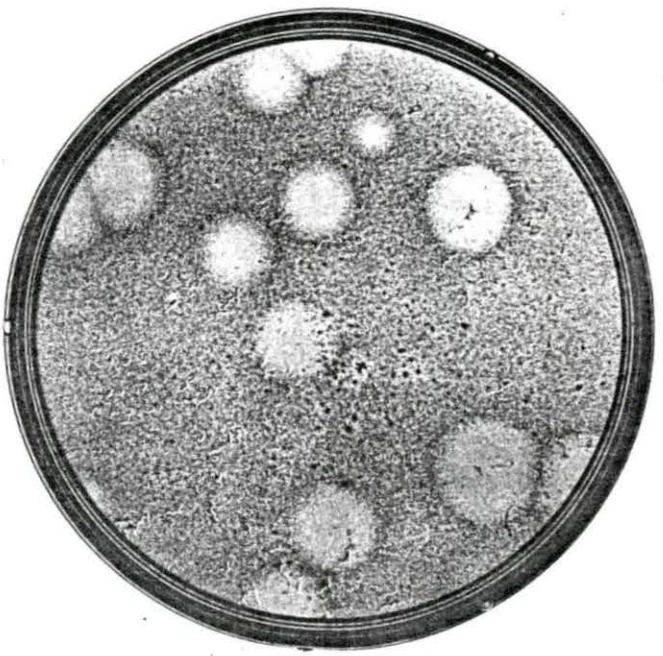


Figure 39. Distribution with respect to diameter of 100 plaques from the cell culture passage illustrated in Figure 38

TCF #4A PARENT STOCK

⊙ Observed Values

⊡ Calculated Mean

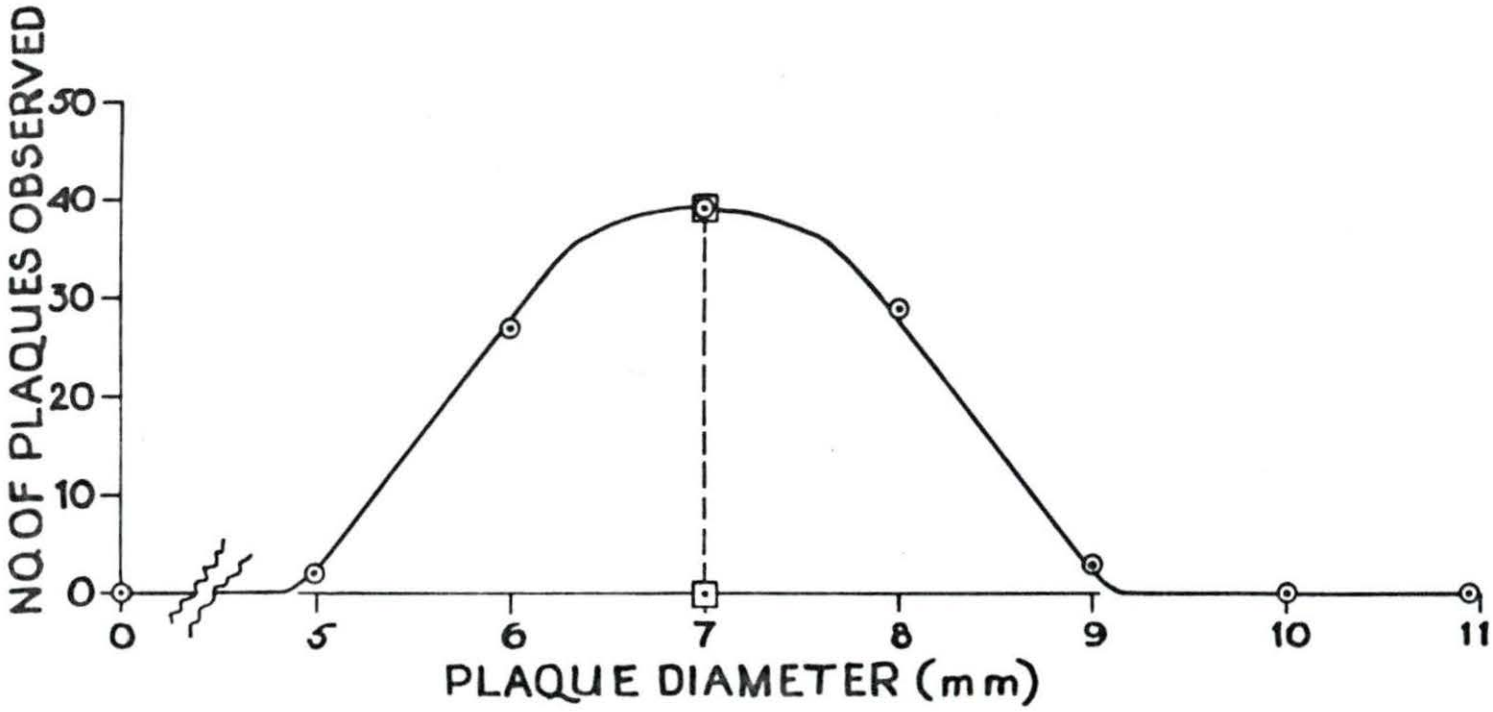
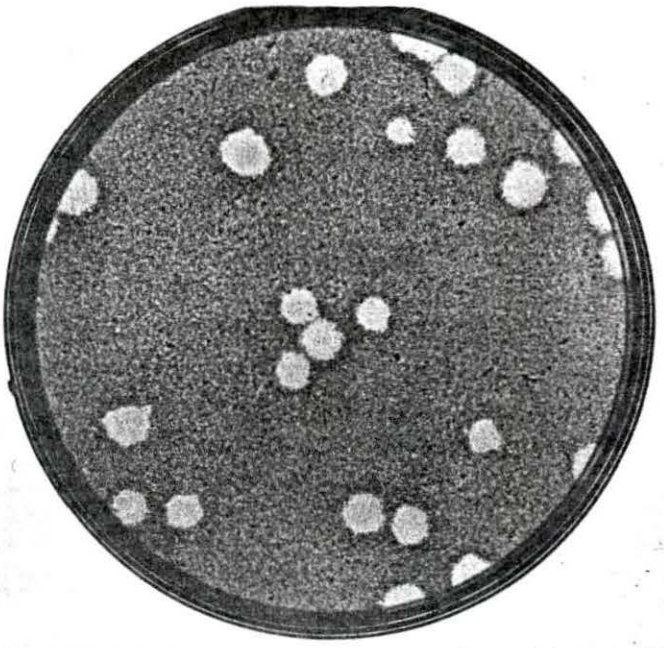


Figure 40. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of WEE 5783E virus after four serial passages at 10^{-5} dilution and one final passage at 10^{-1} dilution of the parent TCF #4A virus in chick embryos

Mean plaque diameter = 2.3 mm.

Figure 41. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of WEE 5783E virus after five serial passages at 10^{-5} dilution of the parent TCF #4A virus in chick embryos

Mean plaque diameter = 3.4 mm.



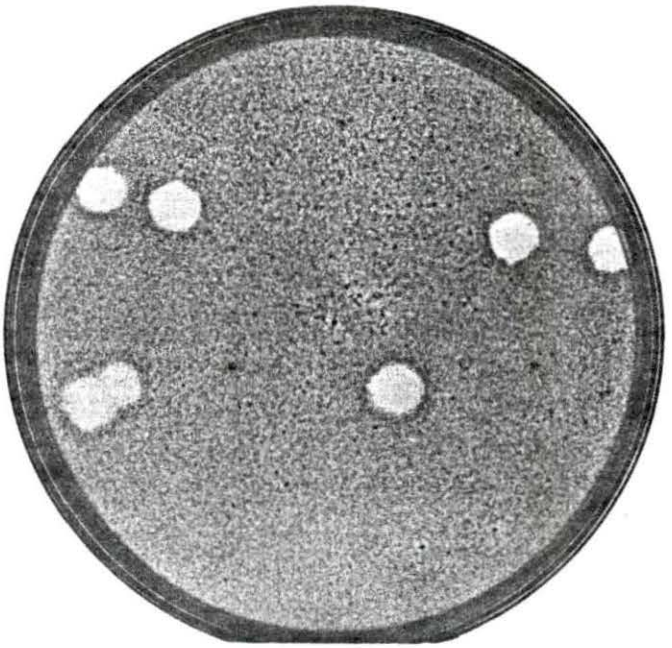


Figure 42. Distribution with respect to diameter of 100 plaques from the cell culture passages illustrated in Figure 40 and in Figure 41

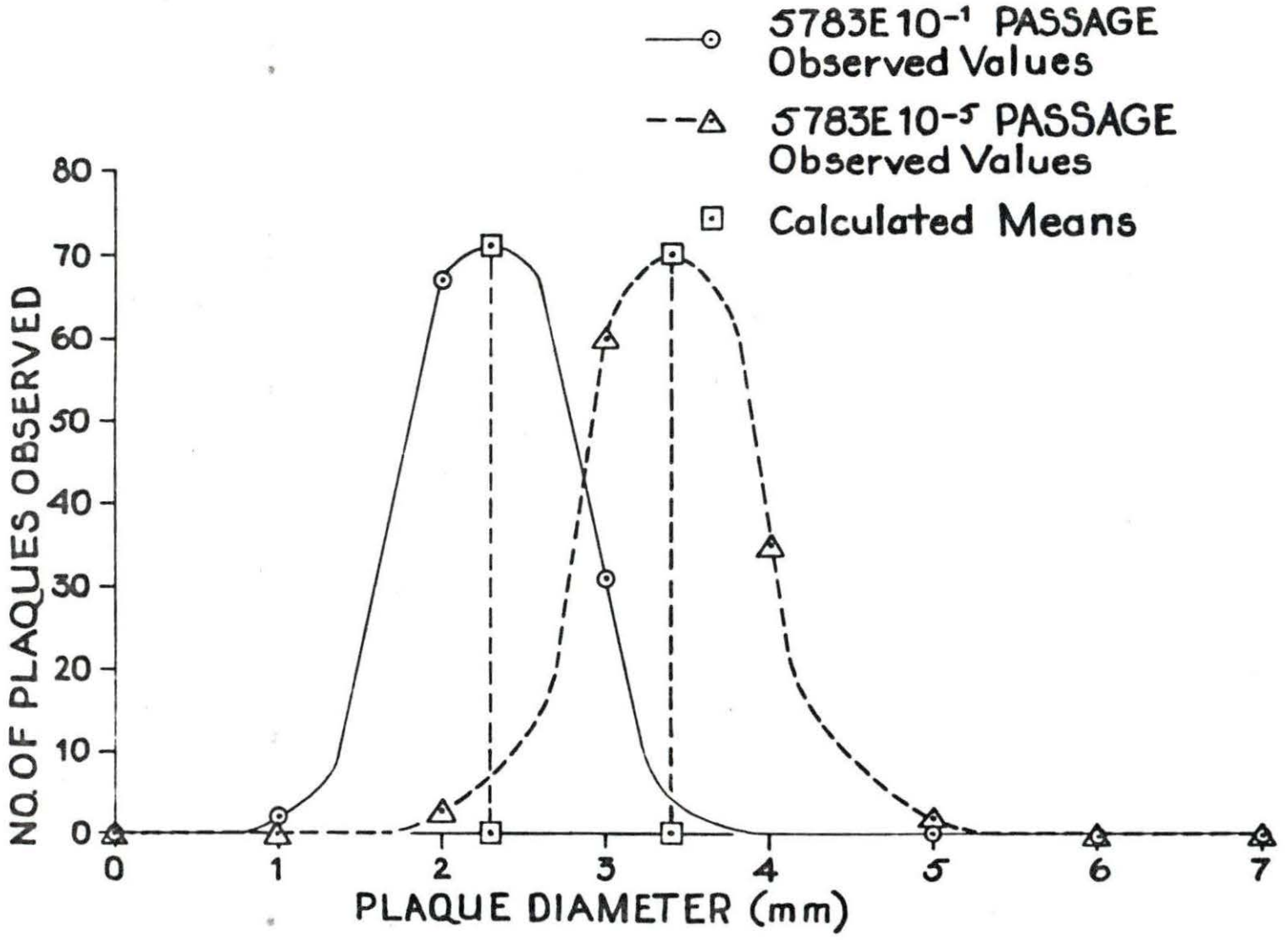
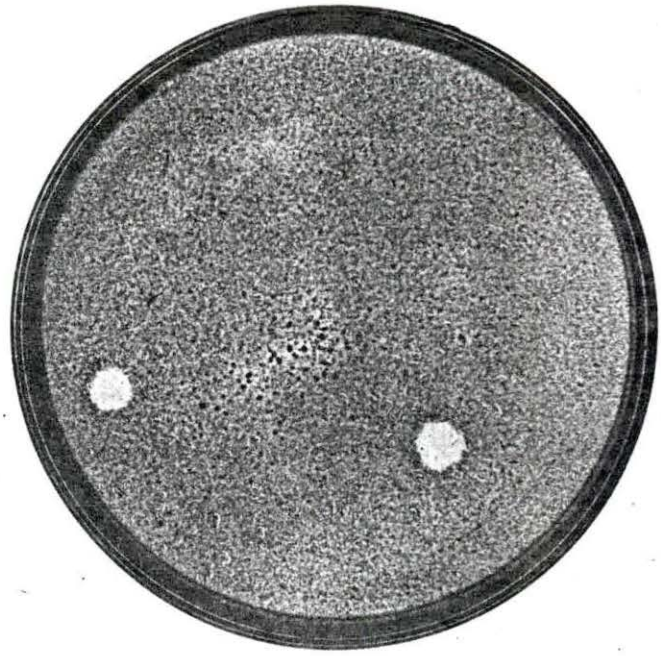

5783E 10⁻¹ PASSAGE
Observed Values
5783E 10⁻⁵ PASSAGE
Observed Values
Calculated Means
NO. OF PLAQUES OBSERVED
0
10
20
30
40
50
60
70
80
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

Figure 43. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-6} dilution of WEE 5782E virus after five serial passages at 10^{-1} dilution of the parent TCF #4A virus in chick embryos

Mean plaque diameter = 2.6 mm.

Figure 44. Plaques observed 48 hours after inoculation of a chick embryo cell culture with 0.5 ml. of a 10^{-7} dilution of WEE 5782E virus after four serial passages at 10^{-1} dilution and one final passage at 10^{-3} dilution of the parent TCF #4A virus in chick embryos

Mean plaque diameter = 2.6 mm.



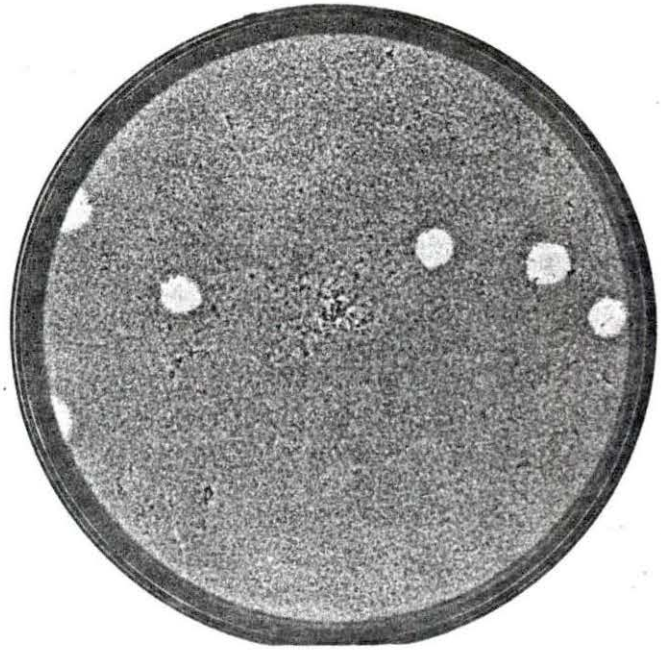
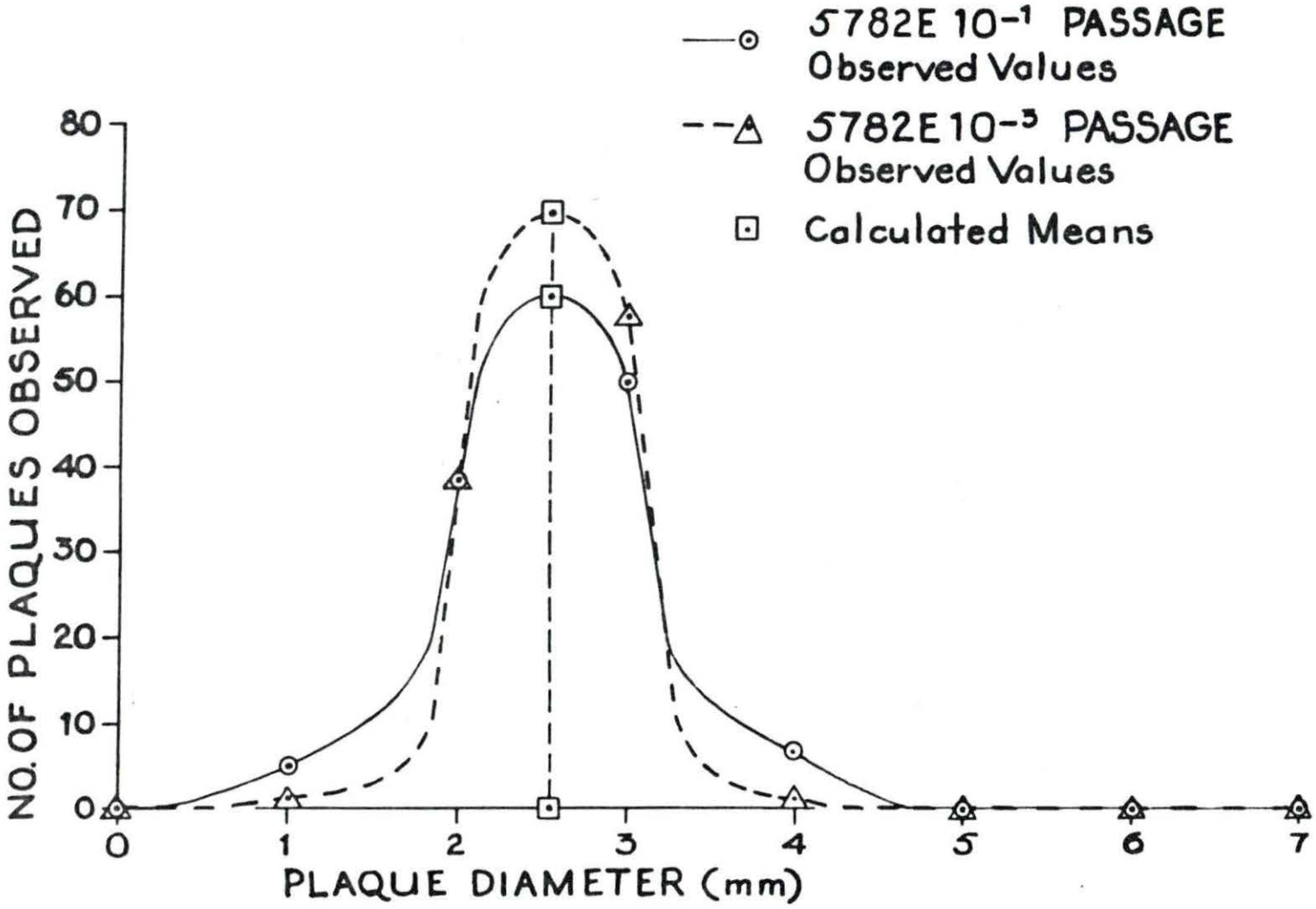


Figure 45. Distribution with respect to diameter of 100 plaques from the cell culture passages illustrated in Figure 43 and Figure 44


5782E 10⁻¹ PASSAGE
Observed Values
5782E 10⁻³ PASSAGE
Observed Values
Calculated Means
NO. OF PLAQUES OBSERVED
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
PLAQUE DIAMETER (mm)

5782E 10^{-3}. Comparison of the two curves indicates that no significant change in mean plaque size of the WEE 5782E 10^{-3} occurred as a result of one final passage at 10^{-3} dilution instead of at 10^{-1}.

Passage at low dilution tended to more effectively increase the proportion of small plaques observed when the resulting virus population was again plaqued. Figure 42 indicates that just one low dilution passage was observed to reduce the mean plaque diameter of the WEE 5783E 10^{-1} pool by a factor of more than 1 mm. in comparison to the mean plaque diameter of the WEE 5783E 10^{-5} pool obtained from the identical inoculum at higher dilution.

Although all five passages of the population of WEE virus resulting in the WEE 5783E 10^{-5} pool were at limiting dilution in chick embryos, the mean plaque diameter obtained in cell cultures from this inoculum was 3.4 mm. in comparison with a much larger mean diameter of 7.0 mm. for the WEE TCF #4A control. Possibly this was a host adaptation effect, since the 3.4 mm. mean for WEE 5783E 10^{-5} still significantly exceeded the 2.6 mm. mean diameter for those populations passed at 10^{-1} dilution.

Significance of results after chick embryo passage

A WEE virus population, which consisted of predominantly small-plaque-producing virus particles, resulted from

five serial 10^{-1} dilution passages in chick embryos of a parent WEE virus population originally known to produce mostly large plaques. These findings agreed with the hypothesis that the WEE TCF #4A parent virus pool may not have consisted entirely of large-plaque-forming particles. In retrospect, the one significantly smaller plaque occurring in the cell culture photograph reproduced in Figure 38 might lend support to this viewpoint. It was estimated that such small plaques represented less than 1.0% of those developing after WEE TCF #4A virus inoculations of cultures. This small minority appearing, however, indicated that complete purification of the large plaque type was not achieved from a genetically mixed population of WEE virus.

The failure of the large plaque type to appear in pools propagated at low dilution in chick embryos suggested that the small-plaque-type virus multiplied more rapidly than the large-type virus in the allantoic cavity of chick embryos.

Plaque diameters measured on plates from chick embryo cell culture Lot #12A could not validly be compared with the diameters of plaques produced by mouse or cell culture propagated WEE viruses measured on Lot #14A. This fact was demonstrated by the difference in size of the plaques produced by WEE TCF #4A control virus aliquots in these two lots of cell cultures.

Results of Immunization of a Laboratory Worker

No published reports concerning immunization of humans against WEE virus were found in the available literature, despite the fact that infection by WEE virus can cause a serious disease in human beings, and accidental infection of laboratory workers has been known to occur. Results of vaccination of one laboratory worker have been followed in this case by determining his antiserum titer before and after immunization, using the serum neutralization test in chick embryo cell cultures. The method of vaccination has been previously described.

Results

Figure 46 graphically illustrates the results obtained when serum neutralization tests were performed in chick embryo cell cultures using three serum samples from the same individual. One sample was collected prior to vaccination, one was taken 14 days after the last in a series of three vaccine injections, and the third was withdrawn approximately one year later. A significant reduction in titer of WEE TCF #4A virus was observed in comparison with the control inoculum when cell plates were inoculated with WEE virus dilutions incubated for 30 minutes at room temperature in the presence of a 1:10 dilution of the 14 day serum sample. The serum sample taken one year subsequent to

Figure 46. Neutralization of WEE virus by complement-free serum samples collected from a laboratory worker before and after immunization with a bivalent EEE and WEE virus vaccine

WEE challenge virus inoculum used: TCF #4A

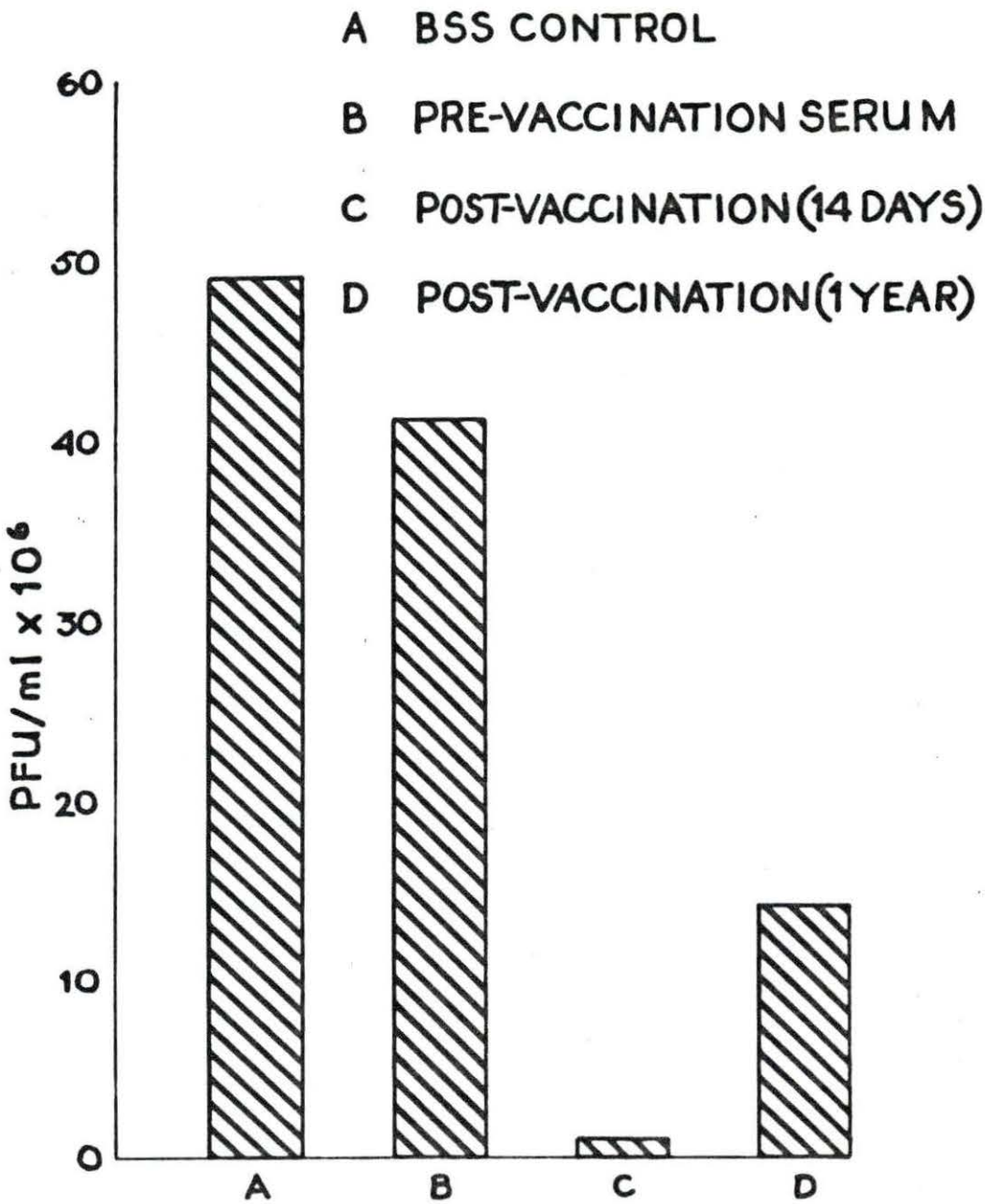
Letters designate the following:

A. Titer of challenge virus in PFU/ml. after incubation with an equal amount of BSS diluent for 30 minutes prior to inoculation

B. Titer of challenge virus in PFU/ml. after incubation for 30 minutes with an equal amount of a 1:10 dilution of normal human serum collected from the worker immediately prior to immunization

C. Titer of challenge virus in PFU/ml. after incubation for 30 minutes with an equal amount of a 1:10 dilution of serum collected from the worker 14 days following the last vaccine injection of the series

D. Titer of challenge virus in PFU/ml. after incubation for 30 minutes with an equal amount of a 1:10 dilution of serum collected from the worker approximately 1 year following immunization


A BSS CONTROL
B PRE-VACCINATION SERUM
C POST-VACCINATION (14 DAYS)
D POST-VACCINATION (1 YEAR)
PFU/ml x 10⁶
60
50
40
30
20
10
0
A
B
C
D

vaccination apparently was not as effective in reducing the infective titer of the WEE challenge virus.

Significance

Little significance can be attached to immunological data collected only from one individual. It is interesting, however, to note that the results obtained do not suggest serious exposure of the worker to WEE virus during the course of a year in which manipulation of suspensions of WEE virus was almost a daily occurrence. Should accidental infection have occurred, it no doubt would have been reflected in the results by an increase rather than a decrease in specific neutralizing antibody titer. These limited findings indicate that regular booster injections of WEE vaccine might afford more complete protection to laboratory workers handling WEE virus preparations for any extended period.

CONCLUSIONS

The original chick-embryo-propagated WEE virus used in this study appears to have existed as a genetically mixed population. The variations in plaque size observed throughout this investigation were associated with the existence of two distinctly different virus types. They have been designated as large-plaque-forming (5 to 7 mm. in diameter) and small-plaque-forming (1 to 2.6 mm. in diameter) types, based on plaque measurements 48 hours subsequent to inoculation on chick embryo cell cultures. The type responsible for the small plaques represented the majority of the population on initial cell culture passage.

When protamine sulfate was added to the usual overlay medium, the small-plaque-producing virus caused formation of plaques with diameters equal in size to those of the large type. Protamine sulfate chemically inactivates a polysaccharide-sulfate inhibitor of plaque development reported to be present in the Noble agar of the overlay medium. The small diameter of the majority of plaques could best be explained by hypothesizing the susceptibility of this type WEE virus to the retarding effects of the inhibitor. The type which produced large plaques could possibly be a mutant strain of WEE virus whose plaque development was only slightly affected by the inhibitor's presence.

The WEE TCF #4A virus pool referred to in this report was a population derived by three successive clonings in which isolated large plaques were selected for propagation. Although aliquots from this TCF #4A pool repeatedly produced predominantly large and uniform plaques, occasionally a very small plaque would appear. Spontaneous mutation from large to small type might explain the appearance of small plaques on titration of the supposedly purified large-plaque-type virus pool. A more likely assumption is that complete purification was not obtained by this attempt.

After five serial passages of aliquots from the WEE TCF #4A virus pool in mice, in chick embryos, or in cell cultures with fluid maintenance, the derived populations were again titrated on cell cultures using the plaque technique. These passages of the population consisting of predominantly large-plaque-forming particles were made at nearly terminal dilution in each host, and the derived populations were observed to contain only a slight minority of the small-plaque type when titrated.

In contrast, after concurrent serial passage of the same WEE TCF #4A aliquots in the same hosts, this time at a much lower (10^{-1}) dilution, all of the derived populations were observed to have a majority of the small-plaque type.

These experimental results confirmed preliminary observations indicating that the relative numbers of large and

small plaque types in a given WEE virus population were related to its propagation history. It is evident also that the type responsible for small plaques is capable of more rapid multiplication in these hosts than is its large-plaque counterpart. Since this is the reverse of the apparent replication rates under agar overlays, it is further indirect evidence indicating the agar component as a selective inhibitor of plaque development.

Interferon could not be detected in any of the WEE virus preparations used as inocula during this study. The same titration technique was successful in demonstrating the presence of interferon in SIV preparations. Although interferon can be shown to effectively retard development of WEE virus plaques in chick embryo cell cultures, its apparent absence indicates that it had no role in producing the plaque size variations observed in this investigation.

Negative results were also obtained when an attempt was made to correlate the presence of high levels of heat-inactivated WEE virus particles in an inoculum with plaque size reduction. It was determined that autoinhibition of cell infection by heat-inactivated particles was not a major factor affecting plaque size, since inocula consisting of high proportions of heat-inactivated virus were observed to produce plaques with diameters equivalent to those resulting from unheated inocula.

SUMMARY

When a pool of chick embryo propagated WEE virus was initially plaqued in primary chick embryo fibroblast cell cultures, a significant number of the plaques achieved diameters more than twice the size of the smaller majority. This phenomenon was repeatedly observed despite inauguration of rigidly controlled experimental methods. The purpose of the present investigation was to determine which of several possible factors, suggested by a review of the literature, were most likely responsible for the observed plaque size variations.

The mean diameter of the smaller plaques could be increased to approximate that of the larger plaque controls when both were allowed to develop in cell cultures changed only by the addition of protamine sulfate to the agar overlay medium. In light of this result, it was hypothesized that the development of small plaques was inhibited by a polysaccharide sulfate compound previously reported to be found in Noble agar (50, 51). The addition of protamine sulfate to overlays reportedly increases the mean plaque diameter of susceptible viruses by chemically inactivating the inhibitor (51). Large plaque producers are apparently much less affected by the inhibitor.

Depending on the concentration of interferon present

in chick embryo cell cultures, development of WEE virus plaques is totally arrested or only retarded (54). The presence of an interferon-like substance carried with WEE virus inocula conceivably could have been responsible for the small plaques observed in the current study. However, such was not the case, since assay of several types of WEE virus preparations for interferon did not indicate its presence. The negative results obtained are made more significant since interferon could be detected when identical assay procedures were applied to SIV preparations.

The effects of heat inactivation of WEE virus suspensions and resulting decrease in mean plaque size on chick embryo cell cultures have been reported by other workers (41). In the present experiment, no decrease in mean plaque diameters accompanied a significant fall in titer upon extended heat inactivation of aliquots from a pool of large-plaque-producing WEE virus. Therefore it was concluded that autoinhibition by heat-inactivated WEE virus particles was not related to plaque variations being investigated.

A pool of WEE virus which repeatedly produced predominantly large plaques was prepared by isolation and propagation of the progeny of one large-plaque-forming WEE virus particle. Five serial passages of aliquots from this pool at 10^{-1} dilution in mice, chick embryos or cell cultures significantly reduced the mean diameters of plaques produced

by the derived WEE virus populations. Five serial passages in these hosts at dilutions of 10^{-5} or above caused lesser alteration of plaque-producing properties.

As a final analysis of experimental results obtained, several conclusions are suggested. It can be hypothesized that the original WEE virus pool from chick embryos existed as a genetically mixed population. The majority of WEE virus particles in the population produced small plaques in the presence of an inhibitor in the overlay medium. A significant number of virus particles, perhaps mutants, were uninhibited by polysaccharide sulfate, and developed large plaques in chick embryo cell cultures. When a single large plaque was harvested, a mixed population was again obtained because of incomplete purification of the large-plaque-producing type. The small-plaque-producing type, existing as a minority of the new population, again reached significant proportion during serial passage, especially at low dilution, in other hosts where the inhibitor was not present.

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APPENDIX A

Media

1. Ion Exchange Water

Distilled water was further purified by passage through an ion exchange column. This yielded water containing inorganic impurities no greater than that amount equivalent to 0.1 ppm. NaCl by conductivity meter measurement.

2. Bovine Serum

At the time of their slaughter, whole blood from healthy young beef animals was collected in chemically-clean, stainless steel buckets. The blood was allowed to clot. The clots were incised and the serum collected as soon as it could be separated. Residual cellular elements were removed by centrifugation for 20 minutes in an angle-head centrifuge. After preliminary clarification, the serum was filter sterilized (Selas 03) and stored at -40° C.

3. Chemicals

Only A.C.S. reagent-grade chemicals were used.

4. GKN (10X)

NaCl	80.0 gm.
KCl	4.0 gm.
$NaHCO_3$	3.5 gm.
Glucose (anhydrous)	10.0 gm.
Ion exchange water	1,000.0 ml.

Bottled in 100 ml. amounts with 0.1 ml. chloroform per 100 ml. Stored at 5° C.

5. Phenol Red Stock Solution (100X)

Phenol red	0.4 gm.
N/20 NaOH	22.0 ml.

Final volume brought to 200 ml. with ion exchange water and sufficient N/20 NaOH to adjust to pH 7.0. Stored at 5° C.

6. Antibiotic Solution (100X)

Streptomycin sulfate	1.0 gm.
Penicillin G (Na or K salt)	2.0 million units
Earle's BSS (1X)	100.0 ml.

Filter sterilized (Selas 02), dispensed in 20 ml. amounts and frozen.

7. 2.8% Sodium Bicarbonate Solution

$NaHCO_3$	28.0 gm.
Ion exchange water	1,000.0 ml.

Tubed in 20 ml. amounts. Sterilized by autoclaving and stored at 5° C.

8. Earle's Solution (10X)

NaCl	68.0 gm.
KCl	4.0 gm.
$CaCl_2 \cdot 2H_2O$	2.0 gm.
$MgSO_4 \cdot 7H_2O$	2.0 gm.
$NaH_2PO_4 \cdot H_2O$	1.25 gm.
Dextrose (anhydrous)	10.0 gm.
Ion exchange water	q.s. 1,000.0 ml.

Dispensed in 100 ml. amounts with 0.1 ml. chloroform per 100 ml. and stored at 5° C.

9. 5% Lactalbumin Hydrolysate (10X)

Lactalbumin enzymatic hydrolysate[1]	5.0 gm.
Earle's BSS (1X)	100.0 ml.

Each 100 ml. bottle made separately. Lactalbumin hydrolysate was suspended by shaking. Autoclaved at 118° C. for 10 min. Frozen.

10. GKN (1X)

10X GKN	100.0 ml.
Ion exchange water	900.0 ml.

Filter sterilized (Selas 02). Bottled in 100 ml. amounts and stored at 5° C.

11. Trypsin Solution

Ion exchange water	885.0 ml.
Bacto-trypsin (1:250)[2]	2.5 gm.
10X GKN	100.0 ml.
Phenol red solution	5.0 ml.
Antibiotic solution	10.0 ml.

pH adjusted to 8.0 with 2.8% $NaHCO_3$ solution. Filter sterilized (Selas 02) and frozen in 400 ml. amounts.

12. Growth Medium

#199 medium (10X) without $NaHCO_3$[3]	30.0 ml.
Ion exchange water	300.0 ml.
Bovine serum	30.0 ml.

Sterile ingredients were mixed aseptically and buffered to pH 7.5 with sterile 2.8% $NaHCO_3$, and used immediately.

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[1]Nutritional Biochemicals, Inc., Cleveland, Ohio.

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

[3]Microbiological Associates, Inc., Bethesda, Maryland.

13. Fluid Maintenance Medium

#199 medium (10X) without $NaHCO_3$[1]		30.0 ml.
Ion exchange water		300.0 ml.
Bovine serum		15.0 ml.

Sterile ingredients were mixed aseptically and buffered to pH 7.5 with sterile 2.8% $NaHCO_3$, and used immediately.

14. Agar Overlay Maintenance Medium

Part 1.

Lactalbumin hydrolysate solution (10X)		200.0 ml.
Earle's BSS (10X)		160.0 ml.
Bovine serum		120.0 ml.
Antibiotic solution (100X)		20.0 ml.
$NaHCO_3$ solution (2.8%) approx.		40.0 ml.
Ion exchange water	q.s.	1,000.0 ml.

Adjusted to final pH 7.4 with $NaHCO_3$ solution. Filter sterilized (Selas 02), bottled in 200 ml. amounts and frozen. Warmed to 41° C. prior to use.

Part 2.

Noble agar[2]		3.0 gm.
Ion exchange water	q.s.	200.0 ml.

Autoclaved at 115-120° C. for 15 minutes. Cooled to 41° C. in a water bath before use.

Final Mixture

Parts 1 and 2 were mixed aseptically in equal volumes. Dispensed 5 ml. per plate, carefully controlling

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[1]Microbiological Associates, Inc., Bethesda, Maryland.

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

temperature of mixture at 41° C.

15. Second Agar Overlay (Neutral Red Stain)

Part 1.

Neutral red	1.0 gm.
Ion exchange water	1,000.0 ml.

Neutral red was added to a small amount of water and mixed, then the remaining water was added and the solution was allowed to stand overnight with occasional stirring. Bottled in 66 ml. amounts and autoclaved at 115-120° C. for 10 minutes. Stored at room temperature. Warmed to 48° C. in a water bath just before use.

Part 2.

Noble agar[1]		2.0 gm.
Ion exchange water	q.s.	100.0 ml.

Autoclaved at 115-120° C. for 15 minutes. Cooled in a water bath to 48° C. before use.

Final Mixture

66.0 ml. of Part 1 and 100 ml. of Part 2 were aseptically mixed. Dispensed 1 ml. per plate, carefully controlling temperature of mixture at 48° C.

16. Special Protamine Overlay Medium

Part 1.

Identical to Part 1 of regular overlay maintenance medium. Prepared and used as described previously.

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

Part 2.

Noble agar[1]		3.0 gm.
Ion exchange water	q.s.	190.0 ml.

Prepared and used as described for regular agar overlay medium.

Part 3.

Protamine sulfate (Salmine)[2]		1.6 gm.
Ion exchange water	q.s.	100.0 ml.

Filter sterilized (Selas 02) and dispensed in 10 ml. amounts. Stored at 5° C.

Final Mixture

Warmed 200 ml. of Part 1, 190 ml. of Part 2 and 10 ml. of Part 3 to 41° C. in a water bath. Aseptically mixed in these proportions and dispensed 5 ml. of the resulting mixture to each culture plate. Final protamine concentration was 0.4 mg. per ml. of medium.

17. Diluent

Earle's BSS (10X)		89.0 ml.
Lactalbumin hydrolysate solution (10X)		100.0 ml.
Antibiotic solution (100X)		10.0 ml.
Phenol red stock solution (100X)		10.0 ml.
$NaHCO_3$ solution (2.8%)	approx.	20.0 ml.
Ion exchange water	q.s.	1,000.0 ml.

Adjusted to pH 7.4 with 2.8% $NaHCO_3$ solution. Filter sterilized (Selas 02) and tubed in 9.0 ml. blanks for

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

[2] Nutritional Biochemicals, Inc., Cleveland, Ohio.

use as diluent or in 5 ml. blanks for use in plaque harvest. Incubated 24 hours at room temperature or overnight at 37° C. to ascertain sterility. Stored at 5° C. Warmed to room temperature prior to use in serial dilution schemes.

Source of Special Equipment

1. Ion exchange column

Purified water for use in cell culture media and for final rinsing of glassware was prepared by allowing distilled water to pass through a De-em-a-jet[1] ion exchange device.

2. Conductivity meter

Purity of the ion exchange water was determined before use by measuring its content of residual inorganic ions with a Barnstead purity meter.[2]

3. Cell culture containers

Sterile disposable plastic tissue culture dishes,[3] 60 x 15 mm. in diameter, were used for chick embryo cell culture vessels.

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[1]Crystal Research Laboratories, Hartford 4, Connecticut.

[2]Barnstead Still and Sterilizer Co., Boston, Massachusetts.

[3]Falcon Plastics, Los Angeles 45, California.

Glassware Preparation

1. Soiled glassware, immediately after use, was rinsed in tap water, and then completely immersed in distilled water until it could be washed.
2. All glassware items were thoroughly brushed in distilled water containing Microsol[1] detergent. They were then rinsed free of detergent and loosened debris by total immersion in two successive lots of distilled water.
3. Completed rinsing was accomplished by passage of glassware in sequence through three separate lots of ion exchange water.
4. Rinsed glassware was simmered for 15-20 minutes in a fourth ion exchange water bath, and inverted to dry while still hot to insure rapid evaporation of residual water.
5. Dry glassware was wrapped and sterilized in a hot air oven whenever possible, but in some cases autoclaving was a necessary alternative.
6. Sterile glassware was stored in a dust free cabinet, until it was used, to reduce chances of recontamination.
7. Stainless steel and rubber items were prepared by a similar technique.

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[1]Microbiological Associates, Inc., Bethesda, Maryland.

8. Pipettes were rinsed using tap water in a mechanical washer followed by three rinses manually in ion exchange water.
9. Contaminated instruments and glassware were disinfected in a quaternary ammonium solution prior to washing.

APPENDIX B

Table 7. Relation of experimental procedure to plaque size

Virus inoculum	Virus dilution[a]	Number of plaques observed per plate[b] 1	2	3	Plaque size (mm.)	Plaques[c] (No.)
WEE 5766E 10^{-5}	4	too many to count			1	0
	5	68	54	69	2	0
	6	4	8	4	3	3
	7	1	0	2	4	96
	8	0	0	0	5	1
WEE 5766E 10^{-2}	5	too many to count			1	0
	6	32	24	24	2	83
	7	4	3	2	3	17
	8	0	0	0	4	0
WEE #2M 10^{-5}	6	56	70	45	1	6
	7	14	6	8	2	94
	8	2	0	1	3	0
	9	0	0	0	4	0

[a]Negative log to the base 10.

[b]Three replicate platings per dilution.

[c]First 100 observed plaques classified as to diameter.

Table 8. Effect of special protamine overlay medium

Virus inoculum	Virus dilution[a]	Number of plaques observed per plate[b] 1	2	3	4	5	Plaque size (mm.)	Plaques[c] (No.)
WEE	3	confluent lysis					2	0
TCF #4A	4	confluent lysis					3	0
Regular	5	confluent lysis					4	16
overlay	6	31	28	23	39	31	5	51
medium	7	1	5	2	6	1	6	33
	8	1	0	1	1	0	7	0
WEE	3	confluent lysis					3	0
TCF #4A	4	confluent lysis					4	1
Protamine	5	confluent lysis					5	11
overlay	6	59	47	48	60	51	6	74
medium	7	5	4	3	6	5	7	14
WEE	1	71	68	74	79	65	1	5
M_3TCP #6A	2	4	9	7	9	5	2	0
	3	0	1	0	1	0	3	6
Regular	4	0	0	0	0	0	4	81
overlay	5	0	0	0	0	0	5	8
medium								

[a]Negative log to the base 10.

[b]Five replicate platings per dilution.

[c]First 100 observed plaques classified as to diameter.

Table 8. (Continued)

Virus inoculum	Virus dilution[a]	Number of plaques observed per plate[b]					Plaque size (mm.)	Plaques[c] (No.)
		1	2	3	4	5		
WEE	1	61	70	76	77	68	3	0
M_3TCP #6A	2	4	5	9	6	6	4	10
Protamine	3	0	1	0	0	1	5	46
overlay	4	0	0	0	0	0	6	35
medium	5	0	0	0	0	0	7	9
WEE	1	16	8	19	11	14	1	51
5769C TCP #7A	2	1	2	2	0	1	2	49
Regular	3	0	0	0	0	0	3	0
overlay	4	0	0	0	0	0	4	0
medium	5	0	0	0	0	0	5	0
WEE	1	17	18	12	21	19	3	0
5769E TCP #7A	2	3	2	1	1	2	4	2
Protamine	3	0	0	0	0	0	5	14
overlay	4	0	0	0	0	0	6	70
medium	5	0	0	0	0	0	7	14

Table 9. Titers of infective SIV in fluids prepared by different methods for assay of interferon-like activity

Virus inoculum	Treatment	Virus dilution[a]	Embryo death time[b] (Hours after inoculation) 1	2	3	4	5	Titer[c] (MLD_{50}/ml.)
SIV #8S 10^{-1}	A, Figure 19	1	48	84	84	HA+[d]	HA+	
		2	36	60	60	72	84	
		3	36	36	48	60	60	
		4	36	36	36	36	36	
		5	36	36	36	36	36	
		6	48	60	60[e]	60	60	
		7	60	60	-	-	-	$10^{7.83}$
SIV #8S 10^{-1}	B, Figure 19	1	60	60	72	72	84	
		2	36	36	48	48	60	
		3	36	36	36	36	36	
		4	36	36	48	48	60	
		5	36	48	48	60	84	
		6	48	60	60	-	-	$10^{7.17}$

[a]Negative log to the base 10.

[b]Results from five embryos per dilution candled at 12 hour intervals following inoculation.

[c]50% end points calculated according to the method of Reed and Muench (46).

[d]SIV hemagglutinins indicated by rapid HA test.

[e]Embryo not dead at the end of 84 hours.

Table 9. (Continued)

Virus inoculum	Treatment	Virus dilution[a]	Embryo death time[b] (Hours after inoculation) 1	2	3	4	5	Titer[c] (MLD_{50}/ml.)
SIV #8S 10^{-1}	C, Figure 19	1	60	72	84	HA+	HA+	
		2	36	48	60	60	72	
		3	36	36	36	36	36	
		4	36	48	60	60	60	
		5	48	60	60	60	60	
		6	60	60	60	60	72	
		7	72	-	-	-	-	$10^{7.67}$
SIV #8S 10^{-1}	A, Figure 19	1	84	84	HA+[d]	HA+	HA+	
		2	36	36	48	60	72	
		3	36	36	48	60	60	
		4	24	24	36	36	48	
		5	36	36	36	60	72	
		6	36	48	60[e]	60	84	
		7	60	60	-	-	-	$10^{7.83}$
SIV #8S 10^{-1}	D, Figure 19	1	48	60	60	60	72	
		2	36	36	48	60	72	
		3	36	36	36	48	72	
		4	36	36	36	36	36	
		5	36	48	60	84	-	$10^{6.375}$
SIV #8S 10^{-1}	E, Figure 19	1	36	48	60	60	60	
		2	36	48	60	60	60	
		3	48	60	60	60	72	
		4	60	60	60	60	60	
		5	60	-	-	-	-	$10^{5.625}$

Table 10. Relationship of autoinhibition by heat inactivated WEE virus particles to WEE virus plaque diameter

Virus inoculum	Virus dilution[a]	Number of plaques observed per plate[b] 1	2	3	4	5	Plaque size (mm.)	Plaques[c] (No.)
WEE TCF #4A 37° C. 0 hours[d]	3	confluent lysis					2	0
	4	confluent lysis					3	0
	5	confluent lysis					4	14
	6	31	28	23	39	31	5	51
	7	1	5	2	6	1	6	35
	8	1	0	1	1	0	7	0
WEE TCF #4A 37° C. 12 hours	2	confluent lysis					2	0
	3	confluent lysis					3	4
	4	confluent lysis					4	13
	5	nearly confluent lysis					5	50
	6	19	18	18	15	9	6	34
	7	1	3	2	2	0	7	0

[a] Negative log to the base 10.

[b] Five replicate platings per dilution.

[c] First 100 observed plaques classified as to diameter.

[d] Incubated in 37° C. water bath for time specified.

Table 10. (Continued)

Virus inoculum	Virus dilution[a]	Number of plaques observed per plate[b] 1	2	3	4	5	Plaque size (mm.)	Plaques[c] (No.)
WEE TCF #4A 37° C. 24 hours	1	confluent lysis					3	3
	2	confluent lysis					4	20
	3	confluent lysis					5	54
	4	confluent lysis					6	23
	5	too many to count					7	0
	6	9	7	5	10	9	8	0
WEE TCF #4A 37° C. 36 hours[d]	1	confluent lysis					3	4
	2	confluent lysis					4	9
	3	confluent lysis					5	49
	4	confluent lysis					6	45
	5	32	35	29	18	29	7	2
	6	2	1	3	0	2	8	0
WEE TCF #4A 37° C. 48 hours	1	confluent lysis					3	1
	2	confluent lysis					4	15
	3	confluent lysis					5	26
	4	51	42	46	30	45	6	57
	5	7	6	9	14	6	7	1
	6	0	0	1	0	0	8	0

Table 11. Genetic changes related to WEE virus plaque size

Virus inoculum	Virus dilution[a]	Number of plaques observed per plate[b] 1	2	3	4	5	Plaque size (mm.)	Plaques[c] (No.)
WEE TCF #4A 10^{-7}	3	confluent lysis					2	0
	4	confluent lysis					3	0
	5	confluent lysis					4	14
	6	31	28	23	39	31	5	51
	7	1	5	2	6	1	6	35
	8	1	0	1	1	0	7	0
WEE TCF #13A 10^{-7}	5	confluent lysis					1	3
	6	too many to count					2	0
	7	14	13	13	8	10	3	0
	8	1	0	0	0	4	4	3
	9	0	0	0	0	0	5	88
	10	0	0	0	0	0	6	6

[a]Negative log to the base 10.

[b]Five replicate platings per dilution.

[c]First 100 observed plaques classified as to diameter.

Table 11. (Continued)

Virus inoculum	Virus dilution[a]	Number of plaques observed per plate[b] 1	2	3	4	5	Plaque size (mm.)	Plaques[c] (No.)
WEE	4	confluent lysis					1	51
TCF #13A	5	confluent lysis					2	14
10^{-1}	6	too many to count					3	0
	7	17	19	19	25	25	4	9
	8	3	4	1	0	3	5	26
	9	0	0	0	0	0	6	0
WEE	3	confluent lysis					1	3
#12M	4	confluent lysis					2	0
10^{-6}	5	confluent lysis					3	34
	6	too many to count					4	61
	7	31	39	41	35	37	5	2
	8	1	7	1	2	8	6	0
WEE	3	confluent lysis					1	34
#11M	4	confluent lysis					2	0
10^{-1}	5	too many to count					3	65
	6	49	52	50	47	55	4	1
	7	4	1	3	6	5	5	0
WEE	4	confluent lysis					5	2
TCF #4A	5	confluent lysis					6	27
10^{-7}	6	too many to count					7	39
	7	9	22	17	14	17	8	29
	8	1	1	3	2	2	9	3
	9	0	0	0	0	0	10	0

Table 11. (Continued)

Virus inoculum	Virus dilution[a]	Number of plaques observed per plate[b] 1	2	3	4	5	Plaque size (mm.)	Plaques[c] (No.)
WEE 5783E 10^{-1}	4	confluent lysis					1	2
	5	confluent lysis					2	67
	6	too many to count					3	31
	7	11	7	12	28	12	4	0
	8	7	2	1	1	2	5	0
WEE 5783E 10^{-5}	4	confluent lysis					1	0
	5	confluent lysis					2	3
	6	too many to count					3	60
	7	9	8	10	4	10	4	35
	8	4	0	0	0	1	5	2
WEE 5782E 10^{-1}	4	too many to count					1	7
	5	40	48	49	56	52	2	39
	6	8	4	3	1	3	3	49
	7	0	0	1	0	0	4	6
WEE 5782E 10^{-3}	4	confluent lysis					1	3
	5	nearly confluent lysis					2	39
	6	too many to count					3	58
	7	8	6	7	6	4	4	0
	8	0	1	1	0	0	5	0

Table 12. Results of immunization of a laboratory worker

Virus inoculum	Serum sample	Virus dilution[a]	Number of plaques observed per plate[b] 1	2	3	4	5	Titer (PFU/ml.)
WEE TCF #4A	BSS diluent	3	confluent lysis					
		4	confluent lysis					
		5	too many to count					
		6	23	24	33	29	27	
		7	2	1	3	2	3	
		8	0	0	0	0	0	
								4.9×10^7
WEE TCF #4A	pre-vaccination	2	confluent lysis					
		3	confluent lysis					
		4	confluent lysis					
		5	16	13	21	24	30	
		6	1	2	1	3	3	
								4.1×10^7
WEE TCF #4A	14 day post-vaccination	2	confluent lysis					
		3	confluent lysis					
		4	54	63	55	61	58	
		5	4	7	4	6	8	
		6	1	0	1	0	1	
								1.2×10^6
WEE TCF #4A	1 year post-vaccination	2	confluent lysis					
		3	confluent lysis					
		4	confluent lysis					
		5	67	69	73	78	70	
		6	7	9	6	6	7	
		7	2	2	1	0	1	
								1.4×10^7

[a] Negative log to the base 10.

[b] Five replicate platings per dilution.