THE DEMONSTRATION OF A CELL-ATTACHMENT-AND-GROWTH FACTOR AND COMPARATIVE STUDIES OF INFECTIOUS BRONCHITIS VIRUS BEFORE AND AFTER SEPARATION FROM THE FACTOR

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ABSTRACT

Infectious bronchitis virus, present in the allantoic fluid of infected chicken embryos, was adsorbed to and eluted from anionic resins. The concentration of the virus was increased ten fold. Electron micrographs indicated the viral particles were relatively uniform and spherical in shape.

A factor was present in the infected allantoic fluid which markedly stimulated the attachment to a glass surface and the growth of whole embryo cell cultures otherwise unable to establish themselves. The factor was not serologically or cytopathically identical to the virus. The factor was present only in infected allantoic fluid and the production appeared to be independent of the amount of the factor or virus employed in the inoculum.

The virus was separated from the factor by anionic resins and limiting dilutions. In the absence of the factor, the virus was capable of producing cytopathic effects on whole embryo cell culture monolayers after fewer serial passages than if the factor were present. In addition, more embryos infected with the virus free of the factor were dwarfed. It was not determined conclusively if this were due to interference by the factor or resulted from a selective action by the resin for a form of the virus which dwarfs the embryos.

Thermal inactivation rates indicated that the embryo

lethal form of the virus was inactivated more rapidly than the dwarfing form. These forms were not distinguishable as O and D phases. The heat inactivated forms did not detectably interfere with the expression of the viable virus.

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INTRODUCTION

The initial thesis of this study was an exploratory search for susceptible cells which would evidence the infection and multiplication of avian infectious bronchitis virus by detectable cytopathic effects. During the investigation, the observation was made that allantoic fluid, harvested from embryonating chicken eggs infected with the virus, contained a substance or substances which produced a detectable effect on cell cultures. The effect differed from that elicited by the virus and was not neutralized by specific antiserum. Further studies of the substance were made. In obtaining the virus free of the substance, a facile method of viral purification and concentration was found. Comparative studies were made of the effects by unpurified and purified virus on chicken embryos and cell cultures.

LITERATURE REVIEW

The recognition of a virus and the subsequent studies most frequently follow a general pattern. The initial and continued observations of diseased hosts activate the investigation of methods for the isolation and identification of the causative agent. As these methods are developed and improved, studies of the nature of the agent and the host-agent relation become more detailed. The recognition and study of Tarpia pulli[81], the agent of infectious bronchitis (IBV), follows that pattern. The known properties of IBV have been reviewed by Cunningham[12,14].

The chicken is the only known natural host of IBV[80]. The disease was first described by Schalk and Hawn[66] in 1931. It is now recognized as a highly contagious respiratory disease of widespread distribution and high incidence. There is a low mortality of infected mature birds but the decreased egg production by the infected birds[3] and high mortality of chicks[4,66] are of considerable economic importance.

Histological studies indicate that intratracheal inoculation of chickens with IBV induces a three step change in the tracheal mucosa[46]. The three phases are: (1) acute - epithelial hyperplasia and marked edema, (2) reparative - epithelial hyperplasia and marked cellularity of the propria, and (3) immune - restoration of the epithelium and either follicular or mild focal, diffuse infiltration of

the propria. No inclusion bodies are observed[37].

The virus can be isolated from the lung and tracheal tissues of naturally or experimentally infected birds during the respiratory stages of the disease which may vary from two to 28 days after infection[19,28,29,38,39]. Ovarian transmission of the virus by infected birds may occur[29]. Specific antibodies have been detected in eggs from immune birds[12,47].

The virus may be propagated in the embryonating chicken egg. Only negligible or undetectable changes are observed on primary inoculations. Continued passage produces readily observable and characteristic effects on the embryo and tissues. Beaudette and Hudson[2] cultivated the virus on the chorio-allantoic membrane (CAM). Delaplane and Stuart[21] emphasized that continued passage in the CAM produced death of embryos more rapidly, an increased number of dead embryos, and dwarfing of the surviving embryos. Delaplane determined that adaptation was more rapid when embryos were inoculated by the allantoic sac route[20]. Cunningham and Jones[16] demonstrated that higher mortality rates were obtained in earlier passages when the inoculations were via the amnionic cavity route. Yolk sac inoculations were successful if no maternal antibodies specific for the virus were present.

The gross lesions which may be detected in the embryonating chicken egg following inoculations of IBV have been described by Loomis, et al.[52]. Living embryos may be dwarfed, curled with the feet tightly compressed over the head by the thickened and closely adhering amnionic membrane,

and are sluggish in their movements. Other findings may be liver congestion, deposition of urates in the kidney, dermal petechiation, thickening of the CAM, excessive urates in the allantoic fluid, and distension of the cloaca with fat-like droplets[36,40].

Inclusion bodies have not been detected by electron microscopy. Intracytoplasmic, spherical elementary bodies may be observed in the infected CAM[23]. They average 200 mu in size and vary considerably in shape, arrangement and number. Virus in allantoic fluid averages from 60 to 100 mu and is spherical with filamentous projections[62]. When amorphous material in the allantoic fluid is removed by trypsin, the virus is approximately 120 mu. Some of the particles are cylindrical[7].

Strains of IBV which have become egg-adapted by repeated passages exhibit a high and rapid lethality for the chicken embryo. The number of passages required for adaptation varies with the strain[28]. The Beaudette strain is the classical example of completely egg-adapted IBV. It has undergone innumerable egg passages and is lethal for chicken embryos within 24 to 36 hours after inoculation. It is relatively thermolabile, is avirulent for chickens, and it is not capable of immunizing or eliciting neutralizing antibodies in the natural host[2,20]. This strain is regarded as a genetically stable, completely egg-adapted strain and is employed frequently in comparative studies with strains recently isolated from naturally infected birds.

A recent study of the Beaudette strain led to the

hypothesis that a variant exists[72]. When passaged intracerebrally in suckling mice, the virus was lethal for mice and for chicken embryos if the embryos were incubated at 34 C. If inoculated eggs were incubated at 38 C, the optimum temperature for embryo-passaged virus, no effects were elicited by the mouse-adapted virus. Previous reports of the failure of the virus to propagate in mice were based on egg titrations incubated at 38 C[63,71]. This illustrates that conflicting results may be reflections of limitations imposed by the methods employed.

Recently isolated strains of influenza virus convert from a filamentous form to a spherical form as they are propagated in chicken embryos[49]. The filamentous form is maintained by the limiting dilution technique which demonstrates that adaptation is dependent upon conditions favoring the selection of a minority component, whether it is present initially or results from mutation, and is not dependent upon the number of egg passages.

Genetic studies of influenza virus indicate that the morphology, growth rate, and ultimate yield of virus are exchangeable genetic traits which distinguish between egg-adapted and unadapted forms of the virus[49]. The unadapted virus is filamentous, and has a slower rate of growth and limited growth capacity. The adapted virus is spherical and multiplies more rapidly with a higher yield than the unadapted virus. Inoculation of chicken embryos with mixtures of the two forms results in recovery of both the two original and two recombinant forms. Similar studies of IBV

have not been reported.

The relation of virulence of a virus to temperature requirement is discussed by Lwoff[53]. There is no disagreement with the conclusion of Dubes and Wenners[25] based on temperature-virulence relation studies of poliomyelitis that the relative avirulence of a strain adapted to propagate at 23 C appears to be due to its reduced capacity to propagate at 37 C or above. Growth rate figures[53,54] of these thermal dependent strains led to the following conclusions: (1) the development of each viral strain exhibits a characteristic curve of temperature sensitivity; (2) each viral strain exhibits a specific rate of multiplication; and (3) mutations can shift the curve in one direction or in another.

Infectious bronchitis virus may contain at least two or more types of thermal sensitive viral particles[73,74]. Inactivation at 56 C of viruses in low egg passage occurs over a range of 30 to 150 minutes at 56 C. The reaction is bimodal and indicates that two phases of the virus exist: (1) original (O) - non-egg-adapted phase which is relatively thermostable, and (2) derivative (D) - egg-adapted which is thermolabile. It is possible tc retain selectively the O phase virus by heat inactivation of the D phase. By means of the limiting dilution technique, the O phase can be maintained through at least 13 serial passages in the allantoic cavity of the chicken embryo. The relation of the relative concentrations of O and D to dwarfing and lethality for chicken embryos was not determined. Studied were not made to determine if lower or higher

temperature-requiring variants were present.

At least two distinct antigenic[41,45] and immunogenic types exist, Connecticut and Massachusetts. Both produce typical histopathological conditions in chickens but differ serologically when measured by neutralization tests.

Growth studies of IBV indicate that propagation is rapid in all tissues of the embryonating chicken egg[15,32,36]. Low-passage virus exhibits a log phase of growth approximately six hours after inoculation and the maximum concentration is attained between 24 to 30 hours. Egg-adapted strains enter the log phase two hours earlier and reach the maximum concentration at 12 hours. Dilution of the inoculum delays the time intervals indicated but the final concentrations are equal.

The allantoic sac route of inoculation of 9 to 12 day embryonating chicken eggs is frequently employed for propagation of IBV. Procedures for the inoculation and harvest are relatively rapid and facile, and $10^{7.0}$ to $10^{9.0}$ infectious doses per ml are obtained without difficulty. It must be recognized however, that the allantois receives the urates from the kidneys, and the allantoic fluid is heterogenous.

By means of specific antisera produced in rabbits, normal allantoic fluid is known to contain no less than five precipitating antigens of a protein nature[7]. Seven precipitating antigens are present in IBV infected allantoic fluid. The two additional antigens cannot be identified by serological means as infective or incomplete viral particles.

The antigens are heat labile and are precipitated with trichloroacetic acid. Trypsin reduces the number of zones of reactions but the infectious particles are unaffected by trypsin. Ficin reduces the density of the zones but not the number of zones. Extraction with ether or oxidation by potassium periodate has no effect on the number of antigens.

Some attempts have been made to purify and concentrate IBV. Other viruses have been purified with considerably more success than IBV. Varying degrees of purification and concentration have been obtained by ultracentrifugation, chemical precipitation, filtration, adsorption to red blood cells or chemically inert substances and subsequent elution, or a combination of two or more methods.

Allantoic fluid containing IBV is commonly clarified by low speed centrifugation to sediment the gross debris. The virus is only partially sedimented by ultracentrifugation at 105,000 X G for two hours.

Aluminin phosphate gel or protamine sulfate does not remove the virus from suspension. Trypsin does not reduce the number of infectious particles but does remove the amorphous material as determined by electron microscopy [7].

A method for obtaining a purified suspension of IBV from large volumes of allantoic fluid was outlined by Buthala [7]. Following low speed centrifugation, the supernatant fluid was treated with ammonium sulfate to 70 percent saturation at 4 C for two hours. The supernatant was discarded and the slurry centrifuged at 4000 rpm for 30 minutes

at 4 C. The precipitate was dissolved in distilled water and dialyzed against saline for 24 hours. The remaining suspension was treated with two mg trypsin per ml and after two hours, was centrifuged at 40,000 rpm for two hours.

The sediment of the last centrifugation was resuspended in 1/100 the original volume. The original titer was approximately $10^{9.0}$ infectious particles per ml; the final product contained $10^{7.0}$ per ml.

Untreated IBV does not agglutinate erythrocytes. Trypsin-treated virus will agglutinate chicken erythrocytes but loses its infectivity for the embryonating chicken[11]. The agglutination cannot be inhibited with specific antiserum.

The use of synthetic resins has augmented purification and concentration of some viruses by adsorption of the virus to the resins and subsequent elution. A wide variety of cationic and anionic resins are available commercially. As an example, Dowex[24] resins are made in many combinations of sizes, expressed as mesh, and various percentages of divinyl benzene, expressed as linkage.

The resins are linear copolymers of styrene linked with divinyl benzene to form the matrix of spherical, porous beads. The divinyl benzene contributes the third dimension to the polymer network and makes it insoluble. The percentage of divinyl benzene, expressed as 10X, 12X, etc., determines the number of reactive groups which may be added and thus, the reactive capacity of the resin. The kind of reactive group added governs the resin's function as a

cation or anion. Beads to which reactive groups are added possessing negative charges and which attract cations, are cationic resins; those which attract anions are anionic resins. They are generally employed in a sodium, hydroxide, or chloride form which ionize in solution. The reactive groups serve as sites for ion-exchanges during adsorption and elution, and the reactions which will occur are somewhat dependent on the selective affinity of the reactive groups.

The selectivity of one resin over another is largely a function of the size of the ion. When cross-linkage is decreased, the bead is more porous and possesses a greater swelling capacity. The selective differences decrease. As the resin swells, diffusion of ions within the resin is more rapid. The converse is true. Therefore, separation of two components of a given solution depends on differences of ionic size.

One Dowex resin, 21K, is a quaternary ammonium anion exchange resin in which the cross-linkage is increased by an amine. Thus, the percentage of divinyl benzene does not represent the total cross-linkage. The amine linkage exhibits the rapid reaction advantages of low cross-linkage resins and the higher physical stability of greater linkage resins.

The size of the bead, or mesh, also influences the rate and amount of reaction which will occur. Dowex resins of 50-100, 100-200, or 200-400 mesh will have diameters of 297-149 u, 149-74 u, and 74-38 u, respectively. Smaller size resins reach an equilibrium with a contacting solution

more rapidly than larger beads. The rate of diffusion within the resin particle is inversely proportional to the cube of the diameter of the particle.

The porosity of a given resin will depend upon the combination of the linkage and the mesh. It is inversely related to the cross-linkage and will exert a physical influence on the rate and the amount of reactions.

Mathews and Hofstad[57] reported that IBV was not adsorbed to Dowex 1 (anionic). Buthala[7] did not find a significant reduction of the virus present in the supernatant fluid of suspensions of IBV treated with Dowex 50 (cationic) in the sodium form.

A simple, expeditious method for the isolation of poliomyelitis virus from fecal specimens utilized synthetic resins[48]. Fecal specimens were mixed with serum for stabilization and added to a given resin in a centrifuge tube, shaken for a short period of time, and the supernatant fluid discarded after low speed centrifugation. The virus was eluted from the resin in the sediment and was present in the supernatant fluid after low speed centrifugation. Partial purification and a ten-fold concentration of the virus was obtained.

The phenomenon of interference has been described as the antagonistic or inhibitory effect produced by one virus or components thereof upon the production of another virus or the same virus[34,69,82]. It may occur between related and unrelated viruses, active and inactive virus particles or other products related to viral multiplication. When the

interference is between two different viruses, it is heterologous interference. It if is limited to the effect of some component of a viral suspension on the multiplication of the same virus, it is termed auto-interference.

Auto-interference of IBV was observed by Groupé[32]. The development of the interfering component and its presence in the viral-infected allantoic fluid was dependent upon the continued incubation of the dead embryos at 37 C for 24 hours. The death rate of embryos was markedly decreased if inoculations of IBV were preceded with inoculations of the interfering components from other embryos. There was no decrease in the quantity of interfering substance if fluids containing IBV were heated prior to inoculations. Therefore, it was not identical to heat inactivated viral particles. The substance was not produced by incubating infected allantoic fluids at 37 C for 24 hours, incubating the infected CAM at 37 C, or normal CAM in infected allantoic fluid at 37 C. The component was produced only when the infected embryos were incubated two hours or more at 37 C following death due to infection.

A study of influenza virus by Groupé and Pugh[33] indicated a similar interfering component was associated with chicken embryos infected with influenza virus. However, if the viral harvest containing the interfering substance were undiluted or highly diluted when injected into embryonating chicken eggs, no interference occurred. Dilutions of $10^{-2.0}$ of the inoculum resulted in decreased mortality of embryos and decreased production of hemagglutinating units. It was

concluded that the presence of large amounts of the virus overcame the interference. The study indicated the interference may result from a quantitative, not qualitative, increase of a substance, and posed the question of whether the post-death incubation initiates or increases the production of the interfering substance, or reduces the number of the active particles. It may imply that interfering substances are present in all viral harvests but in undetectably small amounts. Indirectly, this may be supported by the observation that many more particles are detected by electron microscopy in a given suspension than can be detected by infectivity tests[70]. It has not been demonstrated that the excess particles are or are not the interfering forms.

In most instances, it is assumed that an interfering substance is a defective, incomplete or inactive form of the virus[34]. Interference results from competition or blockade of some metabolic system of the host cells during some period of viral production.

There is evidence to suggest that some interfering substances are non-viral in nature[5,42,43,44,51]. If cells are exposed to heat inactivated or ultra violet irradiated influenza virus, an interfering substance is produced which has been termed interferon.

Interferon is a macromolecular substance produced by and liberated from chicken embryo cells when exposed to inactivated influenza virus. It possesses no detectable viral properties and is not neutralized by specific antiserum. It is not inactivated by ribonuclease, only partially

inactivated by trypsin, and is heat labile at 60 C for one hour. Interferon is probably protein and does not contain nucleic acids. Sedimentation is not accomplished by ultracentrifugation but the component is retained by gradocol membranes of an average pore diameter 0.6 u and is assumed to be asymmetrical.

Interferon is liberated from CAM cells *in vivo* or *in vitro* after interaction with heat inactivated or irradiated influenza virus over a period of 24 hours after inoculation. When interferon precedes inoculations with active virus, active virus production is inhibited and more interferon is produced. Introduced alone, interferon is not capable of inducing production of more interferon by the cells. Stent[77] has suggested that if at the onset of infection, nucleic acids of virus transfer genetic information to a newly synthesized protein, interferon may be an analog of the new protein. Normally, the active virus particle supplies the pattern and stimulus. When interferon is present, it supplies the pattern and the virus functions only as the stimulus. Thus, interferon has not definitely negated that interference is initially viral induced or a form of the virus.

Tyrrell, *et al.*[51,54] determined that interferon was produced *in vitro* during the second 24 hours after inoculation of calf kidney cell cultures with active virus. Adequate oxygenation was necessary for the production of interferon which implied that it is actively produced by the cells in some manner of viral deflection.

Cooper and Bellett[10] have demonstrated the presence of

a transmissible interfering component in vesicular stomatitis preparations, designated as "T". It resembles interferon in numerous ways.

Schlesinger[68] noted interference of multiplication of eastern equine encephalomyelitis virus in chicken embryo cell cultures inoculated with a strain of inactivated influenza virus. Subsequent studies by Wagner[82] demonstrated that the interfering substance was produced as the result of infection with an untreated influenza virus. The interferon was separated from the virus by ultracentrifugation. In addition, substantial evidence was obtained that cell cultures infected with the influenza virus produced two interfering agents, (1) the heat labile virus and (2) the heat stable, nonsedimentable interferon. The conclusions were that interferon was not a degraded viral particle, but interfered with the intracellular synthesis of eastern equine encephalomyelitis.

Wagner[82] found an interferon produced by cell cultures infected with a strain of influenza virus which could be separated from the hemagglutinating viral particles by four hours of centrifugation at 100,000 X G. Interferon was not sedimented and was found to have the following properties: non dialyzable; 90 percent precipitated at 60 percent saturation with ammonium sulfate; destroyed by trypsin or chymotrypsin; unaffected by ribonuclease, deoxyribonuclease, papain, plasmin, or receptor destroying enzyme from Vibrio cholerae; stable at pH 3.0-11.0, destroyed at pH 12.5, and 65 percent destroyed at pH 1.0; completely adsorbed to bentonite, 25 percent elution by pyridine at pH 9.0 or above;

no UV absorption at any wave length; and probably a basic protein of the histone variety or polypeptide.

Of further interest was the finding that a degree of species specificity of protection was demonstrated by interferon. If the source of interferon was the chicken embryo, it was protective for cell cultures of chick origin, but not for cultures of duck or mouse origin. Likewise, if it were of duck embryo origin, it was protective only to duck embryo cell cultures.

The summary presented by Wagner[82] justifies repetition.

> "Interference appears to be one mechanism by which the bacterial or animal cell can defend itself against viral infection. It is unlikely that resistance to superinfection takes place at the surface of the cell, but it almost certainly does so intracellularly. There is insufficient evidence to implicate genetic or metabolic factors as explanations for competitive antagonism between nucleic acid moieties of two viruses within the same cells. However, the nucleic acid of the interfering virus may well be the essential for initiating the cellular response that leads to interference. If this be the case, the virus contains in its nucleic acid the potential information for its own destruction, mediated by the cellular defenses of the host. Certain interfering viruses stimulate the cell to elaborate protein-like substances of nonviral origin that prevent superinfection with homologous or heterologous viruses. These substances, the interferons, can be secreted by an infected cell and transmitted to other cells, thereby rendering them resistant to infection. Presumably, similar events can transpire in a persistently infected cell culture or an intact animal. It can be predicted with confidence that a considerable amount of future research will be directed toward giving the host an added advantage by passive transfer of the antibiotic-like interfering substances, the interferons."

The demonstration of the effect of interferon in intact animals has been inconclusive[82]. Demonstration of the presence and action of interferon rests, as yet, on studies

made with tissue and cell cultures.

Tissue and cell culture techniques have provided new means for propagating and studying viruses. The tissue and cell cultures and environmental conditions which will support viral production must be determined for each virus to be studied. The nutrition of tissue and cell cultures has been reviewed by Morgan[58]. The contribution of a medium to the propagation of viruses may be distinctly different with each virus, and possibly, characteristic of each virus.

Synthetic media have been developed to serve two tissue- and cell-growth purposes, (1) long term survival or maintenance and (2) rapid multiplication or growth. Failure to distinguish between the two may contribute to conflicting results since one may not be suitable for both purposes .

Synthetic media are lacking in a factor, or factors, not yet identified which is not synthesized sufficiently by cells to maintain cell function and integrity over a prolonged period of time[58]. Recently, the emphasis for cell growth has shifted from survival to rapid growth and as many as possible of the nutritional requirements known for animal and bacterial cells have been incorporated into any one of the many balanced salt solutions.

Media are frequently supplemented with serum or serum fractions homologous or heterologous to the species of cells. The specific function of the supplement is not known but studies of extracts have demonstrated that one fraction is able to markedly accelerate the attachment of single cells on glass surfaces and stimulates growth[31,61]. The fraction,

fetuin, is an alpha globulin fraction, macromolecular substance isolated from fetal calf serum. A strong stimulatory action is exercised on the growth of single mammalian cells which would otherwise fail to produce good colonies in conventional media. Fetuin is antitryptic, contains 0.043 mg sialic acid per mg protein, is inhibited by 10^{-3} Versene, is insoluble in 45 percent ammonium sulfate and soluble in 40 percent ammonium sulfate and is non-dialyzable. The exact mode of action in promoting cell attachment and growth has not been determined. This may be due to an antitryptic action.

Numerous studies of embryonic tissue extracts have established that a nucleoprotein fraction, embryonin, exhibits a remarkable growth-stimulating activity for cell cultures[58]. Extracts of adult tissues also promote cell growth but have not been fractionated or identified[67].

Higuchi[75] found that the supernatant fluid obtained from monolayer cultures of mouse fibroblasts (L cells) grown in a serum-free medium, contained a factor designated as L cell stimulation factor (LCSF). The LCSF possesses a marked ability to increase attachment and growth of Chang's human cells or HeLa cells. It is non-dialyzable, inactivated at 65 C in five minutes, inactivated by N/10 HCl or NaOH, and is precipitated with nine volumes of ethanol.

Other proteins, and protein degradation products, have proved to be stimulatory to cell growth[58]. Their function or means of action are unknown.

Innumerable combinations of tissue or cell cultures and

viruses have been reported. Studies of animal viruses in tissue cultures were reviewed by Robins and Enders[65] in 1950. It has been an exceedingly active field and has been reviewed more recently by Madin[56]. Only those reports of IBV will be considered which are summarized in tables 1 and 2.

Chorioallantoic membranes support multiplication of strains of IBV such as the Beaudette[27,30,60,75] strain, Connaught R[27] strain and New York[8] strain. Chicken embryos were employed for detection and titration of the virus. Minced CAM was superior to whole CAM for maximum virus production[30]. A general pattern of viral multiplication of the Beaudette strain in the 16th passage in minced CAM cultures was established[60]. The stages were found to be as follows: (1) a decline or lag phase during the first eight hours after inoculation with a decrease of titer from $10^{6.3}$ to $10^{3.5}$; (2) progressive increase or log phase to a maximum titer of $10^{6.5}$ at the 60th hour; (3) gradual decline during the next two and one-half days to $10^{5.3}$; (4) a stationery phase during the next three days; and (5) a marked decline after the eighth day. It was also determined that 37 C and pH 7.0 were optimal. The best yields were obtained if the minced CAM were incubated at 37 C for 24 to 81 hours pre-inoculation.

Monkey kidney cells support multiplication of strains of IBV but HeLa cells or Earle's L cells (mouse origin) do not[27].

Chicken-embryo-kidney- (CEK) cell cultures support the Beaudette strain[9,75,84], Massachusetts-41[75], and New York[8]

strains. Serial passage of the virus resulted in cytopathic effects (CPE)[76,84]. The Massachusetts-41 strain required more passages than the Beaudette strain[75] for adaptation to cell culture.

The reported studies of viruses in cell cultures have used cells which had established growth. Larkin and Deutcher[50] found that CPE by Adeno virus was detected more readily if the virus were added to the cells prior to their attachment to a glass surface. In addition, the titers were 10^1 to 10^2 higher. No similar studies of IBV have been reported.

Growth studies of the Beaudette strain of CEK cultures[9] indicated an eclipse period which lasted four hours, and the virus reappeared in 16 hours with a maximum titer obtained at 48 hours. The number of infectious doses as determined by chicken embryos or cell cultures was $10^{4.7}$ and $10^{7.0}$ per ml, respectively, by the 10th passage on CEK cells. Growth studies of the same strain of virus in CEK cells show the reverse relation of numbers of infectious doses of the virus for cell cultures to chicken embryos[76].

Wright and Sagik employed the plaque technique successfully with monolayers of CEK cultures treated with the Beaudette strain[84]. Plaques of three to four mm were visible macroscopically in 16 to 18 hours after the monolayer was infected by the virus. A comparison of the titers of the virus as determined by the plaque method and by embryo infectivity indicated a four to one relation.

Thermal inactivation at 56 C of the minced-CAM adapted virus^{60} after 24 passages indicated that the embryo infectious virus had retained approximately the same inactivation rate as the original strain. At 45 C, the inactivation rate was bimodal. The thermolabile component was inactivated in 30 minutes but 90 minutes were required for inactivation of the thermostable component.

It is obvious that much remains to be done to understand the many facets of the virus of infectious bronchitis.

TABLE 1

Cultivation of infectious bronchitis virus in tissue cultures.

Virus Strain	Tissue Culture	Medium	Results Propagation	CPE
Beaudette (27)	CAM-minced	597	Good	None
	CE-liver minced	597	Poor	None
	CE-whole minced	597	Fair	None
	CE-heart minced	597	Fair	None
Beaudette (30,60,75)	CAM-minced	BSS	Good	None
Connaught R (27)	CAM-minced	597	Fair	None
	CE-liver minced	597	None	None
	CE-whole minced	597	None	None
Beaudette (7)	CAM-intact	lacto-albumen	None	None
Beaudette (26)	Mouse or rat liver		None	None
	CE		Fair	None
	Monkey kidney		Fair	None

TABLE 2

Cultivation of infectious bronchitis virus in cell cultures.

Virus Strain	Cell Culture	Medium	Results Propagation	Results CPE
Beaudette (27)	Monkey kidney Earle's L	597 597	Poor None	None None
Connaught R (27)	Monkey kidney Earle's L	597 597	Poor None	None None
Beaudette (9,75)	CE-kidney	lacto-albumen	Good	2nd pass.
Beaudette (84)	CE-kidney		Good	Plaque
Mass-41 (75)	CE-kidney	lacto-albumen	Fair	12th pass.
New York (8)	CE-kidney		----	None
Beaudette, Hofstad 33 and 97 (18)	HeLa		None	None
Beaudette, Hofstad 33 and 97 (7)	CAM CE-kidney CE-lung	lacto-albumen	None None None	None None None

REAGENTS AND PROCEDURES

I. VIRUS

A Massachusetts strain of IBV was employed which is maintained at the North Central Repository at Michigan State University. The repository code number of the strain is 41. It was isolated in 1956 by H. van Roekel at Amherst, Massachusetts.

A seventh egg passage of IBV was inoculated intratracheally into chickens, three months old. When typical signs of the disease were observed, the birds were sacrificed and the lungs and trachea collected aseptically. After grinding the tissues and preparing a suspension in nutrient broth, nine-day-old embryonating chicken eggs were inoculated via the allantoic cavity, and incubated for 30 hours at 37.5 C. The eggs were chilled for three hours at 4 C, the allantoic fluid harvested aseptically, and stored at -27 C. The viral-infected allantoic fluid was passed in chicken embryos in a similar manner as virus stock was needed. The second passage was employed in all studies unless otherwise indicated.

II. NORMAL ALLANTOIC FLUID

Normal allantoic fluid (NAF) was harvested from chilled, 11-day-old chicken embryos and was employed as a control when warranted.

III. DILUENTS

A. Difco Nutrient Broth was used to prepare dilutions

of IBV for titration in embryos.

B. Hank's Balanced Salt Solution (BSS) was employed to prepare serial dilutions of viral suspensions for titration in cell cultures, for preparing cell culture media and for diluting trypsin[67]. The BSS, 10X, was made as follows.

1.	NaCl	80.0 gm
	KCl	4.0 gm
	$MgSO_4 \cdot 7H_2O$	2.0 gm
	$Na_2HPO_4 \cdot 12\ H_2O$	1.2 gm
	Glucose	10.0 gm
	KH_2PO_4	0.6 gm
	*H_2O	800.0 ml
2.	$CaCl_2$	1.4 gm
	*H_2O	100.0 ml
3.	Phenol red	0.2 gm
	*H_2O	80.0 ml

Mix the phenol red with 10 ml of N/20 NaOH. Grind in a mortar. Bring volume to 100 ml with distilled H_2O*. Adjust pH to 7.0 with N/20 NaOH.

*Double glass-distilled H_2O.

Autoclave 1, 2, and 3 separately at 115 C for 10 minutes. When cool, mix together and dispense aseptically in four ounce prescription bottles. Dilute 1:10 with sterile H_2O* for use.

IV. BUFFER

The pH of BSS and any solutions made with BSS was

adjusted with $NaHCO_3$, 3.5 gm dissolved in 250 ml of water and autoclaved at 121 C for 15 minutes (NaH)[67].

V. MEDIA FOR CELL CULTURES

A. Cell maintenance medium was prepared by adding one percent of each of the following which were obtained from Microbiological Associates, Inc., to BSS: glutamine, amino acid pool and vitamins. Penicillin, 100 units per ml and streptomycin, 100 units per ml were added, and the pH adjusted to 7.2 with NaH.

B. Cell growth medium consisted of the cell maintenance medium plus 10 percent bovine serum.

VI. CELL CULTURES

A. Source: Single comb white leghorn chicken embryos, nine days old, were used. The eggs were obtained from a commercial disease free flock. All incubation of embryos was in a Jamesway Model 252 incubator at 37.5 C.

B. Preparation and trypsinization: Chicken embryos were removed aseptically from the shell to sterile Petri dishes. The viscera, eyes, and appendages were removed and the remaining portions minced with two scalpels opposing each other in a scissor-fashion. Approximately four times during the mincing process, BSS was added to wash the tissues and was removed with a capillary pipette. When the tissues were less than 2 mm^3, final washings were made with BSS until the BSS remained clear, and all visible tinges of blood were removed.

The minced tissues were added to a flask containing

0.25 percent trypsin in BSS which had been sterilized by passage through a Seitz filter. The suspension was magnetically agitated for two hours at room temperature. A pH of 8.0 was maintained by additions of NaH during the two hour period.

The trypsinized suspension was poured through four layers of sterile cheesecloth and then poured into 50 ml graduated centrifuge tubes. The suspension was centrifuged for five minutes at 1500 rpm (437 X G) in an International refrigerated centrifuge, Model PR-1, at 4 C. The supernatant fluid was removed, and the sediment was washed with BSS. Centrifugation and washing were repeated two times. The final packed volume of cells was diluted according to the specific use intended.

If cells were to be employed for detecting CPE, a dilution of approximately 1:400 in growth medium was made. Cells were dispensed in Leighton tubes, 2.0 ml per tube, for observation of living cells, or they were dispensed to quarter-sectioned Petri dishes with cover slips, 3.0 ml per section, for stained cell preparations. Cell cultures were incubated at 37 C for approximately 36 hours prior to use.

If cells were to be employed for blind passage of the virus, 10 ml of the 1:400 cell dilution was dispensed in two ounce prescription bottles, and incubated at 37 C for approximately 36 hours.

If cells were to be employed for determining growth stimulation of cells, a 1:900-1000 dilution was made in maintenance medium. The cells were not incubated prior to

use but were inoculated and dispensed into Petri dishes or Leighton tubes in the amounts stated above.

VII. TITRATIONS AND CALCULATIONS

A. Chicken embryo infectious units: Chicken embryos, 10 days old, received 0.1 ml of a given dilution via the allantoic route, with five eggs being employed per dilution. The eggs were incubated at 37.5 C and were candled 24 hours after inoculation. Death of embryos at this time was attributed to non-specific causes and these embryos were not included in calculations of viral infectivity. Results of the titrations were recorded six days after inoculations. The criteria of infectivity were death, curling and dwarfing of embryos. The titer, expressed as embryo infectious doses ($e.i.d._{50}$) was calculated by the method of Reed and Meunch[C4].

B. Cytopathic effects evinced by chicken embryo cell cultures: The growth medium was removed from 36 hour old Leighton tube cell cultures which, viewed microscopically, were found to have formed a monolayer of fibroblasts. Each of three tubes of cell cultures was seeded with 2.0 ml of a virus dilution. Beginning with a 1:20 dilution of the virus in maintenance medium, serial 10 fold dilutions in maintenance medium were prepared. Since 2.0 ml of the dilutions was added to each tube and the initial dilution was 1:20, the results obtained were in units per 1.0 ml.

The inoculated cell cultures were incubated at 37 C. The cultures were examined microscopically after 48 hour incubation. The titer was recorded as the reciprocal of the

highest dilution in which all three cell cultures exhibited CPE.

The same procedure for dilution and seeding was followed for quarter-sectioned Petri dish cell cultures when permanent stained preparations were desired. Inoculated cultures were incubated at 37 C. Cover slips were withdrawn at different time intervals, stained by a modified May Gruenwald-Geimsa stain[67] and observed microscopically.

C. Cell-attachment-and-growth factor: Freshly trypsinized cells were dispensed into Leighton tubes, 1.8 ml per tube, or 2.7 ml per section of a quartered Petri dish. Serial 10 fold dilutions of viral-infected allantoic fluid were made in maintenance medium and 0.2 ml or 0.3 ml of a virus dilution was added to the tube cultures or Petri dish cultures, respectively. Three tubes of cell cultures and one Petri dish cell culture were inoculated per dilution and incubated at 37 C.

Cover slips were withdrawn at different time intervals and stained for permanent preparations for microscopic observations.

Leighton tube cultures were observed microscopically at 24 and 48 hours. A positive response was scored for a given dilution if all three tube cultures exhibited an unquestionably heavy growth at no later than 24 hours after inoculation which was followed by complete degeneration during the subsequent 24 hours. This type of cellular response was indicative of the presence of a cell attachment and growth factor (CAGF). The titer of the factor was

recorded as the reciprocal of the highest dilution eliciting this cellular response.

VIII. RESIN ADSORPTION AND ELUTION

A. Preliminary investigations

Resins of various linkages and meshes (table 3) were mixed in the chloride and hydroxide forms with both NAF and viral-infected allantoic fluid in centrifuge tubes, shaken by hand for 30 minutes, and centrifuged 15 minutes at 2500 rpm (1210 X G) at 4 C. The supernatant fluid was removed and the resin-sediment resuspended in 10 percent Na_2HPO_4. After shaking 30 minutes and a second centrifugation, the eluate was removed and titrated in chicken embryos.

Employing Dowex 21K, "low x", 100-200, or Dowex 1, 10x, 100-200, in the chloride form, the above procedure was followed but two washes with distilled water preceded elution with Na_2HPO_4. Both washes and the eluate were titrated in embryos. In addition, the number of elutions and water-washed controls which would yield virus was determined by repeated elutions and washings of the resin-virus mixture.

Time intervals of 5, 10, 15, and 30 minutes of adsorption and elution were used with Dowex 21K and Dowex 1, 10x, 100-200, to determine the optimum time for adsorption and elution.

B. Final procedure

Viral-infected allantoic fluid, 10.0 ml, was added to five ml of Dowex 21K, "low x", 100-200, or Dowex 1, 10x, 100-200, in the chloride form, in a 50 ml centrifuge tube.

The mixture was shaken intermittently by hand for 15 minutes at room temperature, and centrifuged at 4 C (121 X G) for 10 minutes. The supernatant fluid was removed and an equal amount of 10 percent Na_2HPO_4 added to the sediment. The mixture was shaken intermittently by hand for 15 minutes. The eluate was removed following centrifugation at 4 C (1210 X G). When a greater quantity was desired, a second eluate was obtained from the resin in a similar manner.

IX. ELECTRON MICROSCOPY

Electron micrographs of untreated and eluted virus were made by Nazerian[59].

X. ADAPTATION OF VIRUS TO CELL CULTURES

Allantoic fluid and eluates containing the virus were diluted in maintenance medium. The growth medium was removed from 36 hour old cell cultures in two ounce prescription bottles. To each of five bottle cultures, 10 ml of a dilution was added to provide 10^6 $e.i.d._{50}$ per ml. The inoculated cultures were incubated for approximately 30 hours at 37 C.

Following incubation of the inoculated cultures, the monolayer of cells was scraped free of the glass surface with a stainless steel spatula. The bottles were shaken vigorously and the contents were pooled. This represented the first cell passage harvest. A 10^{-2} dilution of the harvest was made in maintenance medium for the immediate inoculation of cells for a second passage.

The second set of five 36-hour-old chicken embryo cell

cultures was drained of growth medium and seeded with 10 ml of the 10^{-2} dilution of the first passage. These were incubated and harvested as above, and were second cell culture harvests. This was repeated through the eighth passage. All passages were titrated for infectivity for embryos, for CAGF, and CPE.

The ninth through the 12th passages were made, but the harvested virus was frozen and stored at -62 C between passages.

XI. RATES OF PRODUCTION OF VIRUS AND CELL-ATTACHMENT-AND GROWTH FACTOR IN EMBRYONATING CHICKEN EGGS

Untreated or eluted virus was inoculated into embryos, nine days old, via the allantoic cavity. The inoculum, 0.1 ml per embryo, contained $10^{3.0}$ $e.i.d._{50}$. There were $10^{3.0}$ CAGF units per ml in the untreated viral suspension but none in the eluate.

From each group of embryos which had been incubated for three, six, nine, 12, 24, 36, and 48 hours after inoculation, three were removed and chilled at 4 C for 30 minutes. Allantoic fluid was harvested and titrated for the CAGF units and embryo infectious units.

XII. THE EFFECT OF DILUTION, ELUTION AND EGG PASSAGE ON THE PERCENT DWARFING OF INFECTED CHICKEN EMBRYOS

Untreated and eluted suspensions of the second and 11th passages of the virus were employed to provide $10^{7.0}$ or $10^{3.0}$ $e.i.d._{50}$ per embryo. The amounts of CAGF and $e.i.d._{50}$ in each inoculum were as follows:

passage	e.i.d.$_{50}$	CAGF
11th untreated	$10^{7.0}$	10^{12}
	$10^{3.0}$	10^{8}
11th eluted	$10^{7.0}$	10^{7}
	$10^{3.0}$	10^{3}
2nd untreated	$10^{7.0}$	10^{7}
	$10^{3.0}$	10^{3}
2nd eluted	$10^{7.0}$	10^{3}
	$10^{3.0}$	10^{0}

Each sample was used to inoculate 20 embryos via the allantoic cavity. After six days, the percent of the embryos which were dwarfed was recorded.

XIII. HEAT INACTIVATION OF VIRAL SUSPENSIONS

Viral-infected allantoic fluid or eluate, 1.0 ml, was diluted in an equal amount of nutrient broth and placed in a water bath at 56 C. Sufficient tubes of viral suspensions were employed to allow removal of one tube at given intervals. A two minute delay allowed the suspensions to reach thermal equilibrium which was then considered zero time. When a tube was withdrawn, it was placed immediately in an ice bath. The contents were titrated for the e.i.d.$_{50}$. On the basis of the total number of embryos infected, the percent of the embryos dwarfed was recorded.

Mixtures of equal volumes of heated and unheated

suspensions were also titrated for e.i.d.$_{50}$ and the percent of embryos dwarfed by the mixtures was determined.

RESULTS AND DISCUSSION

I. MONOLAYER FORMATION FROM SINGLE CELLS

Numerous observations were made by light microscopy of cell cultures on cover slips withdrawn at hourly intervals after seeding in quarter-sectioned Petri dishes. If single cells were diluted in a growth medium containing five percent serum, cells attached to the stationary glass surface in 30 to 60 minutes and were uniformly dispersed. Fibroblasts formed a confluent monolayer in approximately 24 to 36 hours. If the medium contained one percent serum or less, a higher concentration of cells was necessary for a similar attachment and growth rate (figure 1).

When a relatively high dilution of cells was used without serum, the attachment and production of fibroblasts occurred only if single cells were able to aggregate into groups of approximately four to eight. If aggregation occurred, a monolayer formed within 12 to 24 hours later than if the medium were enriched or the cells were in a heavier concentration.

The monolayers sloughed from the glass surface approximately two to three days after formation. The time when sloughing occurred was somewhat dependent upon the rate at which the monolayer was formed and the concentration of serum.

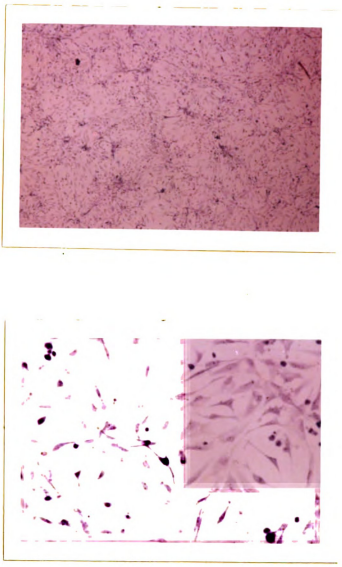

Figure 1. Whole chicken embryo cells in growth medium after 24 hours incubation. May Gruenwald-Geimsa stain, upper X14, lower X80.

II. RESPONSE OF MONOLAYERS TO VIRUS AND NORMAL ALLANTOIC FLUID

A. Single Exposure

Cover slips withdrawn at hourly intervals from cell cultures exposed to 10 fold serial dilution of IBV or NAF revealed no macroscopically or microscopically detectable changes in the cell cultures which could be attributed to the virus. Extensive observations at 100X, 431X, and 970X of cells stained with a modified May-Greunwald Geimsa stain or wet mounts indicated that the growth patterns of cells exposed to IBV or NAF were identical. If the inoculations were made by adding the virus to the medium in which the cells were growing or to a replacement medium, no effect was detectable. If the virus were added directly to the cells, from which the medium had been removed, and allowed to incubate at 37 C for intervals ranging from five minutes to one hour before replenishing the medium, there was no alteration of the cells. Varying the amount of virus employed in the above methods did not alter the results.

Occasionally, the fibroblasts exposed to the virus became relatively indistinct in respect to cellular detail[75]. No consistent correlation to the presence of the virus was established.

Cell cultures sloughed in approximately 48 to 72 hours after exposure. This was apparently due to the contact of cells with each other and a resulting inhibition of growth[1]. The results were consistent with tube cultures prepared and observed concurrently[75].

B. Repeated Passage of Virus on Cell Cultures

Serial passage of the virus on whole embryo cell cultures resulted in a viral suspension capable of producing detectable CPE. Serial transfers of NAF on cell cultures did not produce CPE.

Once the virus had been adapted to the cell cultures so the CPE was evident, monolayers exposed to the cell-adapted virus exhibited a characteristic and consistent overall degeneration 24 to 36 hours after exposure. The progressive degeneration began earlier than the sloughing observed with NAF or untreated cells, and no sloughing of the entire layer occurred. Remnants of the cells remained attached to the cover slips or to Leighton tubes (figure 2).

Microscopy of stained or wet mounts did not establish that any specific alteration was associated with the virus. Viral inclusion bodies were not observed.

The sixth passage of undiluted virus harvested from cell cultures produced CPE. The seventh passage contained 10^3 CPE units per ml and the 10th contained 10^6 per ml. There were approximately $10^{3.67}$ and $10^{4.00}$ $e.i.d._{50}$ per ml, respectively.

Employing the seventh passage cell culture harvest, 100 CPE units were used per tube for the neutralization test by the beta procedure (decreasing serum, constant virus)[13]. The virus was specifically neutralized.

III. RESPONSE OF CELLS PRIOR TO ATTACHMENT TO GLASS

The indistinct appearance occasionally exhibited by

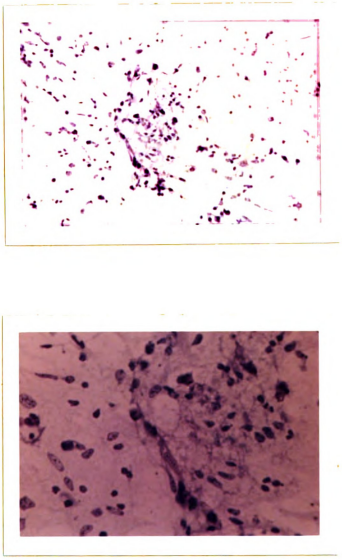

Figure 2. Whole chicken embryo cells 36 hours after inoculation with cell-adapted infectious bronchitis virus. May Gruenwald-Geimsa stain, upper X14, lower X80.

monolayers of cells after primary inoculation of IBV suggested that there might be some slight initial effect by the virus. It was possible that cell cultures maintained a state of refractoriness to the infection by their healthy, rapid-growth condition. Therefore, various media and concentrations of cells were investigated in an attempt to provide a combination of environment and cell which was less than optimal for the cells. The rate of attachment and the fibroblast formation were the criteria selected.

Cells diluted 1:900-1000, approximately 10^6 cells per ml, in BSS containing one percent each of glutamine, amino acids and vitamins were unable to attach and grow on a glass surface, and few or no fibroblasts were formed. If serum were added within 12 hours after seeding, normal cell growth resulted.

When the cells were mixed with allantoic fluid containing IBV, or NAF, prior to the attachment of the cells to the glass, a marked and consistent stimulating effect was elicited by the allantoic fluid containing the virus. Only slight or no effect was produced by NAF and fibroblasts were not produced by the control cells. For detection of the stimulatory effect, it was imperative that the cells be diluted sufficiently in the maintenance medium so that little or no growth was initiated without additives.

The initial effect was the aggregation and attachment of the cells to the glass surface in 15 to 30 minutes and the rapid formation of fibroblasts. Monolayers were complete within 24 to 36 hours. The secondary effect was the

appearance of small, radially enlarging centers of complete cellular deterioration and a rounding and clumping of the cell remnants into centers of dense, deeply staining aggregates. Sloughing was limited to small areas where clearing and clumping were absent (figures 3, 4, and 5).

Dilutions of the allantoic fluid containing the virus added to Leighton tube cultures indicated the CAGF to be present in the 10^{-6} dilution but not in the 10^{-7} dilution. Similar titrations of the 11th egg passage virus indicated that there was 10^{3} times more CAGF.

Neutralization tests with specific antibodies were inconclusive and were the first indication that CAGF was not identical to the viral particles. Detection of CAGF is dependent upon a cell system which is incapable of good growth without the addition of some factor or enrichment. The addition of normal or immune serum in one percent concentration was sufficient to support growth of the cells. Therefore, any effect by CAGF was masked if the serum concentration were this great. The beta procedure (decreasing serum, constant virus) for neutralization[13] did not demonstrate neutralization of CAGF by anti-infectious bronchitis virus serum. Sufficient antibodies were present in dilutions greater than 1:100 and therefore, CAGF was not serologically identical to the cell culture or egg passage virus.

No detectable amount of CAGF was present in fluids harvested from cultures which had produced cell-adapted virus. Ground tracheal suspensions of experimentally infected birds did not contain detectable amounts of CAGF.

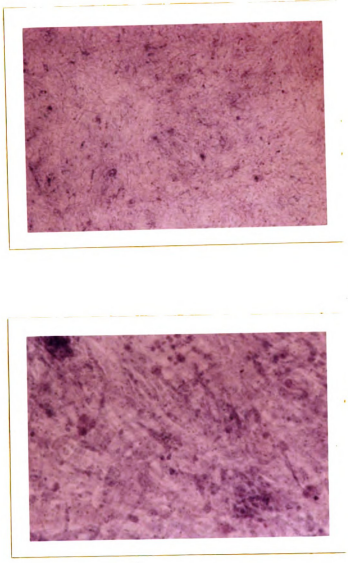

Figure 7. Whole chicken embryo cells 24 hours after the addition of cell-attachment-and-growth factor. May Gruenwald-Geimsa stain, upper X14, lower X80.

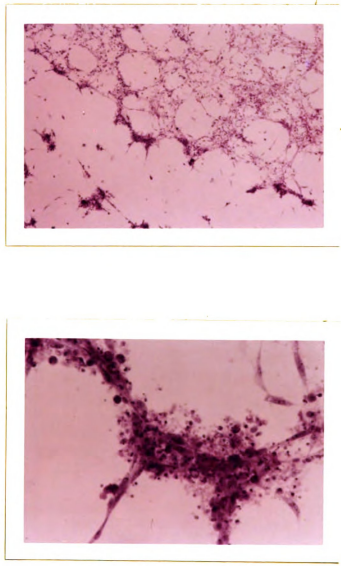

Figure 4. Whole chicken embryo cells 36 hours after the addition of cell-attachment-and-growth factor. May Gruenwald-Geimsa stain, upper X14, lower X80.

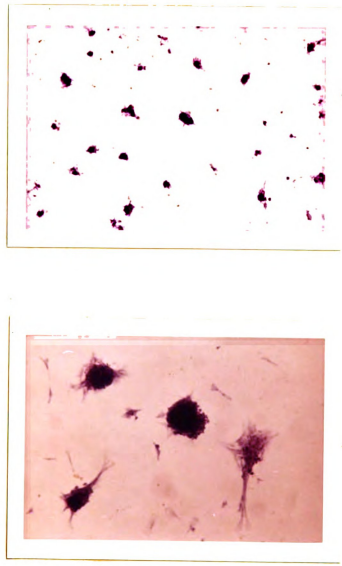

Figure 5. Whole chicken embryo cells 48 hours after the addition of cell-attachment-and-growth factor. May Gruenwald-Geimsa stain, upper X14, lower X80.

Heat inactivated NAF or allantoic fluids containing the virus were toxic to cell cultures. The toxic material could not be completely eliminated by dilution.

IV. PURIFICATION OF VIRUS BY RESIN ADSORPTION AND ELUTION

A. Preliminary

Buthala[7] had demonstrated by gel diffusion that five precipitating antigens exist in NAF and two additional ones are present in allantoic fluid harvested from embryos infected with IBV. None of the seven antigens were identified as the infectious viral particles. The seven antigens and other non-viral waste products from the embryo present in the allantoic harvests might be expected to exert some effect upon cell or embryo systems employed in studying IBV. A purified product, therefore, is most desirable. The procedure of chemical fractionation, centrifugation and enzymatic digestion outlined by Buthala was tedious and time consuming, and yielded one hundredth the original volume with a reduction of 10^2 $e.i.d._{50}$ per ml or a net reduction of 10^4 per ml.

Various resins in differing meshes and linkages were screened to determine if a purified product or concentrated virus could be obtained from the heterogenous viral allantoic fluid by means of adsorption to and elution from resins. The time intervals allowed for adsorption and elution were arbitrarily selected as 30 minutes each. Likewise, the dry, chloride form of resin was employed and mixed with a double volume of allantoic fluid (table 3).

TABLE 3. Virus recovered in supernatant fluids and eluates from resins mixed with allantoic fluid containing $10^{6.50}$ e.i.d.$_{50}$/0.1 ml.

Resin			e.i.d.$_{50}$/0.1 ml	
No.	Linkage	Mesh	Supernatant Fluid	Eluate
1	2X	50-100	$10^{5.78}$	$10^{4.50}$
1	2X	200-400	$10^{5.32}$	$10^{5.63}$
1	8X	50-100	$10^{4.83}$	$10^{6.36}$
1	8X	200-400	$10^{3.83}$	$10^{6.54}$
1	10X	50-100	$10^{3.83}$	$10^{6.50}$
1	10X	100-200	$10^{3.32}$	$10^{7.38}$
1	10X	200-400	$10^{4.17}$	$10^{6.22}$
21K	low	50-100	$10^{3.38}$	$10^{7.31}$
50	2X	50-100	$10^{6.38}$	-
50	2X	200-400	$10^{6.50}$	-
50	12X	20-50	$10^{6.50}$	-
50	12X	200-400	$10^{6.22}$	-

Adsorption and elution of the virus was possible. The anionic resins of choice were Dowex 1, 10x, 100-200 mesh or Dowex 21K, "low x", 100-200 mesh, in the dry chloride form. The latter is newer, more readily available and regarded as a superior product by the producer. No adsorption to cationic resins was detected.

Normal allantoic fluid was processed in a manner similar to the virus. Chicken embryos and monolayer cell cultures were treated with the eluates and supernatant fluids. No adverse effects were observed.

Supernatant fluids from the resin adsorbed virus mixtures were not toxic to chicken embryos but when added to cell cultures, the medium became strongly acid. There was a correlation between the amount of virus removed from the fluid by the resin and the resulting pH of the supernatant fluid. For example, the supernatant fluid from Dowex 1, 10x, 100-200 or 21K was pH 6.0 ± 0.2 and the number of $e.i.d._{50}$ was $10^{2.5}$ per ml. The supernatant fluid of Dowex 1, 2x, 200-400 mesh was pH 7.2 ± 0.2 and $10^{5.32}$ $e.i.d._{50}$ per ml. It is improbable that the lowered pH had an adverse effect on the virus. Subsequent elution provided from $10^{0.5}$ to $10^{1.0}$ more $e.i.d._{50}$ per ml in the eluate in the first case, but there was a decrease of at least $10^{2.0}$ in the second case.

The pH of the supernatant fluid was adjusted by diluting 1:10 in BSS and adding NaH prior to use for titrations in chicken embryos or cell cultures.

Prior to treatment with resins, the viral infected allantoic fluid was pH 8.10; NAF, pH 7.80; 10% Na_2HPO_4, pH 8.85.

When virus was adsorbed and eluted from either of the two resins, the pH of the eluate was 7.85. The supernatant fluid of the NAF control was pH 7.2, the "eluate" pH 8.45. The difference of the pH of eluates from various resins was not as great as that found between different supernatant fluids.

Dowex 21K and 1 were converted to the hydroxide form by treatment with NaOH and subsequent washing with distilled H_2O until the wash was pH 7.0. The amount of virus recovered in the eluate from the hydroxide form of the resins was approximately 10^2 infectious particles per ml less than that obtained from the chloride form. No further studies were made of the hydroxide forms.

The optimal ratio of Dowex 1, 10x, 100-200, to the viral allantoic fluid was determined by mixing different amounts and titrating the resulting eluates and supernatant fluids (table 4).

It was established that a 1:1 ratio was unsatisfactory since browning of the beads indicated that physical breakage had occurred. A ratio of one part resin to two parts of fluid yielded an amount of virus in the eluate equivalent to that from ratios of 1:5 or 1:10. In addition, there was less unadsorbed virus in the supernatant fluid. Therefore, the practical ratio of resin to fluid was 1:2.

The optimal time to be allowed for the mixture of resin and fluid to allow adsorption to occur was determined. The optimal time for elution from the resin with Na_2HPO_4 was also determined (table 5).

TABLE 4. The effect of the volume ratio of Dowex 1, 100-200, chloride form to viral infected allantoic fluid containing $10^{5.5}$ e.i.d.$_{50}$/0.1 ml.

Ratio Resin:Fluid	e.i.d.$_{50}$/0.1 ml Supernatant Fluid	Eluate
1:1	$10^{2.25}$	$10^{4.49}$
1:2	$10^{2.83}$	$10^{6.50}$
1:5	$10^{4.34}$	$10^{6.50}$
1:10	$10^{5.34}$	$10^{6.34}$

TABLE 5. The effect of time with Dowex 1, 100-200, chloride form in 1:2 ratio of resin:viral infected allantoic fluid containing $10^{5.5}$ e.i.d.$_{50}$/0.1 ml.

Adsorption 15 minutes		Elution 15 minutes	
Elution with 10% Na_2HPO_4 in minutes	e.i.d.$_{50}$/ 0.1 ml	Adsorption time in minutes	e.i.d.$_{50}$/ 0.1 ml
5	$10^{5.00}$	5	$10^{5.50}$
10	$10^{6.75}$	10	$10^{6.50}$
15	$10^{6.83}$	15	$10^{6.83}$
30	$10^{6.68}$	30	$10^{6.83}$

Adsorption or elution was not adequate within five minutes. Slightly better results were obtained at 15 minutes than at 10 minutes. Adsorption or elution for 30 minutes did not increase the amount of virus obtained. Therefore, 15 minutes was selected as the optimal time for adsorption and for elution.

Each of the previous studies had entailed only a single elution of the virus from the resin. It was necessary to know if this were possibly a mechanical removal of residual virus trapped in the swollen resins, or if the viral particles were adsorbed to the resin. Therefore, resins were washed with double distilled water after adsorption but prior to the elution. The eluate subsequently obtained by treating the washed resin with Na_2HPO_4 contained as many infectious particles as eluates from resins which were not washed with distilled water. It was also established that washing with water prior to elution did not yield a product any more purified so far as could be determined than if water washings were omitted. Therefore, washing with water prior to elution was not routinely practiced.

Titers of four consecutive washings of resin-virus with distilled water are presented in table 6. Included in the chart also are the titers obtained from four consecutive elutions of virus from a duplicate set of resins with Na_2HPO_4. The first and second elutions are approximately equal in virus but beyond the second elution, insufficient virus remained to justify further elution.

TABLE 6. Virus obtained by treating resin-virus sediment* with 10% Na_2HPO_4 or with distilled water.

Treatment	e.i.d.$_{50}$/0.1 ml	
	Na_2HPO_4	Distilled Water
1st	$10^{7.17}$	$\leq 10^{2.38}$
2nd	$10^{7.00}$	$\leq 10^{1.63}$
3rd	$10^{5.00}$	$\leq 10^{0.50}$
4th	$10^{3.83}$	$\leq 10^{0.50}$

* Untreated viral infected allantoic fluid contained $10^{6.00}$ e.i.d.$_{50}$ IBV/0.1 ml and the supernatant fluid after the adsorption period contained $10^{2.63}$.

B. Final Procedure

The following procedure was adopted for the purification and concentration of IBV harvested in the allantoic fluid of infected chicken embryos:

1. Dowex 21K "low x", 50-100 or Dowex 1, 10x, 100-200 mesh in the dry, chloride form are mixed with a double volume of allantoic fluid in a graduated centrifuge tube and shaken intermittently by hand for 15 minutes at room temperature.
2. The mixture is centrifuged for 15 minutes at 2500 rpm (121 X G), and the supernatant fluid decanted.
3. The sediment is restored to the original volume with 10 percent Na_2HPO_4 and shaken intermittently by hand for 15 minutes at room temperature.
4. The centrifugation is repeated.
5. The eluate is removed with a capillary pipette, avoiding the removal of any resin beads in the sediment or fragments present in the fine surface film.

The eluate which is obtained is crystal clear, and contains $10^{1.0}$ more $e.i.d._{50}$ per ml than the original suspension.

V. COMPARATIVE STUDIES OF UNTREATED AND ELUTED VIRUS SUSPENSIONS

A. Electron Microscopy

Photographs of IBV in untreated allantoic fluid and eluates were made by Nazerian[59]. The untreated virus was

variable in size and shape, probably occurring in aggregates. The eluted virus was spherical, present in greater numbers per unit volume, and considerably more uniform in shape and size. This substantiates that the virus was purified and concentrated by resin adsorption and elution (figures 6 and 7).

B. Serial Passage on Whole Embryo Cell Cultures

Untreated virus and eluted virus were diluted to provide $10^{6.0}$ e.i.d.$_{50}$ per flask of monolayer cell cultures for the first blind passage. The diluted untreated allantoic fluid contained 10^{7} CAGF units and the diluted eluate contained 10^{3}. Neither suspension produced CPE on cell culture. As each passage was made, the harvest was titrated for the number of e.i.d.$_{50}$ and CAGF, and employed as inoculum for tube cell cultures to determine if virus capable of producing CPE were present (tables 7 and 8, figure 8).

It was established in duplicate experiments that the eluted virus adapted more readily than the untreated virus to cell culture as evidenced by the production of e.i.d.$_{50}$ and virus capable or producing CPE in cell cultures. Untreated virus required at least six blind passages before CPE was detected whereas eluted virus exhibited slight CPE on the third passage. After CPE was evident, the increased titer with subsequent passages was approximately equal for both and by the 11th passage the titers were the same.

Titrations of e.i.d.$_{50}$ indicated that there was a slight production of the virus as detected by its effect in chicken embryos, after the first passage. Each cell culture

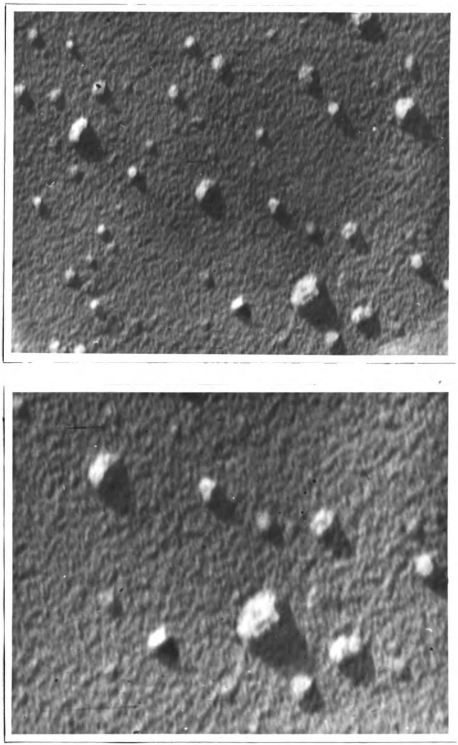

Figure 6. Untreated infectious bronchitis virus. Magnification X26,000. Upper enlargement X4, lower enlargement X12.

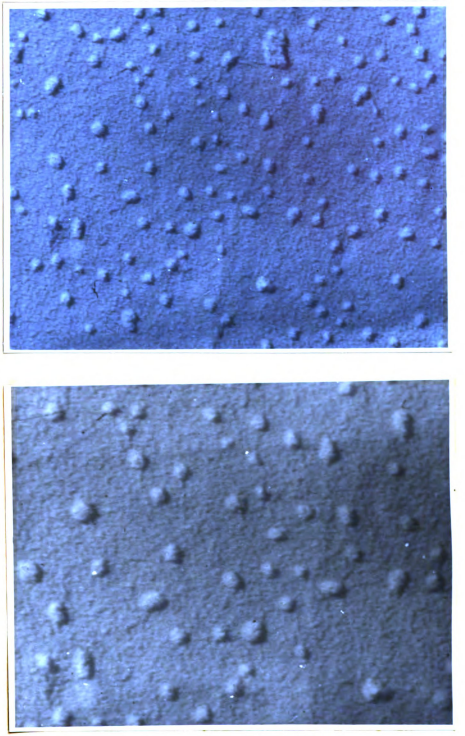

Figure 7. Resin-eluted infectious bronchitis virus. Magnification X26,000. Upper enlargement X4, lower enlargement X12.

TABLE 7. The relation of the cell-attachment-and-growth factor, embryo infectivity, and cytophathic effects of untreated IBV passaged on whole embryo cell cultures.

No. of Passages	CAGF/ 0.1 ml	$e.i.d._{50}$/ 0.1 ml	CPE on Monolayers
Original	10^7	$10^{6.00}$	none
1st	10^4	$10^{2.17}$	none
2nd	10^2	$10^{1.50}$	none
3rd	10^1	$10^{1.32}$	none
4th	none	$10^{1.67}$	none
5th	none	$10^{2.32}$	±
6th	none	$10^{3.00}$	10^0
7th	none	$10^{3.67}$	10^3
8th	none	$10^{3.67}$	10^4
9th	none	$10^{4.00}$	10^4
10th	none	$10^{4.00}$	10^6
11th	none	$10^{4.50}$	10^8

TABLE 8. The relation of the cell-attachment-and-growth factor, embryo infectivity, and cytopathic effects of resin-eluted IBV passaged on whole embryo cell cultures.

No. of Passages	CAGF/ 0.1 ml	e.i.d.$_{50}$/ 0.1 ml	CPE on Monolayers
Original	10^{3}	$10^{6.00}$	none
1st	10^{0}	$10^{3.50}$	none
2nd	none	$10^{3.00}$	±
3rd	none	$10^{3.32}$	slight
4th	none	$10^{3.38}$	10^{0}
5th	none	$10^{3.67}$	10^{2}
6th	none	$10^{4.00}$	10^{4}
7th	none	$10^{4.67}$	10^{4}
8th	none	$10^{4.00}$	10^{6}
9th	none	$10^{4.50}$	10^{7}
10th	none	$10^{4.50}$	10^{7}
11th	none	$10^{4.50}$	10^{8}

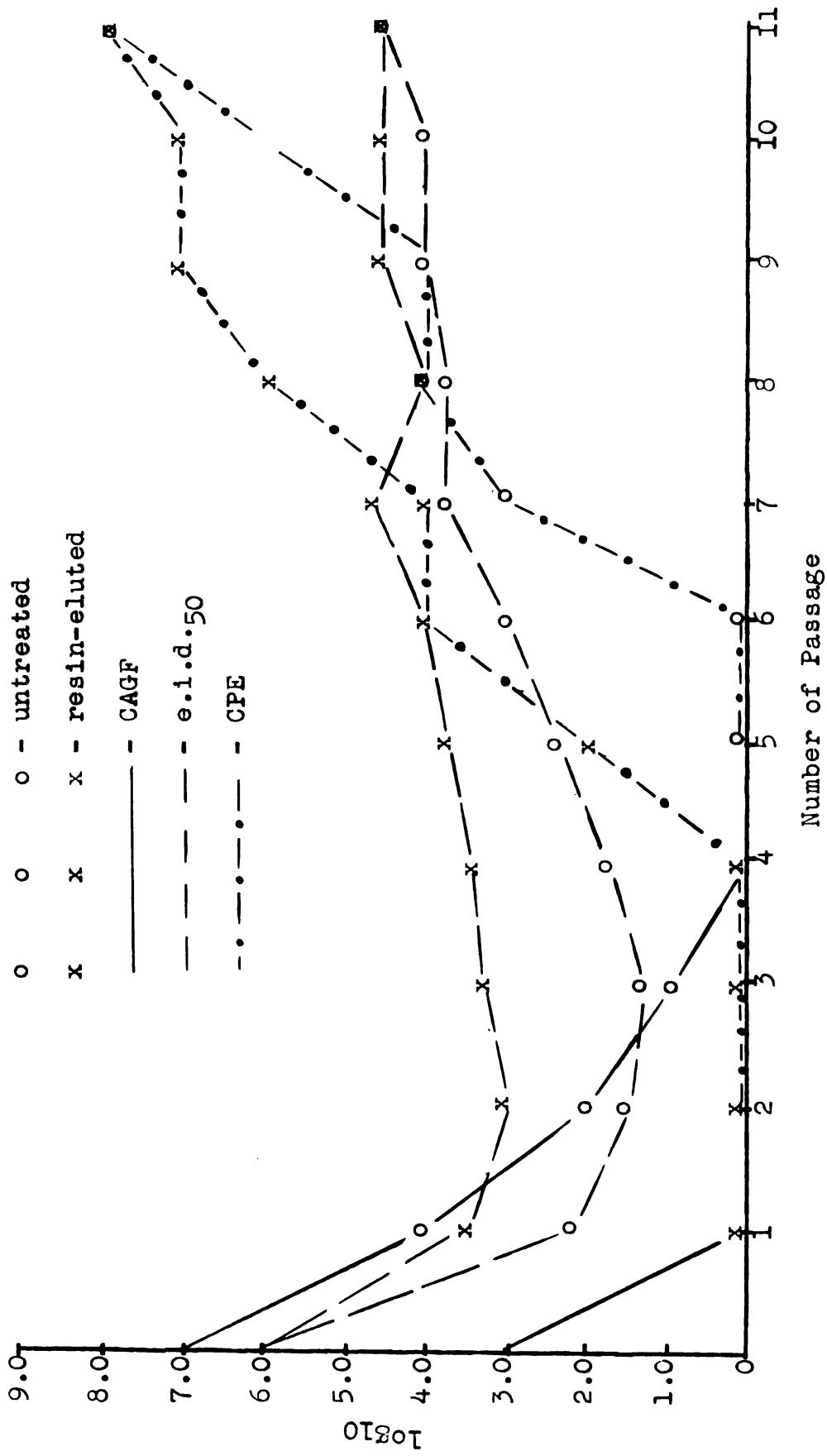

Figure 8. The relation of the cell-attachment-and-growth factor, embryo infectivity, and cytopathic effects of untreated and resin-eluted IBV passaged on whole embryo cell cultures.

harvest was diluted 10^{-2} to serve as an inoculum for the next passage. Since 11 serial passages were made, the cumulative effect of the dilution would have been such that if the virus were not multiplying, there would have been no virus present. Viral reproduction of 10^2 is substantiated when a titer of a harvest is equal to the titer of the previous harvest. From the second through the ninth passage, there were $10^{2.0}$ $e.i.d._{50}$ or more with either virus suspension. The untreated virus decreased much more initially than the eluted virus. The rate of increase after production began yielded an equal titer by the seventh passage. Thereafter, it remained at a relatively constant level of production, approximately $10^{2.0}$ per ml, through the 11th passage.

The CAGF was present in the original untreated suspension of viral allantoic fluid, 10^7 units per ml. There was a 10^{-3} decrease during the first cell culture passage. The original eluted viral suspension contained 10^4 units per ml which decreased equally during the first cell culture passage. In both cases, there was no detectable CAGF in the two passages prior to the appearance of CPE, or thereafter.

The CPE was clearly evident in the second passage after the disappearance of CAGF. This suggests that CAGF may in some way interfere with the production of the virus which can produce CPE, or its presence may interfere with sufficient cells being altered by infection to allow visual detection. The CAGF may have been present in undetectably small amounts through one or more of the three passages

required. In either event, and they may be one and the same, the factor may be functioning analogous to other auto-interfering substances previously reported.

There is also the possibility that CAGF in no way contributes to any effect other than cell attachment and growth. It may function similarly to fetuin[61,31], embryonin[58], LCSF[35], or other cell attachment and growth factors.

If the latter is true, the resin may be semi-selective for a form of the virus which can adapt more readily to cell cultures. However, the fact that adaptation of the virus to monolayer cell cultures, whether untreated or eluted, was apparent in the same number of passages after the CAGF could no longer be detected, would not support the independence of the two events. In addition, if the resin is semi-selective for a particular viral form which will adapt more readily to monolayer cell cultures and this were the only variable involved, the CPE might be expected to appear earlier. More probably, there is some effect on the rate of viral adaptation by the CAGF as well as by some selectivity of the resin for a particular form of the virus. Whether the CAGF is an anamolous form of the virus or an unrelated chicken embryo metabolite can not yet be predicted.

The results also raise the question as to whether or not the viral particles, as measured by e.i.d.$_{50}$ and CPE units, are identical or different forms of the virus. The difference in the rate of production of the virus as detected by the two methods suggests that two forms exist. The production of embryo infectious units during monolayer adaptation

was at a slower rate, and did not yield as high a titer as the CPE units during subsequent passages.

Antiserum obtained from chickens experimentally infected with IBV neutralized the virus in the embryonating chicken eggs or in monolayer cell cultures. Therefore, varying concentrations of e.i.d.$_{50}$ and CPE units were employed to determine if the difference in relative concentrations of e.i.d.$_{50}$, CPE and CAGF units would alter the neutralization index of the antiserum. By the alpha procedure (decreasing virus, constant serum) of serum neutralization[13] the neutralization index of the serum was determined in embryonating chicken eggs when mixed with each of the three viral suspensions.

	e.i.d.$_{50}$/ml	CPE units/ml	CAGF units/ml
A. Untreated viral allantoic fluid	100	10^0	10^3
B. Virus eluted from resins treated with A.	100	10^0	10^0
C. Virus harvested in 10th monolayer cell passage.	100	10^5	10^0

The neutralization indices of A, B, and C were equal. The presence of absence of CAGF or CPE units did not alter the neutralization index of the serum. This might be interpreted to mean that the CPE units were independent and different from the e.i.d.$_{50}$. However, the same antiserum neutralized the virus in monolayer cell cultures. It is more probable that the viral particles were altered in some slight way by continued passage in cell cultures which allowed the

same amount of e.i.d.$_{50}$ to contain an increasing amount of CPE units than for the antiserum to contain antibodies with a distinct and independent specificity for each of the forms. The former may be substantiated also by the constant titer of the e.i.d.$_{50}$ while the CPE titer continued to increase with cell passage. It is possible that the same number of viral particles was present at either an early or later passage after the CPE of monolayers was observed, but that some slight alteration allowed more of those present to produce a detectable effect in cell cultures, and thus be erroneously interpreted as an actual increase in viral particles. This has not been determined, only suggested, by the lack of detection of any serological differences between the two units and the lack of reduction of antibodies available to neutralize the embryo infectious units when CPE units are present.

It might also be assumed that the CAGF units are entirely unrelated to the infectious viral forms since there was no interference of antibody neutralization of the infectious forms. However, this would necessitate the unsubstantiated assumption that specificity and interfering abilities reside within the same portion of the particles.

As noted during the adaptation of the virus to cell cultures, no CAGF was produced. The CAGF apparently is a result of IBV infection of chicken embryos. The rate at which CAGF is produced by the infected embryo and the effect which the presence of CAGF in the inoculum might exert on the rate of production were determined. Harvests of allantoic fluid

from embryos inoculated with untreated viral allantoic fluid and with the eluted viral suspension were made at three hour intervals for the first 12 hours and at 24, 36, and 48 hours after inoculation. The harvests were titrated for the rate of production of embryo infectious virus and CAGF. In addition, the percent of dwarfing of infected embryos was noted, since previous titrations of eluted virus consistently yielded greater numbers of dwarfed than dead embryos (tables 9, 10, and 11, figures 9 and 10).

The results indicated that CAGF was produced concurrently and at approximately the same rate of increase as the embryo infectious virus between six and 24 hours after inoculation. The initial production began at the same time and at approximately the same rate regardless of the amount introduced in the inoculum.

The rate and time of the beginning of production of the virus appeared to be independent of the amount of CAGF introduced when equal numbers of viral particles were introduced. However, the number of embryos dwarfed by the virus produced during the first 24 hours was influenced by the amount of dwarfing particles present in the original inoculum if one form causes dwarfing and another form kills the embryo. This appeared to be independent of CAGF through the 24th hour. After 24 hours, the total number of viral particles did not increase significantly, but continued incubation altered the ratio of dwarfing virus to lethal virus and there was also a slight continued increase of CAGF. The significance is not apparent but does pose the questions of whether

TABLE 9. The relation of virus lethality and dwarfing to the time virus was obtained from chicken embryos inoculated with 10^3 e.i.d.$_{50}$ untreated IBV/embryo.

Hours after inoculation	Responses in Chicken Embryos							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
3	*2+2/4	1+0/4	0+0/4	0+0/4				
6		2+2/4	2+3/5	2+3/5	0+0/5			
9			4+1/5	2+3/5	2+2/5	0+1/5	0+0/5	
12				3+2/5	2+3/5	1+3/5	0+0/5	
24					4+1/5	2+3/5	1+3/5	0+0/5
36					5+0/5	4+1/5	1+2/5	0+1/5
48					5+0/5	5+0/5	3+1/5	0+0/5

* The first number of the numerator indicates the number of embryos dead; the second, the number of embryos dwarfed. The denominator is the total number of embryos inoculated. This designation also applies to tables 1, 14, and 15.

TABLE 10. The relation of virus lethality and dwarfing to the time virus was obtained from chicken embryos inoculated with 10^2 $e.i.d._{50}$ resin-treated IBV/embryo.

Hours after inoculation	Responses in Chicken Embryos							
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
3	0+1	0+0						
6		1+4 / 5	0+4 / 5	0+1 / 5	0+0 / 5			
9		3+2 / 5	0+5 / 5	0+5 / 5	0+1 / 5	0+0 / 5		
12				2+3 / 5	0+3 / 5	0+2 / 5	0+0 / 5	
24				4+1 / 5	1+4 / 5	0+4 / 5	0+2 / 5	0+0 / 5
36				4+1 / 5	1+4 / 5	0+4 / 5	0+1 / 5	0+0 / 5
48				4+1 / 5	2+3 / 5	1+3 / 5	0+0 / 5	0+0 / 5

TABLE 11. The relation of embryo infectivity, cell-attachment-and-growth factor, and percent dwarfing to the time virus was obtained from chicken embryos inoculated with 10^3 $e.i.d._{50}$ IBV/embryo.

Hours after inoc.	$e.i.d._{50}$/0.1 ml		CAGF/0.1 ml		Percent dwarfing	
	Untreated	Eluate	Untreated	Eluate	Untreated	Eluate
3	$10^{1.50}$	$10^{1.16}$	10^3	10^0	60	83
6	$10^{4.50}$	$10^{4.50}$	10^4	10^1	56	90
9	$10^{5.50}$	$10^{5.83}$	10^5	10^2	47	84
12	$10^{6.38}$	$10^{6.50}$	10^6	10^3	56	80
24	$10^{7.38}$	$10^{7.68}$	10^6	10^3	50	85
36	$10^{7.31}$	$10^{7.50}$	10^7	10^4	29	75
48	$10^{7.38}$	$10^{7.38}$	10^8	10^6	7	50

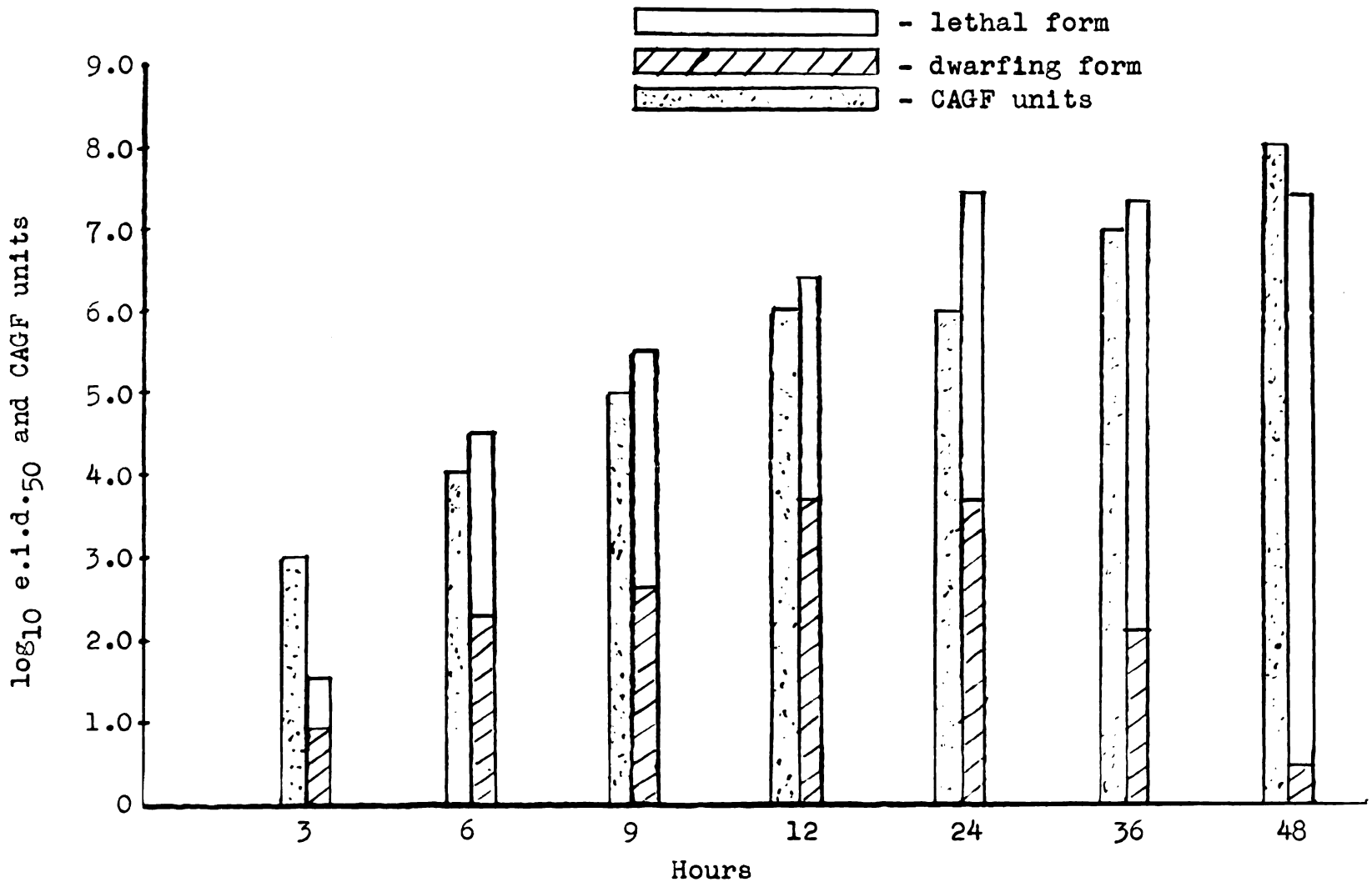

Figure 9. The relation of embryo infectivity, cell-attachment-and-growth factor, and percent dwarfing to the time virus was obtained from chicken embryos inoculated with 10^3 $e.i.d._{50}$ untreated IBV/embryo.

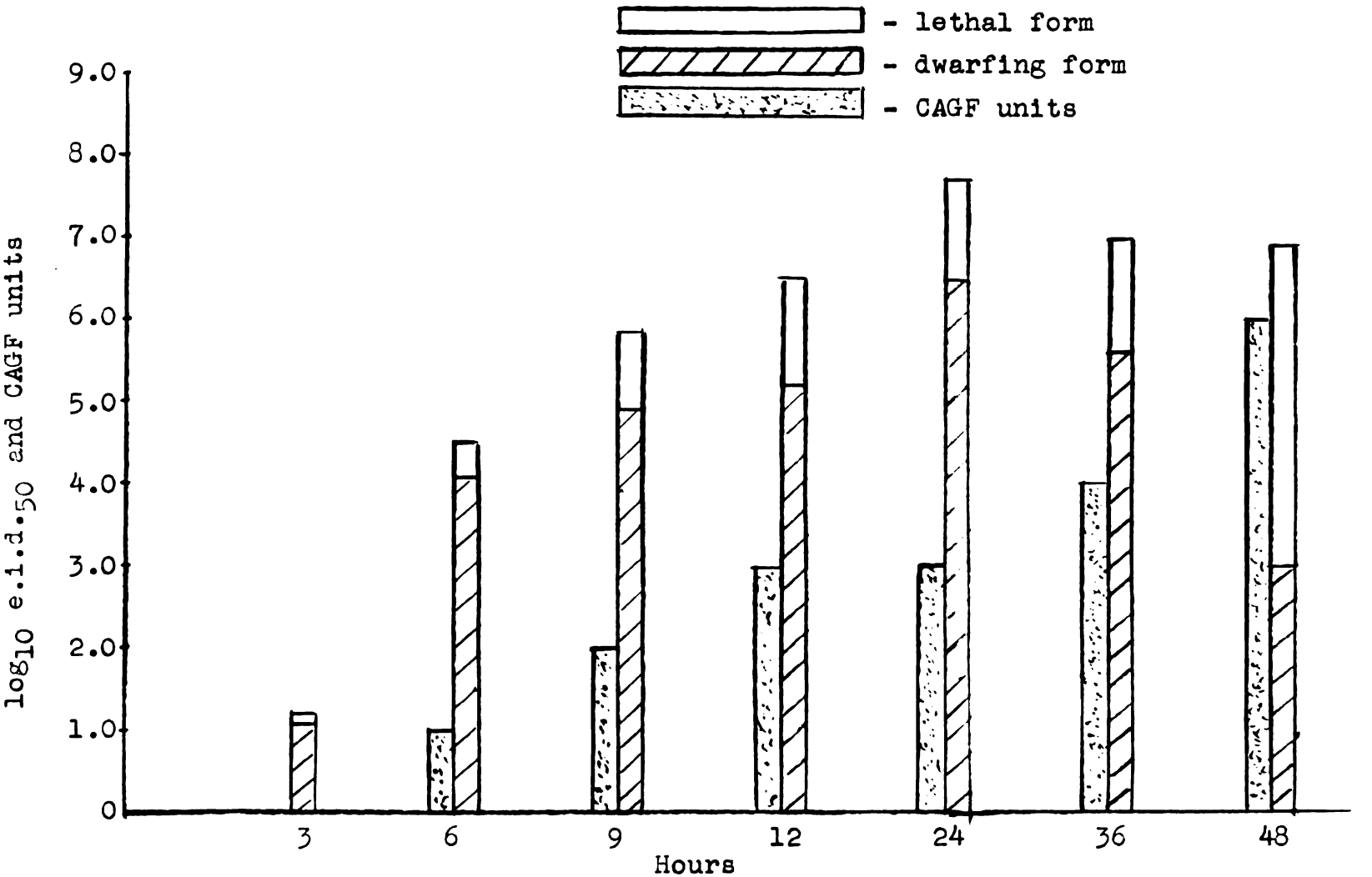

Figure 10. The relation of embryo infectivity, cell-attachment-and-growth factor and percent dwarfing to the time virus was obtained from chicken embryos inoculated with 10^3 e.i.d.$_{50}$ resin-eluted IBV/embryo.

or not the presence of the CAGF may induce the production of more lethal viruses. The increased CAGF could be some altered form of the dwarfing virus which decreases the number of dwarfing particles while more lethal particles are being produced. The presence of the CAGF may somehow inhibit or compete with the dwarfing virus, and when the virus is eluted from resins, the decreased CAGF allows more dwarfing particles to express themselves or to be produced. The resin may possess the capacity to separate preferentially the dwarfing particles not only from CAGF but also from the viral form lethal for the chicken embryo.

Various concentrations of virus and CAGF were inoculated into chicken embryos. An 11th egg passage and a second egg passage of the virus were employed in untreated or eluted suspensions. To alter further the ratios of the different components, dilutions of these suspensions were made. Twenty eggs per viral suspension were inoculated per embryo. The results are expressed as the percent of embryos exhibiting manifestation of infection by dwarfing (table 12). As previously indicated, the percent is based on the number of embryos dwarfed of the total infected, not the total inoculated.

Dilutions of untreated or eluted 11th egg passage fluid increased the amount of embryos which were dwarfed. Virus obtained by elution more markedly increased the percent of dwarfing. This was true also with the second passage untreated and eluted virus.

The presence of CAGF is implicated as somehow

TABLE 12. The relation of dwarfing to the units of embryo infectivity and cell-attachment-and-growth factor.

History	Inoculum/embryo		Percent of dwarfing of 20 embryos
	e.i.d.$_{50}$	CAGF	
11th passage untreated	$10^{7.0}$	$10^{12.0}$	20
	$10^{3.0}$	$10^{8.0}$	35
11th passage eluted	$10^{7.0}$	$10^{7.0}$	50
	$10^{3.0}$	$10^{3.0}$	65
2nd passage untreated	$10^{7.0}$	$10^{7.0}$	50
	$10^{3.0}$	$10^{3.0}$	65
2nd passage eluted	$10^{7.0}$	$10^{3.0}$	85
	$10^{3.0}$	$10^{0.0}$	90

interfering with the expression of the dwarfing virus when present. This was further supported in that the untreated 11th passage contained a relatively high amount of CAGF and produced only 20 percent dwarfing and 80 percent lethality. By dilution, dwarfing was increased to 35 percent. By elution, the dwarfing was equal to that produced by the untreated and undiluted second passage virus. The quantity of CAGF was equal in both cases.

When eluted virus was diluted so that CAGF was reduced from $10^{3.0}$ to 10^{0} per embryo, there is only five percent increase in dwarfing. Apparently 10^{3} CAGF units per embryo was the minimal level at which it can exert its influence. Any slight effect was obscured by the continuous shift from the dwarfing form of the virus to the lethal form as the result of embryo adaptation.

This raises the question of whether or not the dwarfing form and the lethal form believed to be detected and partially separated by resin elution, are the same viral particles which are differentiated by thermal inactivation[74]. From the thermal inactivation rates of the untreated and eluted second passage virus, it is obvious that the inactivation rates are approximately the same for either untreated or eluted virus (tables 13 and 14, figure 11). This indicates that the difference between the untreated and eluted virus as detected by dwarfing is not to be considered as identical to the "O" and "D" phases of IBV. However, heating affected the percent of embryos dwarfed among those infected. Untreated virus increased dwarfing from 47 to 94 percent after

TABLE 13. Inactivation rates of untreated IBV at 56 C.

Minutes	Responses in Chicken Embryos									e.i.d.$_{50}$ per ml	% Embryos Dwarfed
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}		
0			4+0/5	4+1/5	3+2/5	2+3/5	0+4/5	1+3/5	0+0/5	$10^{7.38}$	47
5		2+3/5	1+4/5	1+4/5	4+1/5	1+2/5	0+2/5	0+0/5		$10^{5.50}$	64
10		2+3/5	2+3/5	0+5/5	1+4/5	0+3/5	0+0/5			$10^{5.17}$	80
15		2+3/5	3+2/5	0+5/5	0+5/5	0+2/5				$10^{4.83}$	79
20		0+5/5	1+4/5	0+5/5	0+3/5	0+0/5				$10^{e.17}$	95
25	1+4/5	0+5/5	0+4/5	0+3/5	0+0/5					$10^{4.00}$	94

TABLE 14. Inactivation rates of resin-eluted IBV at 56 C.

Minutes	Responses in Chicken Embryos									e.i.d.$_{50}$ per ml	% Embryos Dwarfed
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
0					1+4/5	0+5/5	0+5/5	0+4/5	0+1/5	$10^{8.50}$	95
5			1+4/5	0+5/5	0+4/5	0+3/5	0+0/5			$10^{6.00}$	94
10			1+4/5	0+4/5	0+3/5	0+2/5	0+0/5			$10^{5.50}$	92
15		0+5/5	1+4/5	0+5/5	0+2/5	0+0/5				$10^{4.83}$	94
20	1+4/5	0+5/5	0+4/5	0+4/5	0+0/5					$10^{4.25}$	95
25	1+4/5	0+5/5	0+3/5	0+0/5						$10^{4.17}$	92

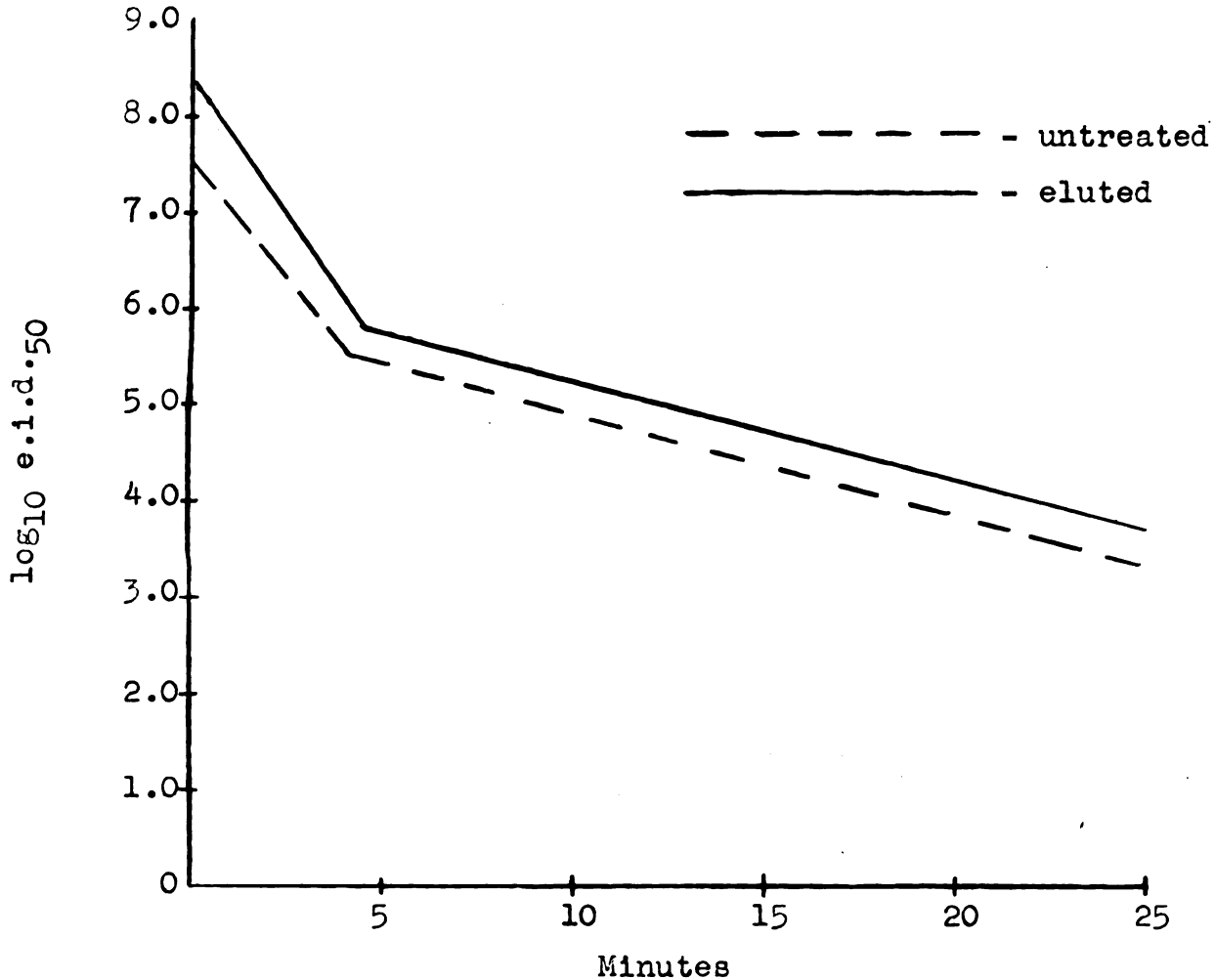

Figure 11. Inactivation rates of untreated and resin-eluted IBV at 56 C.

by exposure to 56 C for 25 minutes. Eluted virus was relatively constant in the percent of dwarfing it produced which was independent of the amount of virus inactivated. From these results, two conclusions may be drawn. First, the five percent of the embryos which were killed by the eluted virus, regardless of the time heated, is repeated evidence of the shift in population from the dwarfing form to the lethal form of the virus during the period of incubation of the embryos for titration and not evidence of the presence of the lethal form in the eluted suspension. Second, the marked reduction of mortality of embryos evincing infection of heated non-resin treated viral suspension established that the lethal form of the virus was inactivated more rapidly that the dwarfing form.

It was noted previously that the CAGF may interfere with the expression of production of the dwarfing form of the virus. To determine that the effects noted above were not due to a more rapid rate of inactivation of CAGF during the heating period and thereby allowing more of the dwarfing forms to exert their effect, embryos were inoculated with various mixtures of unheated and heated viral suspensions.

Each of the following mixtures were inoculated into 20 embryos:

A. Equal volumes of unheated eluted virus and heated eluted virus, 10^4 $e.i.d._{50}$ per embryo, containing no CAGF initially.

B. Equal volumes of unheated and heated non-resin treated virus, 10^4 $e.i.d._{50}$ per embryo, containing

10^4 CAGF units.

C. Equal volumes of unheated non-resin treated virus and heated eluted virus, 10^4 $e.i.d._{50}$ per embryo, containing 10^4 CAGF units.

D. Equal volumes of unheated eluted virus and heated non-resin treated virus, 10^4 $e.i.d._{50}$ per embryo, containing 10^4 CAGF units.

Mixtures of unheated and heated NAF were employed as controls.

The percents of dwarfing elicited by mixtures A, B, C, and D were approximately equal to the dwarfing observed in the embryos inoculated with control mixtures. The percent of dwarfing was an expression of the unheated viral suspension and was not altered by the presence of heated viral suspensions whether non-resin-treated or eluted (table 15).

Two inferences are made. If CAGF can interfere with the dwarfing of the embryo by the virus, it is inactivated at the same rate as the virus is inactivated. If this were not so, the presence of the heated CAGF in mixture D should result in fewer dwarfed embryos than in the control embryos. Secondly, the mixtures containing heat inactivated viral particles did not alter the percent of dwarfing. Therefore, heat inactivated viral particles do not interfere with the dwarfing form of the virus, and are not identical to CAGF.

It has been established that CAGF is present in the allantoic fluid harvested from chicken embryos infected with IBV. The most readily detectable effect is the ability of CAGF to accelerate cell attachment to and growth on a glass

TABLE 15. Embryo dwarfing effect by mixtures of untreated and resin-eluted IBV, unheated or heated to 56 C for 25 minutes.

Mixture	56 C, 25 Min.	Unheated	% of 20 embryos dwarfed	% of dwarfing of control embryo inoc.
A.	Eluate	Eluate	95	95
B.	Non-resin-treated	Non-resin-treated	60	65
C.	Eluate	Non-resin-treated	60	60
D.	Non-resin-treated	Eluate	95	90

surface of the cells which are otherwise unable to establish themselves. The CAGF is not neutralized by antibodies specific for the virus and is not present in tissues of infected birds.

The CAGF is partially separated from the infectious viral particles by adsorption of the virus to anionic resins and subsequent elution. Limiting dilutions of the eluate provide a viral suspension free of CAGF.

The presence of CAGF is associated with a slower rate of viral adaptation to monolayer cell cultures as determined by the cytopathic effects. It also appears to interfere with either the expression or production, or both, of the infectious form of the virus which dwarfs chicken embryos.

The possibility exists that the presence of CAGF is coincidental with the action of the virus on monolayer cell cultures or to the effect of the virus on chicken embryos. If so, resin purification and concentration of the virus may be semi-selective for a particular form of the virus. Neither of these events need be mutually exclusive.

SUMMARY

1. Infectious bronchitis virus propagated in the allantois of embryonating chicken eggs does not produce a detectable cytopathic effect on whole chicken embryo cell cultures on primary inoculation.

2. The virus produces a detectable cytopathic effect on whole chicken embryo cell cultures after serial passage. The effect is one of a generalized deterioration of the monolayer, and can be specifically neutralized by anti-infectious bronchitis virus serum.

3. Allantoic fluid harvested from chicken embryos infected with the virus contains a component designated as cell-attachment-and-growth factor (CAGF), which enhances cell attachment and growth of cells otherwise unable to establish themselves. The rapid formation of monolayers is followed by radially enlarging areas of complete cellular destruction and accumulating large, dense centers of debris.

4. The factor is not produced by cell cultures infected with the virus and it is not present in tissues of infected chickens. It is produced in infected chicken embryos concurrently with production of virus from six to 24 hours after inoculation. The rate of production of CAGF is independent of the amount of the product present in the inoculum.

5. A concentrated and partially purified suspension of the virus can be obtained from viral-infected allantoic fluid by adsorption of the virus to and subsequent elution from

anionic resins. By limiting dilutions, the virus may be obtained free of CAGF.

6. Eluted virus adapts more readily to monolayers of cell cultures than does non-resin treated virus. This appears to be the result of increasing the ratio of viral particles infectious for the chicken embryo to the cell-stimulating factor by selection of a form of the virus, or allowing expression of the virus, which can exert a detectable effect in earlier passages.

7. Reduction of the amount of CAGF by elution and dilution results in a marked increase in the percent of infected embryos which are dwarfed rather than killed by the virus. It is probable that the factor interferes with the expression and production of the form of the virus which is responsible for the dwarfing of the embryo.

8. Thermal inactivation rates indicate that a bimodal inactivation of virus occurs in either eluted or non-resin treated suspensions. Adsorption and elution do not separate the O and D forms. When non-resin treated virus is heat inactivated, the embryo-lethal form is inactivated more rapidly than the dwarfing form. The heat inactivated forms of the virus do not alter the percent of dwarfing of embryos by viable viruses.

9. The relation of CAGF to that of the infectious viral particle and the role it plays is undetermined. It may function as fetuin[61,31], embryonin[58], or LCSF[35] in cell suspensions otherwise unable to establish themselves. It appears to interfere with the elicitation of embryo-dwarfing

and cytopathic effects on monolayers by a form of the virus. Whether it is an incomplete, altered, defective, or abortive viral particle or protein, some form of the host's defensive mechanisms which attempts to maintain a state of refractoriness by cellular proliferation and sloughing, or some left-over metabolic product of altered cellular functions remains to be determined. Whether it is offensive, defensive, or inadvertent is unknown.

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