SOME PROPERTIES OF INFECTIOUS BRONCHITIS VIRUS AS DETERMINED BY THERMAL AND FORMALIN INACTIVATION

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SOME PROPERTIES OF INFECTIOUS BRONCHITIS VIRUS AS DETERMINED BY THERMAL AND FORMALIN INACTIVATION

By

INDRA PAL SINGH

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This thesis is

respectfully dedicated

to

MY FATHER

ABSTRACT

Infectious bronchitis virus (IBV) exists in Original (O) phase and Derivative (D) phase. This diphasic characteristic resulting from egg propagation, is reflected in several properties of the virus. In the present study the differences in thermal sensitivity as reflected by bimodal inactivation at 56 C., were the basis for characterization of the two phases.

Isolation of thermostable O phase from O - D populations was accomplished by differential inactivation of thermolabile D phase at 56 C. The isolated O phase was inactivated exponentially with a reaction-rate constant similar to that for O phase in the respective O - D populations.

The isolated O phase was maintained through 13 serial egg-passages by using the limiting dilution technique, but thermostability decreased directly with egg-passage.

Bimodal inactivation at 56 C., of mixtures of isolated O phase and the Beaudette egg-adapted strain considered to be in D phase, emphasized that inactivation is due to heterogeneity of thermal sensitivities of the virus.

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The O phase virus was antigenic and pathogenic for chickens. Growth characteristics of O phase were similar to those of IBV in low egg-passage.

Inactivation of IBV in O - D population by 1:2,000 and 1:4,000 formalin at pH 7.5 and 37 C., followed a curvilinear rate. The Beaudette egg-adapted strain was inactivated exponentially by 1:4,000 formalin. The O phase was more resistant to 1:4,000 formalin and the inactivation rate was less curvilinear as compared to the O - D population. Since these features were in common with thermal inactivation, the curvilinearity of the inactivation rate was considered to be due to the heterogeneity of the two virus phases to formalin.

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INTRODUCTION

Infectious bronchitis virus (IBV) undergoes modification of several of its characteristics as a result of serial propagation in embryonating chicken eggs. Previous studies of thermal inactivation showed that IBV is heterogeneous in its thermal sensitivity and that in early egg-passage the virus possibly exists in thermolabile Derivative (D) phase and thermostabile Original (O) phase.

The objects of the present study were: (1) to isolate O phase by the differential thermal inactivation method and to maintain it in serial egg-passage in order to obtain a homogeneous population of IBV, and (2) to evaluate the characteristics of O phase, and mixed O - D populations by the kinetics of thermal and formalin inactivations.

LITERATURE REVIEW

INFECTIOUS BRONCHITIS

Infectious bronchitis is an acute and contagious disease of chickens characterized by respiratory disturbances. The disease was first recognized by Schalk and Hawn in 1930, in the United States,[86] and has since been reported in England,[1] Canada,[55] The Netherlands,[5] Italy,[76] and Japan.[84]

The etiological agent, a virus, has been classified as Tarpeia pulli in order Virales.[73, 91] According to electron microscopy, the virus in allantoic fluid is spherical with a diameter of about 65 to 135 millimicrons.[78, 79, 80] Intracytoplasmic elementary bodies of an average diameter of about 200 millimicrons are found in the infected chorio-allantoic membrane.[29] An inclusion body has not been reported.[68]

Infectious bronchitis virus multiplies readily in the allantoic cavity, amnionic cavity, and the chorio-allantoic membrane of embryonating chicken eggs.[17, 19, 20, 26, 30, 68] The virus in early egg-passage is rarely lethal to chicken embryos but produces characteristic macroscopic lesions, the most typical of which are stunting and curling of the embryos with deformed feet compressed

over the head; wry neck; and the amnionic membrane is thickened, and resists removal of the embryo.[4, 19, 20, 25, 26, 30, 68]

During the course of serial propagation in embryonating chicken eggs, there is an enhanced virulence of the virus for chicken embryos accompanied by progressive loss of virulence and antigenicity for chickens.[4, 25] Complete adaptation of the virus to the embryo may require 60 or more passages depending on the strain. Completely egg-adapted virus, such as the Beaudette strain, is non-pathogenic and non-antigenic to chickens but highly virulent to chicken embryos, killing all within 24 to 36 hours post-inoculation.[21] Egg-adapted virus still capable of inducing the disease in chickens does not revert to its original upon passage in chickens.[25] Although the completely egg-adapted virus is non-antigenic, it is specifically neutralized by antibodies produced by virus possessing antigenic properties.[32, 74, 75]

Growth rate studies in chicken embryos indicate that IBV in low egg-passages enters the log phase in 6 hours and reaches the maximum concentration in 24 to 36 hours. Completely egg-adapted virus, such as the Beaudette strain, multiplies more rapidly, entering the log phase in 4 hours and attaining maximum titer in 12 to 18 hours.[54]

The Beaudette strain can readily be cultivated, after a few passages, in cell culture from chicken embryo kidney,[12, 23, 33] liver, heart,[33] and whole embryo fibroblasts,[12, 23] and in the isolated chorio-allantoic membranes.[23, 33, 35, 72] The Connaught R and Beaudette strain do not infect mouse liver cells but do multiply in monkey and chicken embryo liver and heart cells without the production of cytopathogenic effects.[33] The Massachusetts and Connecticut strains do not infect chicken embryo kidney cells.[12]

Interference occurs between active IBV and heat inactivated IBV, IBV and influenza, and IBV and Newcastle disease virus in chicken embryo culture.[49, 50, 51, 52, 53]

Infectious bronchitis virus does not possess hemagglutinating activity,[3, 31, 56] but when modified by trypsin, chicken and turkey erythrocytes can be agglutinated.[14] The degree of hemagglutination varies with egg-passage level of the virus. The greatest hemagglutinating activity occurs with low egg-passage virus while the Beaudette egg-adapted strain shows little or no activity. The hemagglutinin of trypsin-modified IBV is probably not associated with enzymic activity. Heat inactivated virus can agglutinate chicken erythrocytes and the cells from which the virus has eluted are reagglutinable.[14]

The ability of IBV to produce neutralizing antibodies is associated with infectivity.[25, 27, 57]

Inactivated or completely egg-adapted virus is non-antigenic for chickens. Antibodies can be measured <u>in vitro</u> by the neutralization test.[22, 24, 74, 75] The lethal $dose_{50}$ neutralizing index ($l.d._{50}NI$) of sera from 99.7 per cent of normal chickens by the alpha procedure (decreasing virus constant serum method) has been found to be within the range of $10^{1.517} \pm 10^{0.0376}$ or approximately 30 neutralizing doses.[18]

Neutralizing antibodies can be measured two weeks after primary antigenic stimulus of chickens with virulent IBV. The maximum neutralizing index is reached at the sixth week, remains constant up to the twentieth week, and then gradually decreases. A secondary stimulus at the twelfth week is followed by a further increase in antibody titer for about three weeks, but at the twentieth week the antibody level is the same as at twelfth week following primary antigenic stimulus.[28, 74, 75]

Immunological differences have been observed between strains of IBV by reciprocal neutralization tests. At the present, there are two sero-types which have been identified as Massachusetts and Connecticut. There is the possibility that a third sero-type may exist.[59, 61]

Antibiotics have no effect on the virus.[21] Egg-adapted virus is inactivated in three minutes by 1 per cent solutions of phenol, tincture of metaphen, formalin; 25 per cent ethyl alcohol; 5 per cent sodium hydroxide; 1:1,000 mercuric chloride and 1:10,000 potassium permanganate.[15]

Polylysine inhibits the infectivity of IBV for chicken embryos if injected prior to inoculation of the virus. The inhibiting effect diminishes significantly with the virus in high egg-passage or completely egg-adapted virus. It has been suggested that the mechanism of inhibition of viral infectivity by polylysine is perhaps due to its combination with the acidic groups of the protein component of the virus particles. The decrease in the inhibiting effect of polylysine on virus in high egg-passage may be considered to be due either to the modification of surface pattern of the virus or to increased virulence of the virus for chicken embryos.[47]

Beta-propiolactone in a final concentration of 0.025 per cent at 37° C., inactivates IBV in two hours as measured in chicken embryos, without destroying its immunogenicity for chickens.[13]

Maximum stability of IBV at 4° C., is at pH 7.8.[16] The isoelectric point is probably at pH 4.05. The density ranges from 1.13 to 1.16 in glycerine and sucrose solutions.[8]

THERMAL INACTIVATION

Considerable information about the kinetics of thermal inactivation of animal viruses has been obtained in recent years. Such studies are of interest because they provide very meaningful information about the basic properties of the virus. Information usually sought from studies of inactivation are: the relationship of infectivity to antigenicity; structural relationship of components and various chemical groups of virus particle; and homogeneity.

Based on experiences with bacteria, bacterial viruses, plant viruses, and the fact that animal viruses are largely composed of protein, it is usually assumed that inactivation of animal viruses by heat and other physical agents follows a first-order reaction. Any deviation from such a course is to be considered in the light of inhomogeneity, aggregation and impure preparations of virus, and possibly technical error. Recent studies with animal viruses, however, have shown marked deviations from a first-order reaction. Such deviations are being viewed as a reflection of a phenomenon common to animal viruses rather than an exception.

There are only a few reports that thermal inactivation of animal viruses is according to a first-order, at different temperatures. The accumulating evidence in

favor of the non-exponential behavior of the thermal inactivation rate of animal viruses has stimulated considerable interest for review and re-evaluation of earlier work. Bronson and Parker[7] reported that inactivation of myxoma virus at 50° and 55° C., is exponential. Fenner[34] reached a similar conclusion with two strains of vaccinia virus. Lauffer, Wheatley and MacDonald[65] reported that thermal inactivation of influenza A virus is interpretable by a first-order reaction. Kaplan[62] pointed out that Fenner[34] and Lauffer et al.,[65] did not continue their experiments to the point where heterogeneity might be observed. Under such circumstances, the validity of the conclusion that these vaccinia and influenza viruses were inactivated in an exponential manner may remain doubtful until further work is done to obtain complete inactivation of the virus.

Bourdillon[6] reported that inactivation of a partially purified mouse brain preparation of columbia SK strain of poliomyelitis virus at 49.5°, 56.5° and 63° C., did not follow a first-order kinetics. There was no conclusion as to the possible reason of this behavior.

Lauffer and Carnelly[64] and Scott and Lauffer[87] showed that thermal destruction of influenza A virus hemagglutinin did not proceed according to first-order kinetics. The data best fit to a three-halves order reaction, which is indicative of a bimolecular reaction. Lauffer and Wheatley[67] re-interpreted the data and showed that the overall reaction on a semilogrithmic plot appeared to consist of two first-order reactions with different slopes. On this basis, they concluded that slow and fast reacting hemagglutinin particles were present, each of which was inactivated according to a first-order reaction.

Bachrach _et al._,[2] studied the inactivation of foot-and-mouth disease virus, type A, strain 119, at temperatures from 4 to 61° C. Inactivation curves at 55° and 61° C showed bimodal patterns similar to the thermal destruction of influenza hemagglutinin. They suspected that this was due to the heterogeneity of the heat sensitivity of the virus. Inactivation at temperatures below 49° C., followed a first-order reaction. Activation energies calculated from the Arrhenius plot of results obtained below 43° C., and above 43° C., were found to be 27.2 and 120.6 K calories/mole, respectively. It was concluded that loss of infectivity by heat may be due to two different processes.

Kaplan[62] reported similar experiences at 50° and 60° C., with the Lister Institute strain of vaccinia virus adapted to the chick embryo. It was concluded that the virus was heterogeneous with respect to its heat stability and represented a mixture of heat sensitive and heat resistant virus particles. The fast inactivation rate of heat sensitive virus particles was temperature dependent, and that of the slow inactivation of resistant survivors was independent of the temperature. Heat resistant virus isolated from a single pock lesion did not differ in its thermal inactivation rate from that of the original virus.

Youngner[98] found that 11 strains of poliomyelitis virus, representing three immunologic types, were not inactivated at 50° C., according to a first-order. The thermal inactivation curves indicated heterogeneity of the heat sensitivity of the virus. Heat resistant virus isolated from a single plaque was more thermostable than of the parental population. Serial passage of the virus in monkey kidney cell culture had no effect on the inactivation rate. The various strains of virus at 36.5° C., were inactivated at identical exponential rate. No resistant variants could be isolated. It was concluded that different mechanisms are responsible for inactivating the virus at the two temperatures.

Page[25] studied inactivation of the Beaudette egg-adapted strain of IBV at 4°, 22°, 37°, and 56° C., and concluded that the rate was exponential with the activation energy for inactivation being of the order of 24 K calories/mole at 40° C.

Singh[88] found that inactivation of 10 strains of IBV at 56° C., proceeded in a two-component fashion. The reaction was considered to be complex and it was assumed that IBV as used in this study represented a mixture of thermolabile and thermostabile virus particles, each of which was inactivated according to a first-order. The thermolabile particles were identified as Derivative (D) phase and thermostabile particles as Original (O) phase.

The D phase particles were considered to be a modified form of the O particles as the result of adaptation to cultivation in the embryo. The O phase particles were those which had not been modified and had retained their original characteristics.

Thermal inactivation of strains of IBV diluted 10^{-2} in nutrient broth with 20 per cent horse serum followed a first-order reaction. This was considered to be due to stabilization of the virus particles by serum proteins. As a result of this, the virus particles were uniformly inactivated and differences in the heat sensivities were not exhibited.[88]

The interesting feature of the thermal inactivation of animal viruses thus far reported has been the similarities of the mode of inactivation rates at different temperatures. Interpretations of the observed deviations from a first-order reaction, in general, have been pointed to the heterogeneity of the heat sensitivity of the virus particles. This may be due to either mutation, host induced modification, or some other factors.

Woese[97] utilized the data of Kaplan[62], Youngner[98], and Bachrach *et al.*,[2] and attempted to postulate a unified hypothesis to explain the complex nature of thermal inactivation of animal viruses at different temperatures. He hypothesized that thermal inactivation of animal viruses is primarily due to the structural destruction of nucleic acid. The conclusion was that nucleic acid is capable of existing in two interconvertible viable forms and inactivation occurs by different mechanisms at low and high temperature ranges: (1) the collapse mechanism involving the secondary structures of nucleic acid, associated with high activation energy; and (2) the chain break mechanism involving a single site of the nucleic acid associated with low activation energy. At 50° C. or above both mechanisms operate with the collapse mechanism predominating. As a result two-component fashion curves are obtained. At 40° C. or below, the chain break mechanism predominates, giving a single component type of inactivation. This hypothesis excludes the possibility that virus particles are heterogeneous with respect to heat sensitivity as has been reported with animal viruses at high temperatures.

The parallel loss of infectivity and serologic specificity at high temperatures has also been explained by Woese[97] on the basis of his hypothesis. He stated that at high temperatures nucleic acid is inactivated by the collapse mechanism; therefore, the protein is readily denatured as a result of loss of its stabilization by the nucleic acid. At low temperatures, nucleic acid is inactivated by a single-point chain break mechanism still capable of stabilizing the protein against denaturation. The serologic properties of a virus are directly associated with its protein component; therefore, any drastic change in protein component, such as denaturation, will destroy antigenicity.

FORMALDEHYDE INACTIVATION

The virucidal activity of formaldehyde is well known and has been extensively employed for inactivation of viruses in vaccines. Only within the last decade has the kinetics of inactivation of viruses by formaldehyde received much attention for interpretation of some of the important functional and structural properties of viruses. At the present time, the kinetics of inactivation of some animal viruses remains controversial and no uniformity of agreement has been reached as to the mode of action of formaldehyde.

REACTION OF FORMALDEHYDE ON VIRUSES

Formaldehyde is used as formalin which is approximately a 37 per cent concentration by weight of the gas. In aqueous solution, formaldehyde exists in many hydrated forms but predominantly as methylene glycol ($HO-CH_2-OH$) which seems to react with viral particles.[46, 93]

The virucidal activity of formaldehyde has been assumed to be due to its reaction with various protein groups. In case of tobacco mosaic virus (TMV) Cartwright and Lauffer[10] suggested that the virus was inactivated as the result of formation of methylene bridges between amino and sulfhydryl groups of protein. Fraenkel-Conrat[44] found that inactivation of TMV occurred even after all the reactive amino and sulfhydryl groups were blocked by acetylation and oxidation, respectively. He also found that formaldehyde can react with nucleic acid in the intact TMV. Nucleic acid showed a greater affinity than did proteins for formaldehyde. From this, he suggested that the loss of functional activity of TMV was associated with inactivation of ribonucleic acid (RNA) by formaldehyde.

Since the findings that infectivity is chiefly a property of the nucleic acid of viruses,[42, 46, 48] it is generally considered that loss of infectivity occurs as the result of some irreversible chemical reaction of nucleic acid with formaldehyde. Staehelin[89] found that the RNA of TMV is very sensitive to formaldehyde, and that two different reactions take place: (1) the greater part of the formaldehyde is reversibly bound to RNA and can be removed by dialysis, and (2) the smaller fraction of formaldehyde is irreversibly bound and cannot be removed by dialysis. The relative amount of irreversibly bound formaldehyde increased directly with time of the reaction. He suggested the possibility of a cross-linking between RNA molecules based on the increase in its molecular weight after the reaction.

The reaction sites of nucleic acid have not been studied extensively, but it is considered that the amino groups of purine and pyrimidine bases, and the hydroxy groups of pentose and secondary groups of phosphoric acid, are the most probable reactive sites.[41, 45, 46]

The reaction sites on viral protein are considered to be amino, imino, sulfydryl, hydroxyl groups, peptide linkages, and several ring structures. The reactions are often initially reversible but later become irreversible, and usually involve the formation of methylene bridges between reactive groups giving rise to new ring structures. The overall effect is the decrease in the permeability, charge, and solubility resulting in chemical inertia of the protein coat.[37, 38, 39, 40, 45, 46, 94]

KINETICS OF INACTIVATION

Ross and Stanley[82] reported that inactivation of TMV by formaldehyde followed a first-order reaction over most of the course. Fischer and Lauffer[36] and Cartwright et al.,[11] reached similar conclusions. Ross and Stanley[82] also mentioned that a much longer time is required to obtain complete inactivation than would be indicated from extrapolation of data. This indicated that the inactivation rate probably deviated from the first-order course in the later stages of the reaction.

Keogh[63] reported inactivation of vaccinia virus by formaldehyde as being of a first-order reaction. The validity of the conclusion appears somewhat doubtful. Gard[45] using Keogh's data showed that four of the five experiments exhibited marked deviations from a first-order reaction.

Lauffer and Wheatley[66] studied PR8 influenza A virus and concluded that inactivation by formaldehyde was probably that of a first-order reaction. Gard[43] reported the possibility of the inactivation of influenza A virus not being of a first-order reaction.

Salk et al.,[83] in their several experiments found a first-order reaction for inactivation of three strains of poliomyelitis virus by formaldehyde. On the contrary, Timm et al.,[90] Wesselén et al.,[96] Lycke,[69] Lycke et al.,[70] and Schaffer[85] have reported that formalin inactivation of poliomyelitis virus is not according to a first-order reaction.

According to Bachrach et al.,[2] foot-and-mouth disease virus was inactivated by formaldehyde in an exponential manner. Wesselén and Dinter[95] found that inactivation showed a deviation from a first-order reaction.

As is apparent from review of the literature the controversial nature of the formaldehyde inactivation of animal viruses has aroused a wide interest to determine the exact nature of the process. This is particularly true with poliomyelitis virus because the safety factor supposedly encompassed in the process of vaccine production is based on the first-order kinetics of formaldehyde inactivation of the virus. Salk et al.,[83] originally attributed the deviation from a first-order reaction of formaldehyde inactivation to the lack of pretreatment of the virus by filtration and adequate mixing with formalin. As a result, the suspension was considered to contain virus aggregates and extraneous protein material which would prevent direct contact between the virus and formaldehyde. Gard and Lycke[44] and Timm _et al._,[90] showed that filtration and mechanical homogenization of the virus suspension had no effect on the course of inactivation. Using a highly purified preparation of poliomyelitis virus, Schaffer[85] also found that inactivation by formaldehyde did not follow a simple kinetics.

Gard[43] stated that deviation from a first-order reaction in the formaldehyde inactivation of poliomyelitis virus is not caused by a decrease in concentration of formaldehyde in the reaction mixture. Although a slight decrease of formaldehyde seemed to occur, the deviation could not be accounted entirely by such a small loss.

An explanation as to the cause of the deviation has been sought in genetically-conditioned-heterogeneity among virus particles. Gard and Lycke[44] stated that this hypothesis does not seem very probable. They tried without success to establish formalin resistant variants.

Gard[43, 46] proposed a theory to explain the deviation from the simple first-order reaction in all cases of chemical inactivation of viruses. According to his theory, the action of formaldehyde on nucleic acid, which is responsible for infectivity, depends upon the ability of formaldehyde to penetrate the protein coat. The rate of inactivation can be assumed to depend upon the rate of penetration by formaldehyde. The rate of penetration, in turn, will depend upon the state of protein coat. As the reaction of formaldehyde with protein progresses, the protein becomes progressively less permeable and chemically inert leading to a decrease in the rate of penetration by formaldehyde. Under these conditions, inactivation of nucleic acid cannot be expected to follow a simple reaction rate. There seems to be some experimental support to his theory. Schaffer[85] from studies using C^{14} found that the uptake of formaldehyde by poliomyelitis virus was not a simple reaction. The rapid initial uptake was followed by a prolonged period with a linear increase.

MATERIALS AND METHODS

The seven strains of IBV employed were selected because they represent a fairly wide geographical distribution in this country, and have been used previously for inactivation studies.

Of the seven strains, six are maintained at North Central Repository, Michigan State University, and are identified as follows:

REPOSITORY CODE NO.	ORIGIN	ISOLATED BY
3	Iowa, 1947	M. S. Hofstad, Ames, Iowa
17	New Jersey, 1956	F. R. Beaudette, New Brunswick, N. J.
19	Alabama, 1956	C. S. Roberts, Auburn, Alabama
40	Michigan, 1956	C. H. Cunningham, East Lansing, Mich.
41	Massachusetts, 1941	H. Van Roekel, Amherst, Mass.
42	New Jersey, 1936	F. R. Beaudette, New Brunswick, N. J.

The seventh strain was received from Anchor Serum Laboratories, St. Joseph, Missouri, and with the designation of A-3-1. This strain was isolated originally by J. F. Crawley, in Canada.

The repository strains are referred to by code number and egg-passage. For example, 3-11 indicates strain 3, 11th egg-passage. The Beaudette egg-adapted strain, 42, does not have the egg passages enumerated. This strain has been through hundreds of passages but the exact number is unknown. All strains were passaged in eggs at least once a month. Single comb white Leghorn embryonating chicken eggs 10 or 11 days old were used. An inoculum of 0.1 of a 10^{-2} or 10^{-3} dilution of virus per egg was employed via allantoic cavity. With the exception of the Beaudette egg-adapted strain 42, allantoic fluids were harvested 36 to 48 hours post-inoculation. In the case of the Beaudette strain, the allantoic fluid was harvested 24 to 30 hours after inoculation. Allantoic fluid was collected from about 10 to 20 eggs per passage and only from living embryos. The fluids were pooled and stored at -30° C. At the time of use, the frozen allantoic fluid was thawed at room temperature and centrifuged at 3,500 r.p.m. for 20 minutes at 4° C. The clear supernatant fluid was removed and dispensed in 2 ml portions in 12 x 75 mm tubes, which were kept in an ice bath.

TITRATION OF INFECTIVITY

Serial ten-fold dilutions of the virus were prepared using Difco nutrient broth, pH 7.2, as the diluent.

Five eggs were inoculated per dilution. The eggs were incubated and candled once daily. Embryos dead within 24 hours post-inoculation were not included in determinations of fifty per cent end points as the deaths were considered to be due to non-specific causes. At the end of the sixth or eighth day, all living embryos were examined for gross lesions characteristic of those produced by infectious bronchitis virus, such as stunting and curling of embryo with deformed feet compressed over the head, and urates in the mesonephros.[68] The criteria for positive responses were mortality and gross lesions of the embryo. The embryo infective $dose_{50}$ (e.i.$d._{50}$) was calculated by Reed and Muench method.[81]

THERMAL INACTIVATION

1. DETERMINATION OF THERMAL INACTIVATION

All samples were incubated simultaneously in a water bath at 56° C. After two minutes of preheating, the time which had previously been determined for the virus to equilibrate at 56° C., samples were removed at predetermined time intervals, chilled immediately in an ice bath, and the virus was titrated for infectivity. None of the samples was stored for titration at a later period.

2. ISOLATION OF C PHASE VIRUS FROM O-D POPULATION

Isolation of C phase virus was attempted by the selective thermal inactivation method based on the differences in heat sensitivities of C and D phase viruses in a mixed population at 56 C. When a mixed population, O-D, of IBV is heated at 56 C., a bimodal rate of inactivation results. The faster inactivation is a property of the D phase virus. Extrapolation of the D phase inactivation rate to the abscissa indicates the time at which D phase would be expected to be completely inactivated. Using this information, the mixed population may be heated for this predetermined period and C phase virus, because of its greater thermostability, may be isolated from the mixture.

Each O-D mixture of virus was incubated at 56 C., for the time previously determined by extrapolation. The virus was removed from the water bath, chilled, diluted ten-fold, and inoculated into eggs. The allantoic fluid was harvested 36 to 48 hours after inoculation, pooled and frozen.

The inactivation rate of the harvested virus was determined at 56 C. The criterion for the isolated O phase was an exponential inactivation similar to that of the C phase in the respective O-D population prior to selective separation.

3. MAINTENANCE OF O PHASE VIRUS IN EGG-PASSAGE

The limiting dilution technique was employed to maintain O phase virus in serial egg-passage. The strains in O phase were passaged in the dilution next lower to that in which fifty per cent end point occurred. The e.i.d.$_{50}$ of the inoculum was determined prior to each of the first five passages. For subsequent passages, a 10^{-5} dilution of the virus was employed, because in the first five passages the e.i.d.$_{50}$ ranged from 10^{-6} to $10^{-6.5}$. Embryos were inoculated and incubated for 48 to 60 hours. They were chilled overnight and the allantoic fluid was harvested, pooled and frozen. After the 5th, 10th, and 13th egg-passages, thermal inactivation rates were determined at 56° C.

The O phase egg-passage level for each strain is designated as follows: 3-12 (O_{13}). This indicates the 13th passage of O phase isolated from strain 3-12.

4. REPRODUCTION OF BIMODAL PATTERN OF THERMAL INACTIVATION BY MIXING O PHASE VIRUS AND D PHASE VIRUS (BEAUDETTE EGG-ADAPTED STRAIN)

The O phase viruses isolated and maintained in serial egg-passage were mixed with the Beaudette strain in different proportions by volume. By reason of the complete adaptation of the Beaudette strain in embryo culture, and its extreme thermal sensitivity as shown by complete inactivation within 10 minutes at 56° C.,[75] at a linear rate, this strain is considered to consist of D phase virus only. The e.i.d.$_{50}$ of the strains in O phase, and the Beaudette strain, were always determined prior to mixing. The mixtures were shaken vigorously, transferred in 2 ml portions to 12 x 75 mm test tubes, incubated at 56° C., and removed at certain intervals. The samples were immediately cooled in an ice bath and titrated for infectivity.

5. DETERMINATION OF ANTIGENICITY OF O PHASE VIRUS FOR CHICKENS

Five groups of two, six-week-old chickens each, were placed in individual Horsfall-Bauer isolation units. After two weeks, the chickens were bled by cardiac puncture for pre-infection sera. Sera from individual chickens were pooled in equal portions for each group. One week later, the chickens of each group were inoculated intranasally with virus, 0.2 to 0.3 cc per chicken, as indicated below:

Group	Strain
1	3-21
2	3-12 (O_{13})

3	19-12 (O_{13})
4	19-21
5	Control

The chickens were observed daily for signs of infection. Sera were collected four weeks after inoculation. Neutralization tests using the decreasing virus-constant serum method,[22] five eggs per dilution, were performed on the pooled pre-infection and post-infection sera, using the Beaudette strain as the antigen.

6. <u>GROWTH RATES OF O PHASE VIRUS</u>

Two preparations of strain 3-12 containing O-D phases were used: (1) undiluted virus, and (2) virus diluted 10^{-2} in nutrient broth. These preparations, 2 ml, were incubated at 56° C., for 30 and 40 minutes, respectively. The period for the undiluted virus had been previously determined by extrapolation of inactivation rates as the time at which D phase virus should have inactivated. With the diluted virus, the 30 minute period was arbitrarily selected as it was reasoned that a more rapid inactivation would occur with the lesser concentration of virus. Each preparation was used to inoculate 50 eggs. At 12 hour intervals, five eggs were removed, chilled, the virus harvested, and frozen. Infectivity titrations were performed later.

FORMALDEHYDE INACTIVATION

PREPARATION OF VIRUS SAMPLES

The frozen allantoic fluid suspension of virus was thawed at 4° C., for about 6 to 8 hours. This procedure was followed because it had been observed that the amount of precipitate formed upon thawing was relatively more following prolonged thawing at 4° C. than at room temperature for a shorter period of time. The allantoic fluid was centrifuged at 3,500 r.p.m. for 30 minutes at 4° C. The supernatant fluid was removed and mixed with an equal volume of phosphate buffer. The pH of such mixtures ranged from 7.4 to 7.5.

PHOSPHATE BUFFER

Sodium phosphate buffered saline, 0.02 M with respect to phosphate, and 0.15 M with respect to NaCl, was used.

FORMALDEHYDE SOLUTION

Formalin, Baker's reagent grade, 36.2 per cent HCHO, was employed to prepare 1:20 and 1:40 dilutions of formalin in sterile distilled water. A sufficient amount of diluted formalin was added to the virus to give final concentration of 1:2,000 and 1:4,000 formalin in the mixture. Fresh dilutions of formalin were prepared for each experiment.

DETERMINATION OF FORMALIN INACTIVATION

Two procedures were employed:

(1) The virus samples, in phosphate buffered saline at pH 7.5, were distributed in 40 or 50 ml quantities into each of two 4 ounce prescription bottles with screw caps. The bottles were placed in a water bath at 37° C. When the temperature of the virus preparations reached 37° C., as determined by thermometry, an initial sample of 5 ml was withdrawn and immediately placed in an ice bath. This was considered to be the control for the virus at zero time before the addition of formalin. Formalin was then added to one bottle of the virus at 37° C., and mixed thoroughly. The other bottle was used as the control. To prevent evaporation of formaldehyde, the bottles were kept tightly closed. At certain time periods, 5 ml samples of virus were removed, and mixed with sufficient amount of sodium bisulfite to provide a molarity equal to that of the formalin in the virus samples to neutralize the free formaldehyde. This was accomplished by adding 0.1 ml containing 1.56 mgm of $NaHSO_3$ to each 5 ml sample of the virus treated with

1:4,000 formalin. These samples were kept in the ice bath until the last sample of virus was removed from the 37° C. water bath. The total period for reaction of formaldehyde with virus at 37° C. varied from 3 to 7 hours depending upon the particular experiment. The samples were then dialyzed against two changes of phosphate buffer at 4° C., for 20 to 24 hours and then titrated for infectivity.

The dialyzing unit consisted of a Visking cellulose dialyzing tubing 24/32 inches in diameter and 3 to 4 inches long. One end of the tubing was tied to form a bag and the other end was tied around one end of a glass tube which passed through the center of a rubber stopper. Phosphate buffer, 60 to 70 ml, was poured into a 32 x 200 mm culture tube and the rubber stopper with the cellulose bag was placed in the tube. The open end of the glass tube was plugged with cotton and the entire unit was sterilized in the autoclave. The virus sample was introduced into the cellulose bag through the glass tube and dialyzed for 10 to 12 hours. The cellulose tube was then transferred to another culture tube containing fresh sterile phosphate buffer solution and dialyzed for another 10 to 12 hours.

(2) This procedure differed from the first one in that the samples consisted of 20 ml in 30 ml screw

cap vials. At each interval, 2 ml samples were withdrawn and immediately placed in an ice bath. No sodium bisulfite was added to neutralize free formaldehyde and the samples were not dialyzed. Chilling alone was relied upon to suppress the reaction of formaldehyde at each sampling period.

Controls were treated in the same manner except for the addition of formalin. No attempts were made to keep the pH of the virus-formalin mixture constant, although it was observed that pH had increased by 0.2 to 0.3 units by the end of the reaction period. No significant loss of infectivity and change of volume of virus samples was observed as a result of dialysis alone and handling of the virus during the experimental procedures.

RESULTS

The results of the effect of 56° C. on IBV, 3-12, 19-12, 40-16, 41-8, 17-35, and A-3-1, are given in table 1, and plotted in figure 1 through 6.

All six strains were inactivated in a two component fashion. The overall kinetics was considered to consist of two consecutive first-order reactions representing: (1) the rapid inactivation of thermolabile D phase, and (2) the slow inactivation of thermostabile O phase. The reaction-rate constants (k) for inactivation of O and D phases, respectively, are given in table 2, as derived from the formula: $\log V°/V = -kt$, where V° is the initial concentration of the virus at time 0, and V is virus concentration at time, t.

The D phase constitutes from 98.75 to 99.92 per cent of the O-D population of the strains used. For calculation of O/D, the inactivation line for O phase was extrapolated to the ordinate to determine the log concentration of O phase. Using antilogs, the number of infective D phase particles was determined by subtracting the number of O phase particles from the total O - D mixture. The O/D and the per cent D phase particles in the O - D mixture were then calculated, and are presented in table 3.

TABLE 1

INACTIVATION AT 56° C. OF INFECTIOUS BRONCHITIS VIRUS

Time of exposure in min.	log $_{10}$ embryo infective dose$_{50}$ of virus					
	strain					
	3-11	19-11	40-16	17-35	41-8	A-3-1
0	7.3	7.5	7.5	7.5	7.2	6.2
5	-	-	-	3.2	3.5	3.3
10	-	-	-	3.0	2.3	2.4
15	4.5	3.0	4.7	2.0	0.8	1.5
20	-	-	-	1.4	0.0	0.7
25	-	-	-	0.6	-	0.0
30	3.8	2.4	4.0	-	-	-
45	2.6	1.0	3.7	-	-	-
60	1.5	0.2	2.7	-	-	-
75	-	-	1.8	-	-	-

FIGURE 1

INACTIVATION AT 56 C. OF INFECTICUS BRONCHITIS VIRUS, 3-12

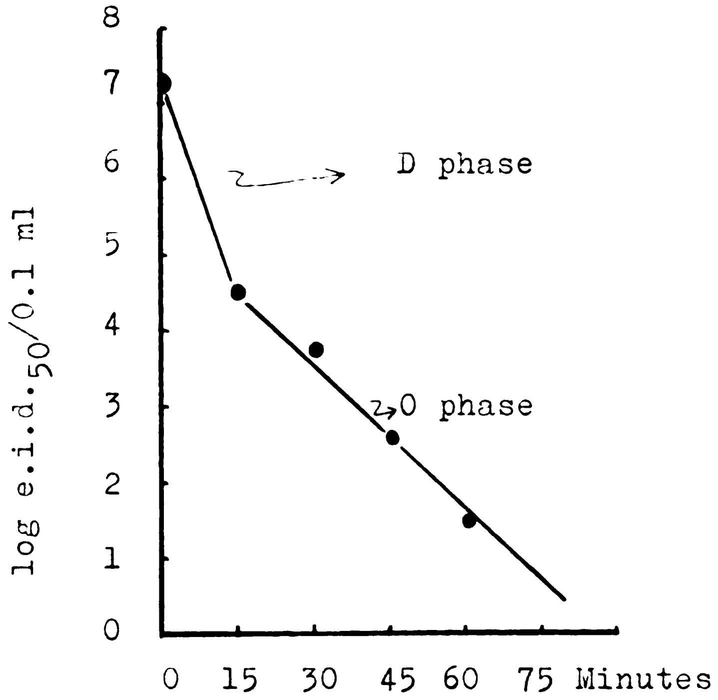


FIGURE 2

INACTIVATION AT 56 C. OF INFECTIOUS BRONCHITIS VIRUS, 19-12

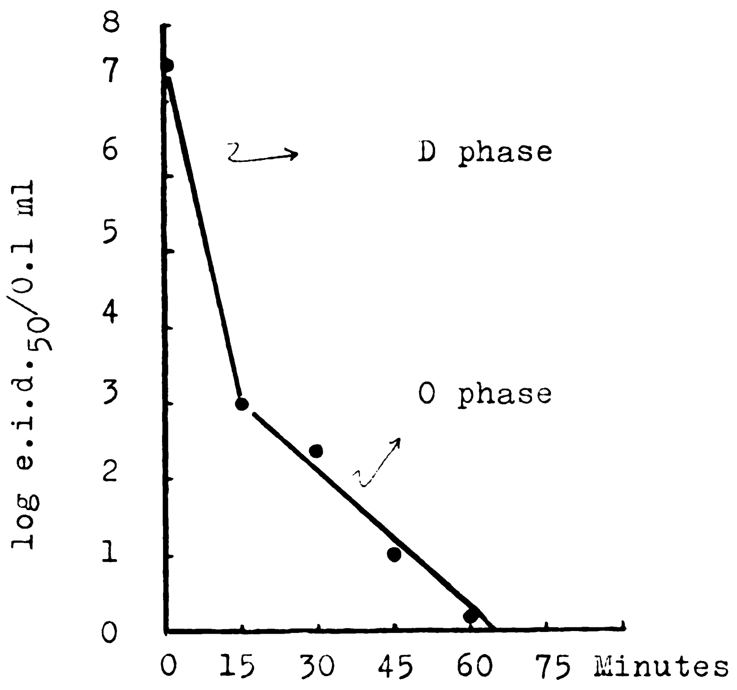


FIGURE 3

INACTIVATION AT 56 C. OF INFECTIOUS BRONCHITIS VIRUS, 40-16

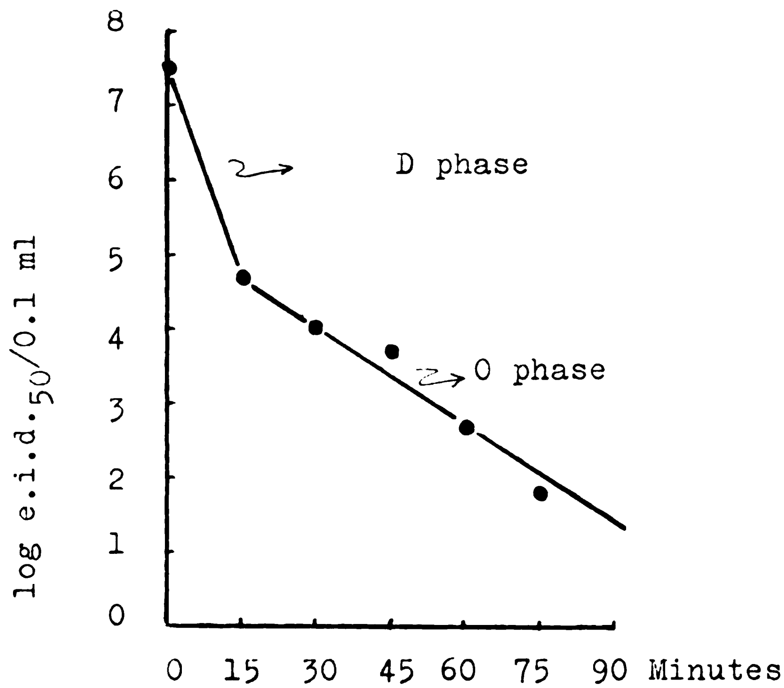


FIGURE 4

INACTIVATION AT 56 C. OF INFECTIOUS BRONCHITIS VIRUS, 41-8

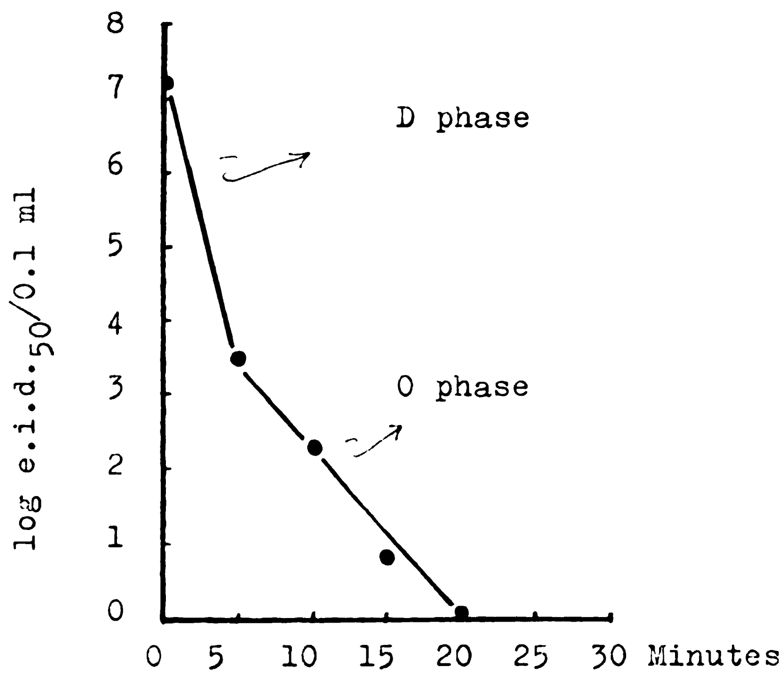


FIGURE 5

INACTIVATION AT 56 C. OF INFECTIOUS BRONCHITIS VIRUS, 17-35

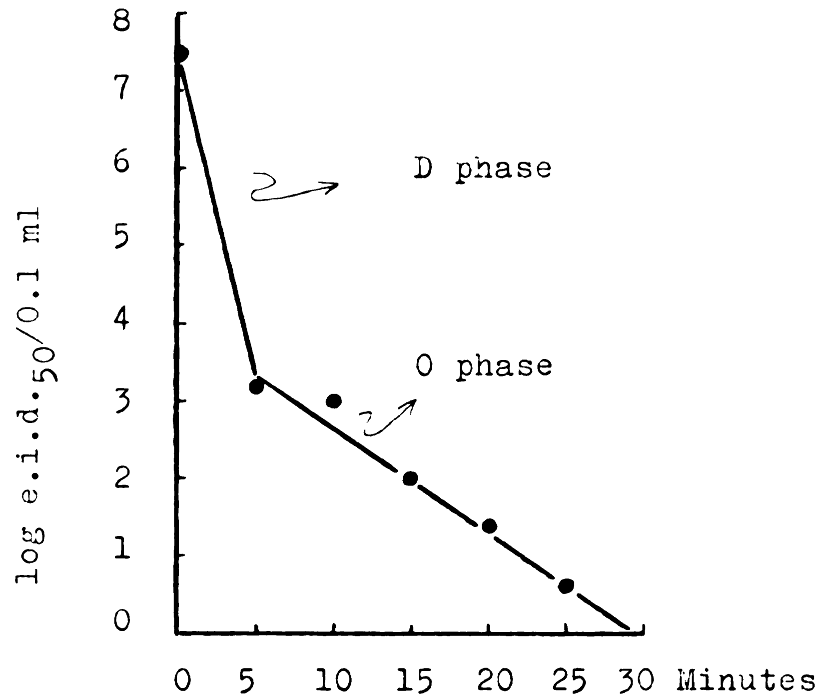


FIGURE 6

INACTIVATION AT 56 C. OF INFECTIOUS BRONCHITIS VIRUS, A-3-1

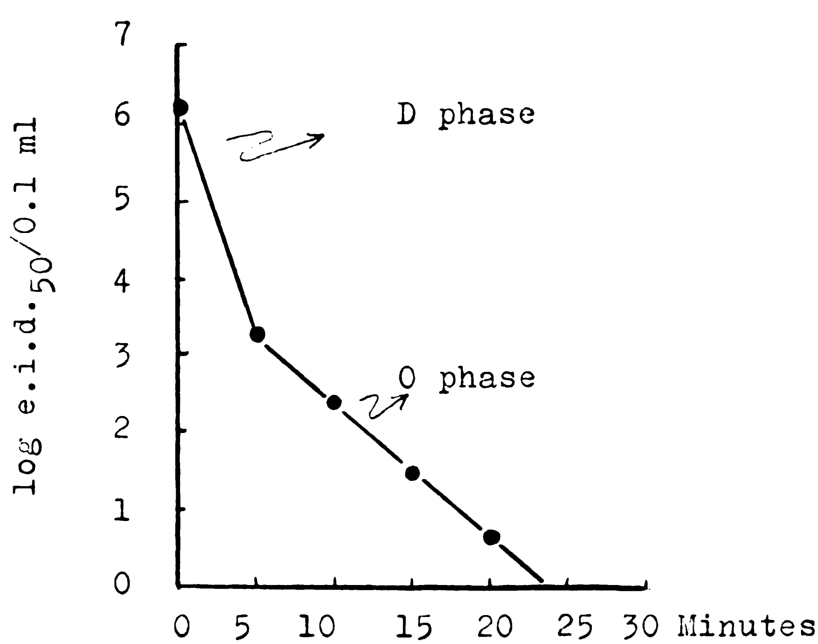


TABLE 2

REACTION-RATE CONSTANTS (k) FOR INACTIVATION OF O AND D PHASES OF INFECTIOUS BRONCHITIS VIRUS IN O - D POPULATION

Strain	k minute^{-1}	
	O phase	D phase
3-12	0.062	0.186
19-12	0.060	0.300
40-16	0.043	0.193
17-35	0.144	0.840
41-8	0.233	0.740
A-3-1	0.174	0.580

TABLE 3

RATIO OF O/D AND PER CENT D PHASE IN O - D POPULATION OF INFECTIOUS BRONCHITIS VIRUS

Strain	O/D	%D
3-12	1/78.4	98.75
19-12	1/3980.2	99.97
40-16	1/157.5	99.46
17-35	1/2508.4	99.92
41-8	1/500.6	99.86
A-3-1	1/99.0	99.00

Comparison of the inactivation rates for strains 3, 19, and 40 of different egg-passages obtained in this study and as reported previously indicate identical patterns. The only difference, with the exception of strain 40, lies in the proportions of O and D phases. Strains 3-12, and 19-12 contained more D phase than did the 3-6 and 19-7, respectively,[88] which indicates that D phase increases in the population with each egg-passage.

ISOLATION OF THERMOSTABLE O PHASE VIRUS

The six strains whose inactivation rates had been previously established (table 1), were used. The time obtained by extrapolation, and the time found effective for selective inactivation of D phase to permit recovery of O phase from the mixed O-D population of different strains of IBV, is shown in table 4.

TABLE 4

THE TIME AT 56 C., FOR INACTIVATION OF D PHASE FROM O - D POPULATION OF IBV

Strain	*Time obtained by extrapolation	*Time found effective
3-12	40	60
19-12	25	25
40-16	41	-
17-35	10	10
41-8	10	15
A-3-1	10	20

* minutes

In the case of 19-12 and 17-35, the time obtained by extrapolation, 25 and 10 minutes, respectively, was effective for inactivation of the D phase. With 3-12, 41-8 and A-3-1, a considerably longer time than that determined by extrapolation was required to inactivate D phase, as shown in table 4. No success was achieved in three attempts to isolate O phase from the mixed population of strain 40-16. Two attempts were made by subjecting 40-16 to 56 C. for 41 and 60 minutes, respectively. For the third attempt, the virus heated for 41 minutes was used to inoculate embryos and the harvested virus was heated at 56 C. for 60 minutes.

The results of the thermal inactivation of O phase in the first embryo passage after isolation from the mixed population of 3-12, 19-12, 17-35, 41-8, and A-3-1 are presented in table 5. In figures 7 through 11, the data are plotted and fitted by the linear regression equation of the least squares method. The reaction-rate constants for the isolated O phase (k_o) and for the O phase of the respective O-D population (k_p) are shown in table 6.

The reaction-rate constants for the isolated O phases and for O phase in their respective O-D populations were exponential and of a similar magnitude. This indicates that the inactivation rate of O phase is

TABLE 5

INACTIVATION AT 56° C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS IN THE FIRST EGG-PASSAGE (O_1)

Time of exposure in min.	$\log_{10}$ embryo infective dose_{50} of virus strains				
	3-12(O_1)	19-12(O_1)	41-8(O_1)	17-35(O_1)	A-3-1(O_1)
0	6.5	6.2	7.0	6.3	6.0
5	-	-	5.5	5.2	4.8
10	-	-	4.3	4.0	4.2
15	5.6	5.5	3.6	3.6	2.7
20	-	-	2.5	3.4	2.3
25	-	-	-	3.2	-
30	4.5	4.0	-	2.5	-
45	3.5	3.7	-	-	-
60	4.2	3.5	-	-	-
75	2.5	2.6	-	-	-

FIGURE 7

INACTIVATION AT 56 C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, 3-12, IN THE FIRST EGG-PASSAGE (O_1)

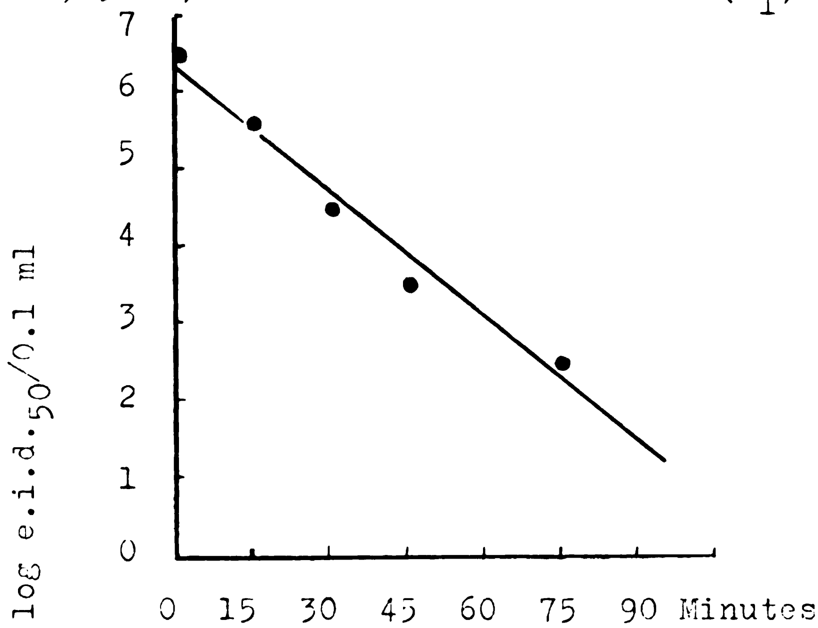


FIGURE 8

INACTIVATION AT 56 C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, 19-12, IN THE FIRST EGG-PASSAGE (O_1)

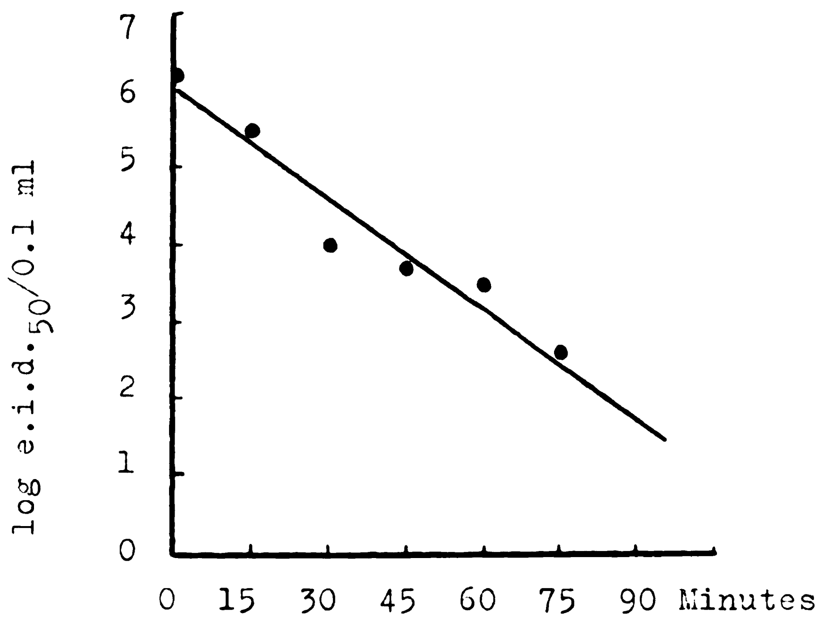


FIGURE 9

INACTIVATION AT 56 C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, 41-8, IN FIRST EGG-PASSAGE (O_1)

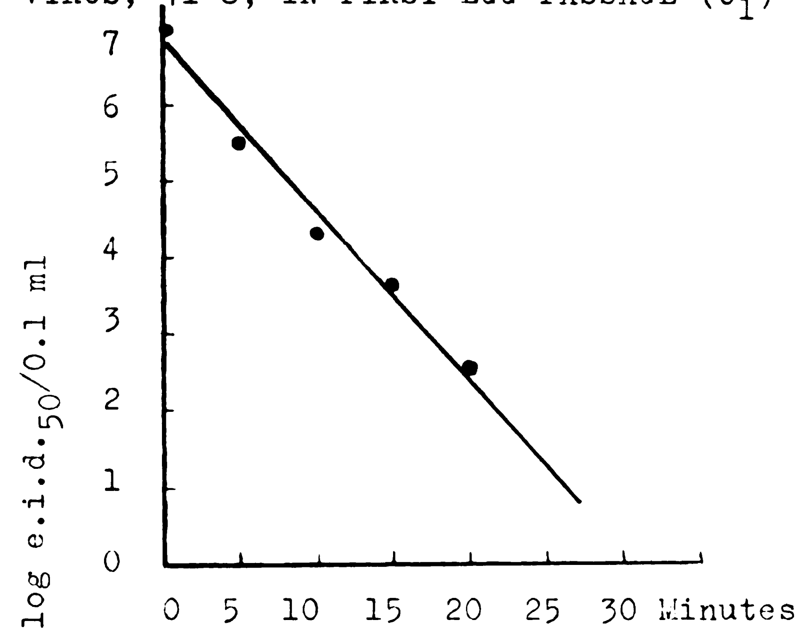


FIGURE 10

INACTIVATION AT 56 C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, 17-35, IN FIRST EGG-PASSAGE (O_1)

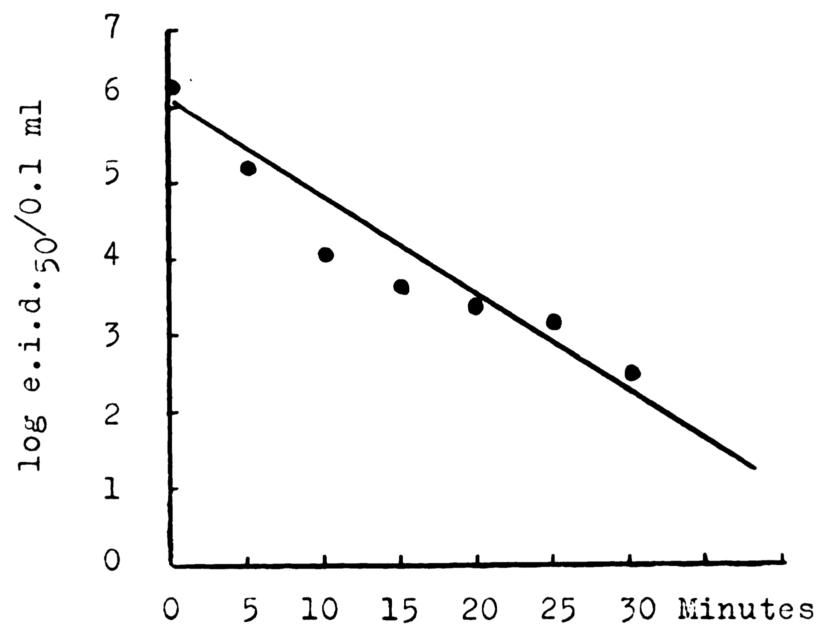


FIGURE 11

INACTIVATION AT 56 C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, A-3-1, IN THE FIRST EGG-PASSAGE (O_1)

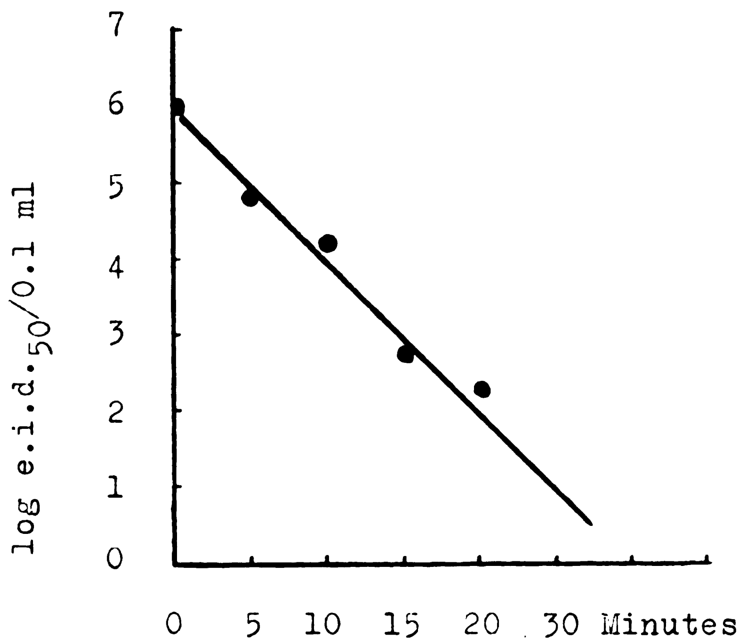


TABLE 6

SPECIFIC-REACTION RATE CONSTANTS FOR O PHASE IN FIRST EGG-PASSAGE VIRUS (k_{01}) AND FOR O PHASE OF THEIR POPULATION (k_p)

Strains	k_{01} minute^{-1}	k_p minute^{-1}
41-8	0.22	0.23
A-3-1	0.19	0.17
17-35	0.13	0.14
3-12	0.05	0.06
19-12	0.05	0.06

independent of the initial concentration of the virus and that the isolated O phase and the O phase in the mixed population of a strain have the same thermal sensitivities at 56 C.

MAINTENANCE OF O PHASE VIRUS IN EGG-PASSAGE

The data for inactivation of O phase of 3-12 and 19-12 in the 5th, 10th, and 13th egg-passages are presented in table 7. In figures 12 and 13, these data and those for first egg-passage (fig. 7 and 8) are plotted as log V°/V versus time, to obtain a single point of origin for comparison of the inactivation rates at different egg-passages.

The O phase of 3-12 and 19-12 in the 5th, 10th, and 13th egg-passages were inactivated according to a first-order reaction. However, it was found that the reaction-rate constants tend to increase directly with successive egg-passages, as shown in table 8.

As shown in table 7, and figure 14, the O phase of 41-8 could not be maintained in serial egg-passage. At the 10th passage the virus had reverted to the bimodal characteristic of the original O-D population.

TABLE 7

INACTIVATION AT 56° C. OF O PHASE FROM IBV, 3-12, 19-12 and 41-8 AT DIFFERENT EGG-PASSAGES

Time of exposure in min.	$\log_{10}$ embryo infective dose_{50} of virus						
	3-12(O_5)	19-12(O_5)	3-12(O_{10})	19-12(O_{10})	41-8(O_{10})	3-12(O_{13})	19-12(O_{13})
0	6.3	6.2	5.5	6.4	7.3	6.7	6.3
5	-	-	-	-	3.5	-	-
10	-	-	-	-	2.5	-	-
15	5.5	5.2	4.6	4.7	2.0	5.0	4.7
20	-	-	-	-	1.2	-	-
30	4.3	4.2	3.5	3.2	-	3.7	2.3
45	3.4	3.0	3.0	2.5	-	2.3	1.8
60	2.4	2.5	-	1.5	-	1.2	0.3

TABLE 8

REACTION-RATE CONSTANTS OF O PHASE AT DIFFERENT EGG-PASSAGES

Number of egg-passages	K, $minute^{-1}$	
	3-12	19-12
O_5	0.07	0.06
O_{10}	0.06	0.08
O_{13}	0.09	0.10

FIGURE 12

INACTIVATION AT 56 C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, 3-12 AT DIFFERENT EGG-PASSAGES

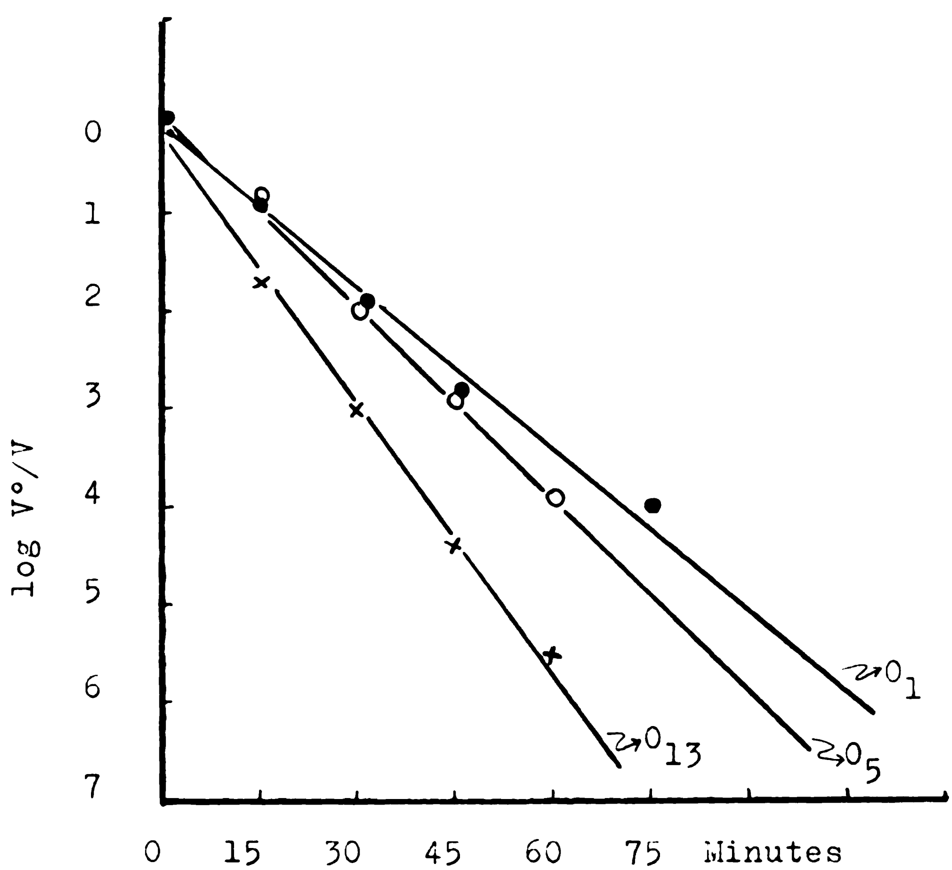
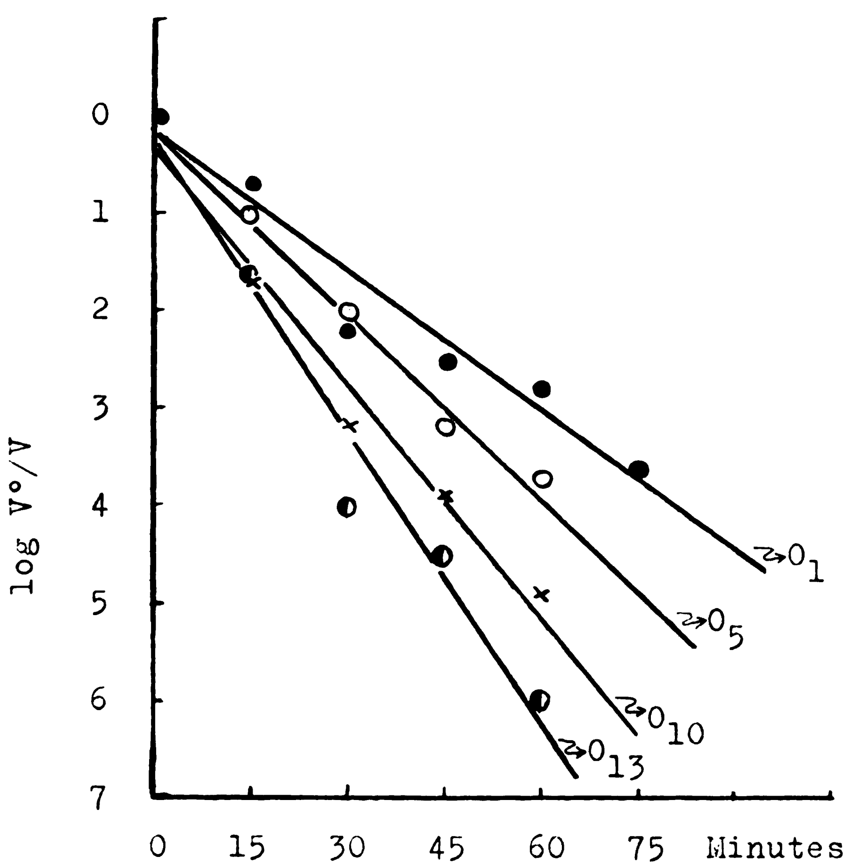


FIGURE 13

INACTIVATION AT 56 C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, 19-12, AT DIFFERENT EGG-PASSAGES



REPRODUCTION OF BIMODAL PATTERN OF THERMAL INACTIVATION

The O phase of 19-12 in the 6th egg-passage and of 3-12 in the 10th and 12th egg-passages were mixed with the Beaudette strain (D phase) in different proportions by volume. No attempt was made to obtain a definite O/D in the mixture.

The thermal inactivation data presented in tables 9, 10 and 11, and plotted in figures 15, 16, 17, indicate that the mixtures were inactivated according to a bimodal rate, which is characteristic of the O - D population of IBV. The inflection between the fast and slow inactivation rates occurs at 10 minutes, which indicates that the majority of the D phase particles were inactivated in the mixture within 10 minutes.

No controls for individual thermal inactivation rates of O phase and the D phase were performed. The O phase virus was always employed in the egg-passage immediately above or below that for which the exponential thermal inactivation rate had been determined. The Beaudette strain, being completely egg-adapted, is fixed in its properties, including thermal sensitivity, and would not be influenced by additional egg-passages.

TABLE 9

INACTIVATION AT 56 C. OF MIXTURE OF 3-12(O_{10}) AND BEAUDETTE STRAIN (D PHASE) IN PROPORTION OF 3:1 BY VOLUME

Time in minutes	$\log_{10}$ embryo infective dose_{50} of virus
0	6.5
15	3.0
30	1.5
45	0.2
60	0.0

TABLE 10

INACTIVATION AT 56 C. OF MIXTURE OF 3-12(O_{12}) AND BEAUDETTE STRAIN (D PHASE) IN PROPORTION OF 1:3 BY VOLUME

Time in minutes	$\log_{10}$ embryo infective dose_{50} of virus
0	5.2
5	4.0
10	2.3
15	2.2
30	1.5
45	0.5
60	0.0

TABLE 11

INACTIVATION AT 56 C. OF MIXTURE OF 19-12(O_6) AND BEAUDETTE STRAIN (D PHASE) IN PROPORTION OF 1:1 BY VOLUME

Time in minutes	$\log_{10}$ embryo infective dose_{50} of virus
0	6.6
10	3.8
15	3.5
30	2.6
45	1.4
60	0.2

FIGURE 14

INACTIVATION AT 56 C. OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, 41-8, AT 10th EGG-PASSAGE, (O_{10})

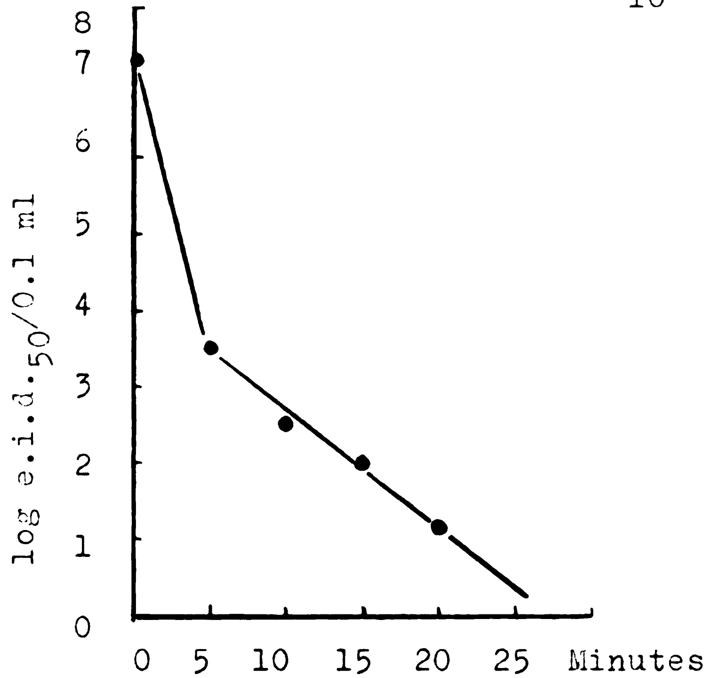


FIGURE 15

INACTIVATION AT 56 C. OF MIXTURE OF IBV, 3-12 (O_{10}) AND BEAUDETTE STRAIN (D PHASE) IN PROPORTION OF 3:1 BY VOLUME

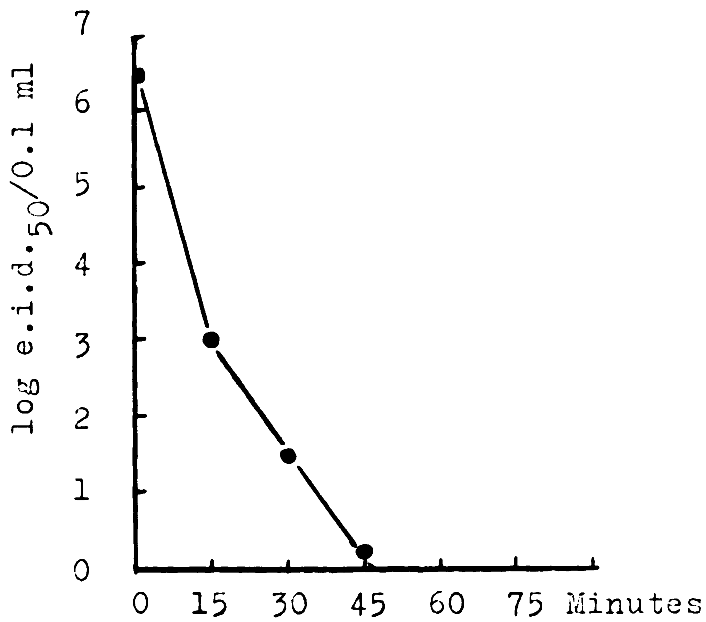


FIGURE 16

INACTIVATION AT 56 C. OF MIXTURE OF IBV, 3-12 (O_{12}) AND BEAUDETTE STRAIN (D PHASE) IN PROPORTIONS OF 1:3 BY VOLUME

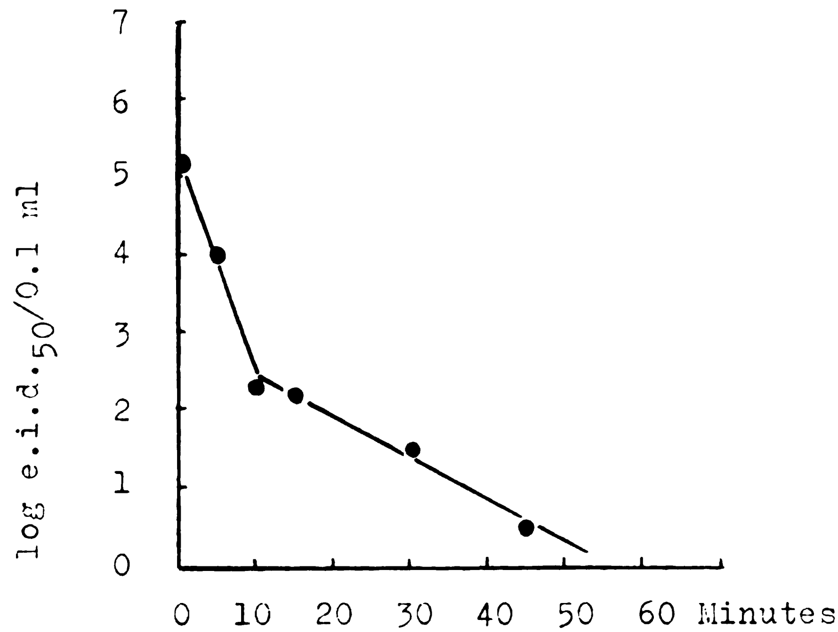
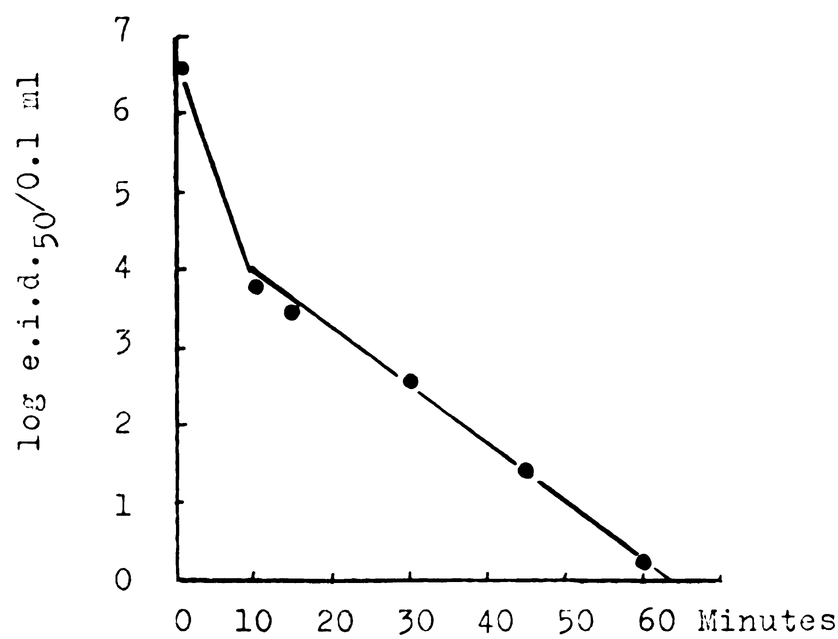


FIGURE 17

INACTIVATION AT 56 C. OF MIXTURE OF IBV, 19-12 (O_6) AND BEAUDETTE STRAIN (D PHASE) IN PROPORTIONS OF 1:1 BY VOLUME



DETERMINATION OF ANTIGENICITY OF O PHASE VIRUS

The infectious dose$_{50}$ neutralizing indexes (e.i.d.$_{50}$NI) of pre-infection and post-infection sera from the chickens inoculated with O phase and the respective O - D mixtures of two strains IBV, are presented in table 12.

TABLE 12

ANTIBODY RESPONSE OF CHICKENS TO O PHASE AND THE RESPECTIVE O - D POPULATION OF INFECTIOUS BRONCHITIS VIRUS

Groups of chickens	Strains used for inoculation	e.i.d.$_{50}$NI of pre-infection sera	e.i.d.$_{50}$NI of post-infection sera
1	3-21 (O-D)	0.1	2.8
2	3-12 (O_{13})	0.8	$\geq$ 3.8
3	19-21 (O-D)	1.1	$\geq$ 3.8
4	19-12 (O_{13})	0.1	$\geq$ 3.8
5	Control	0.4	0.3

In all cases the e.i.d.$_{50}$NI of pre-infection sera was within the range of the values for sera from normal chickens. The post-inoculation sera showed significant increases in e.i.d.$_{50}$NI.

The chickens inoculated with 19-12 (O_{13}) and 19-21 developed severe respiratory disturbance, and one chicken receiving 19-12 (O_{13}) died. All others recovered after about a week. Mild signs of infection were observed with chickens inoculated with 3-12 (O_{13}) and 3-21, without any mortality.

GROWTH RATE OF O PHASE

The growth rates of O phase contained in 3-11 undiluted and diluted 10^{-2}, which were exposed to 56 C., for 40 and 30 minutes, respectively, are presented in table 13 and figure 18. The maximum concentration of the virus using undiluted inoculum occurred at the 36th hour. With diluted inoculum the maximum concentration occurred at 48th hour. In both cases, concentration of the virus declined after the 48th hour. Similar results have been reported with IBV in early egg-passage.[54] The multiplication of O phase was not interfered with by heat inactivated D phase virus which was present in undiluted inoculum. The maximum titer do not appear to be entirely dependent upon the concentration of the virus in the inoculum. This is evidenced from the fact that the undiluted and diluted inocula contained $10^{3.0}$ and $10^{0.8}$ e.i.d.$_{50}$, respectively, and the maximum titers for both were essentially the same.

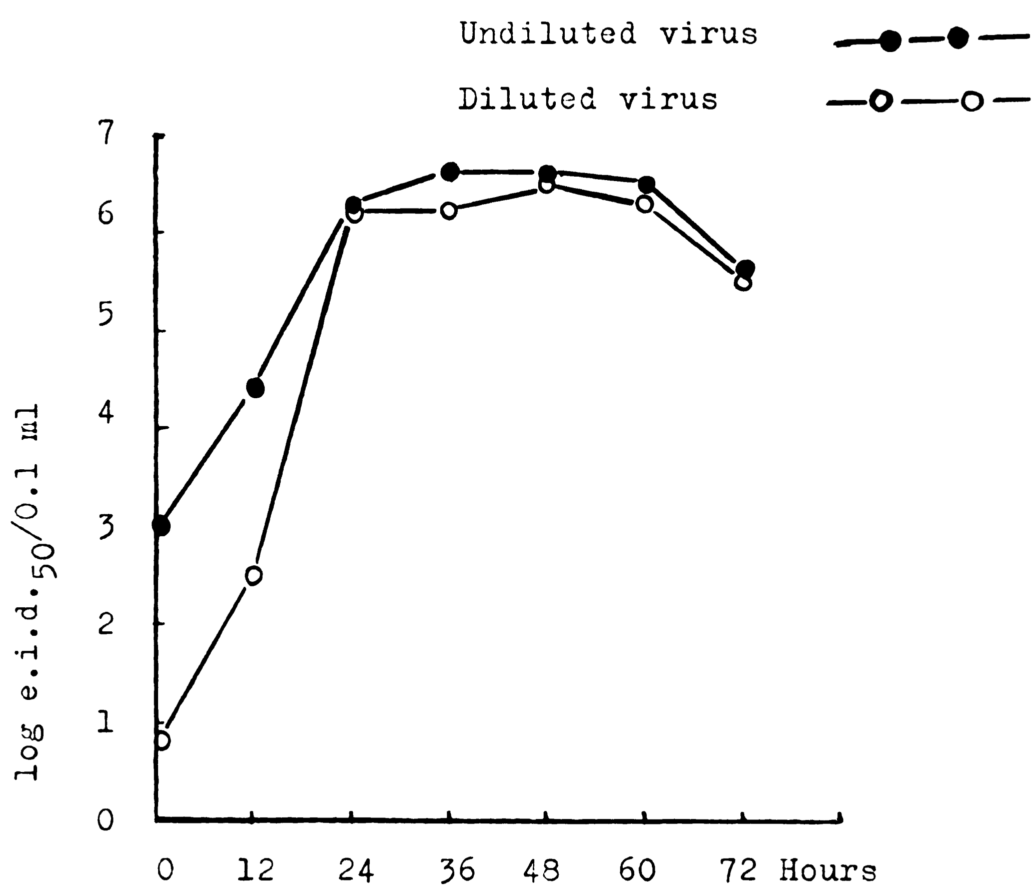
TABLE 13

GROWTH RATE OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS, 3-11

Time in hours	$\log_{10}$ embryo infective dose_{50} of virus: Undiluted 3-11, 40 minutes at 56 C.	$\log_{10}$ embryo infective dose_{50} of virus: 10^{-2} dilution of 3-11, 30 minutes at 56 C.
0	3.0	0.8
12	4.4	2.5
24	6.3	6.2
36	6.6	6.2
48	6.6	6.5
60	6.5	6.3
75	5.6	5.5

FIGURE 18

GROWTH RATE OF C PHASE OF INFECTIOUS BRONCHITIS VIRUS, 3-11



FORMALIN INACTIVATION

The results of inactivation of IBV, 19-21, 40-26, 41-18 and 3-21, 19-21, 40-26, 41-18, 42 (Beaudette egg-adapted strain) by 1:2,000 and 1:4,000 formalin, respectively, at pH 7.5 and 37° C. are presented in tables 14 and 15 and figures 19, 20, 21 and 22.

There was a slight residual infectivity at 6 hours for those strains which were treated with 1:4,000 formalin. This represented as much as 7.0 log units of virus which were inactivated. With the control, not more than one log unit reduction of infectivity occurred during the 6 hour period, illustrating that inactivation was due to formalin and not to heat. Strains 3-21, 19-21, 40-26, and 41-18, were not inactivated according to a first-order reaction. Inactivation, which was curvilinear, was rapid in the initial stage but gradually and progressively decreased with time. The viruses were inactivated at a faster rate by 1:2,000 formalin than by 1:4,000 formalin, but the pattern was essentially the same in both cases.

The Beaudette egg-adapted strain was inactivated by 1:4,000 formalin according to first-order reaction, as shown in table 15 and figure 23. However, the possibility of deviation from the course in the later stages of the reaction may not be entirely excluded because the inactivation was not carried out to the base line. The loss of infectivity due to thermal inactivation at 37° C., amounted to 1.5 log units in three hours, which was considerably higher than that found with the other strains.

The results with 3-12 (O_{14}) and 19 (O_{14}) by 1:4,000 formalin, table 16, and figures 24 and 25, indicate that although inactivation did not follow a first-order reaction, the rate was more exponential and more prolonged than that for the respective O-D populations.

The procedure of neutralization of formalin by $NaHSO_3$ and subsequent dialysis of the virus-formalin mixture was introduced after the majority of the experiments were completed and no direct comparison of this procedure, and that of rapid freezing of the virus-formalin mixture to stop the formalin reaction could be made. Despite this, the results show that with either procedure the pattern of inactivation was essentially the same. The former procedure would be more desirable because of the quantitative neutralization of formalin by $NaHSO_3$.

TABLE 14

INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS BY 1:2,000 FORMALIN* AT pH 7.5, AND 37° C.

Time of exposure in minutes	$\log_{10}$ embryo infective dose_{50} of virus		
	19-21	40-26	41-18
0	6.7	6.3	7.3
10	5.4	-	-
15	-	5.8	-
20	5.2	-	-
30	4.2	-	5.5
45	4.0	4.0	-
60	3.5	-	3.8
75	3.2	3.4	-
90	2.4	-	3.5
105	2.0	2.4	-
120	-	-	1.8
140	-	2.0	-
150	-	-	1.8
180	-	-	1.3
210	-	-	0.4
240	-	-	0.0

* Formalin was not neutralized with Na HSO_3, and the samples were not dialyzed, except in the case of 41-18.

TABLE 15

INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS BY 1:4,000 FORMALIN* AT pH 7.5, AND 37° C.

Time of exposure in hours	$\log_{10}$ embryo infective $dose_{50}$ of virus									
	Strains									
	3-21		19-21		40-26		41-18		42	
	*F	C*	F	C	F	C	F	C	F	C
0	5.5	-	6.8	-	6.5	-	7.4	-	5.8	-
0.25	-	-	-	-	5.7	-	-	-	5.3	-
0.5	-	-	-	-	5.3	-	-	-	5.0	-
1.0	-	-	5.7	-	5.0	6.5	4.8	-	4.3	5.5
1.5	-	-	-	-	4.6	-	-	-	3.5	-
2.0	2.5	5.5	3.4	6.8	3.8	6.2	3.6	6.8	3.4	4.6
2.5	-	-	-	-	3.2	-	-	-	2.4	-
3.0	1.6	-	2.0	-	2.6	6.3	2.0	-	1.8	4.5
4.0	0.6	5.5	1.6	6.4	2.3	-	1.5	7.0	-	-
5.0	0.4	-	1.2	-	-	-	1.2	-	-	-
6.0	0.0	5.4	0.7	6.3	-	-	0.4	6.6	-	-

F-with formalin; C-without formalin

* Formaldehyde was neutralized and the samples were dialyzed, except in 40-26.

TABLE 16

INACTIVATION OF O PHASE OF INFECTIOUS BRONCHITIS VIRUS BY 1:4,000 FORMALIN AT pH 7.5 AND 37° C.

Time of exposure in hours	$\log_{10}$ embryo infective dose_{50} of virus			
	Strains			
	3-12 (O_{14})		19-12 (O_{14})	
	F	C	F	C
0	7.5	-	6.6	-
1	6.2	-	5.8	-
2	5.5	7.5	5.0	6.6
3	4.6	-	4.2	-
4	4.2	6.8	3.8	6.7
5	3.7	-	3.3	-
6	3.3	7.0	3.2	6.5

F - with formalin; C - without formalin

Formaldehyde neutralized and dialyzed.

FIGURE 19

INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS, 3-21, BY FORMALIN (F) AT pH 7.5 AND 37 C.

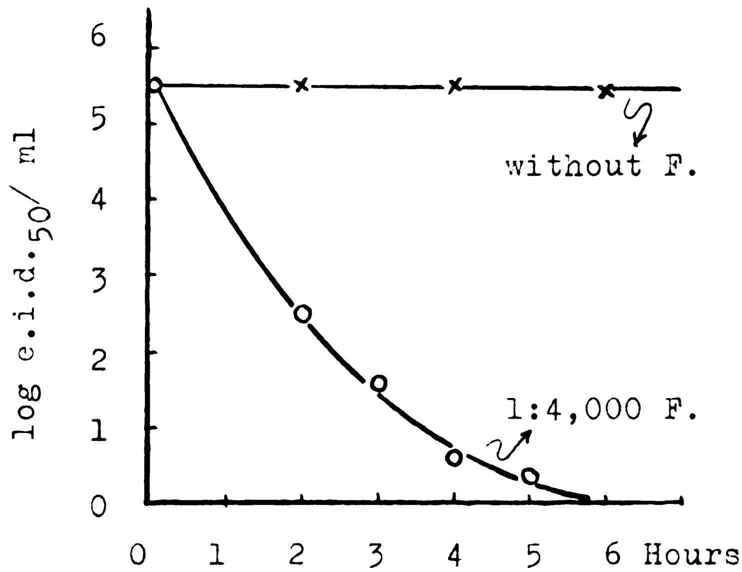


FIGURE 20

INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS, 19-21, BY FORMALIN (F) AT pH 7.5 AND 37 C.

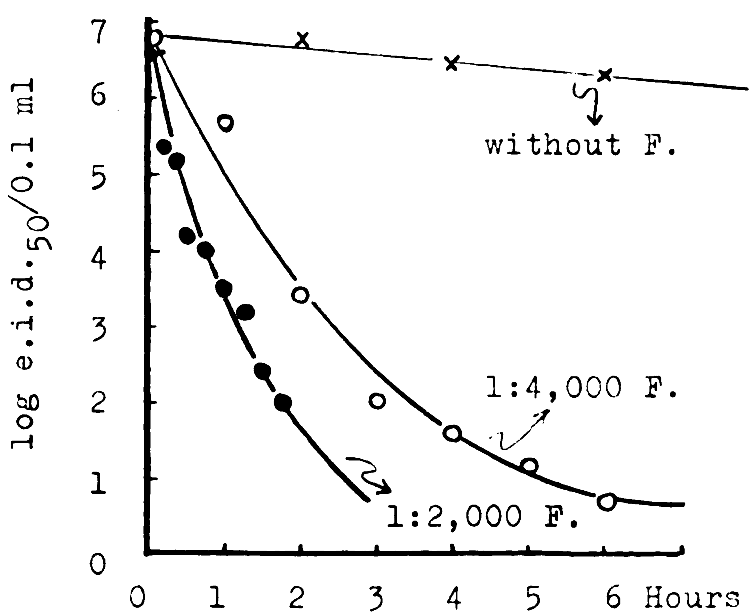


FIGURE 21

INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS, 40-26, BY FORMALIN (F) AT pH 7.5 AND 37 C.

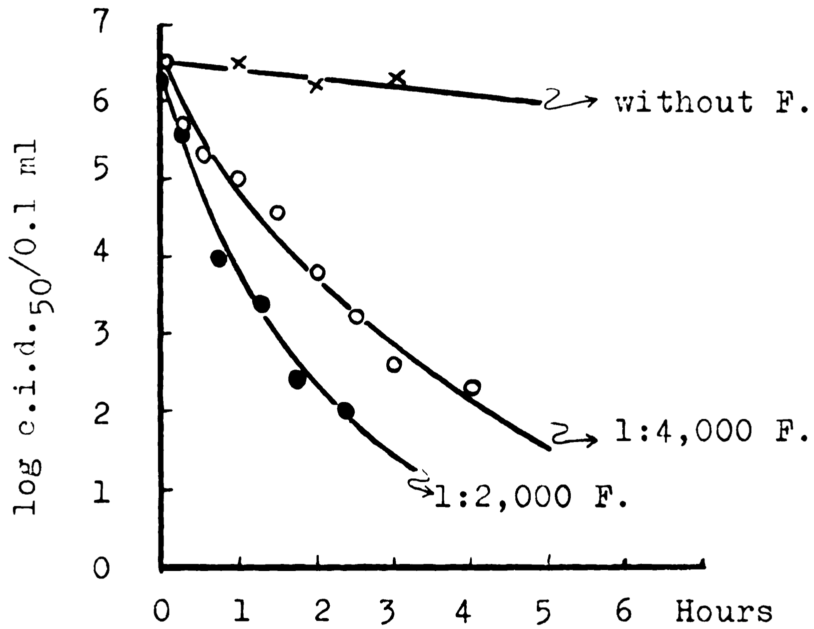


FIGURE 22

INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS, 41-18, BY FORMALIN (F) AT pH 7.5 AND 37 C.

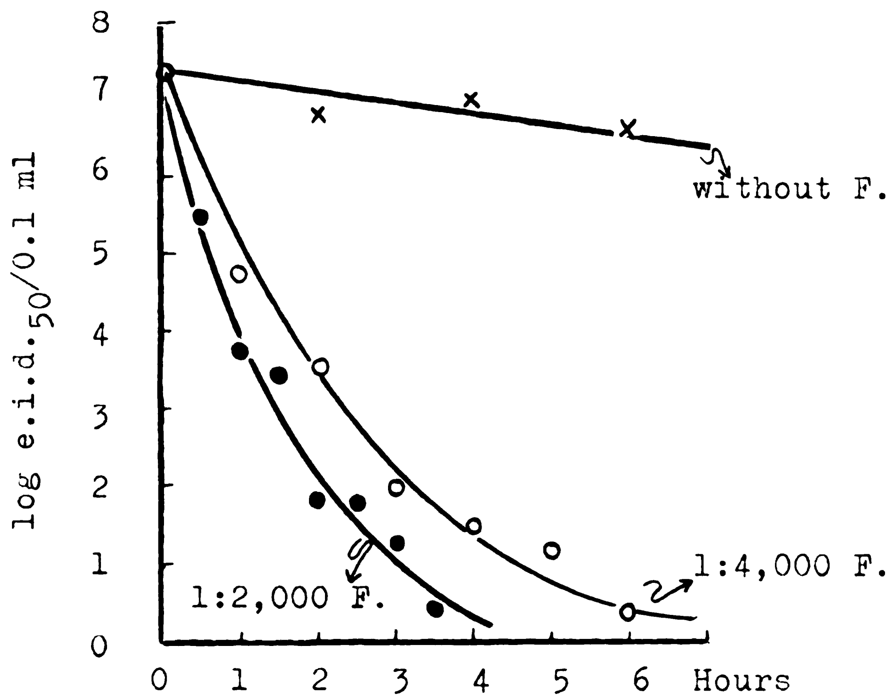


FIGURE 23

INACTIVATION OF BEAUDETTE EGG-ADAPTED STRAIN OF IBV, 42, BY FORMALIN (F) AT pH 7.5 AND 37 C.

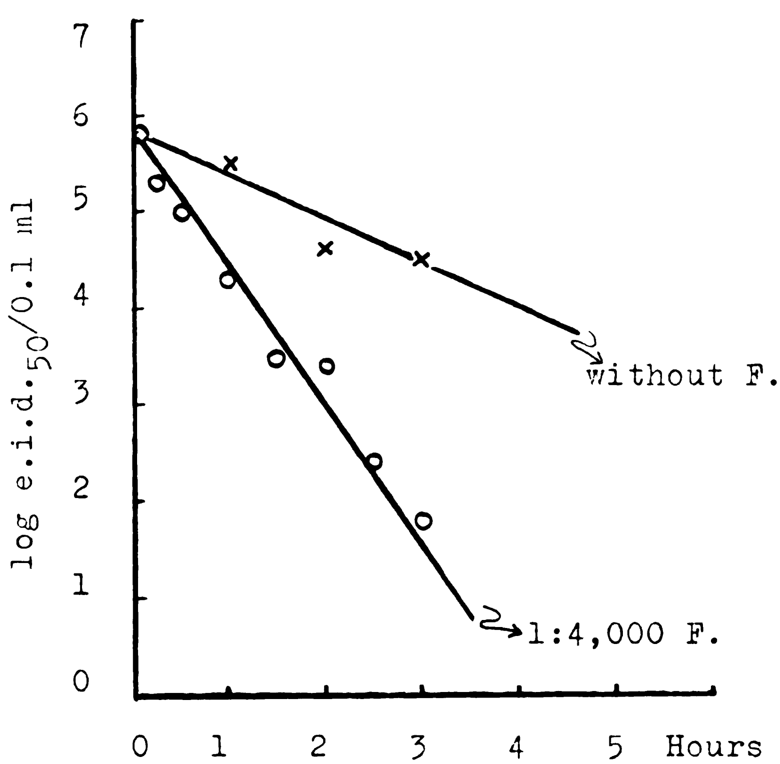


FIGURE 24

INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS, 3-12 (0_{14}), BY FORMALIN (F) AT pH 7.5 AND 37 C.

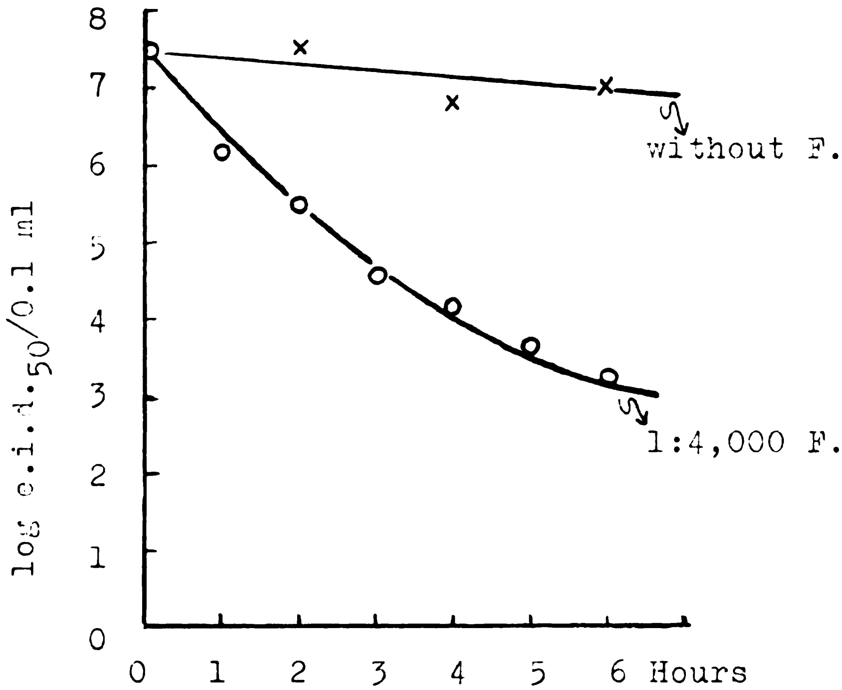
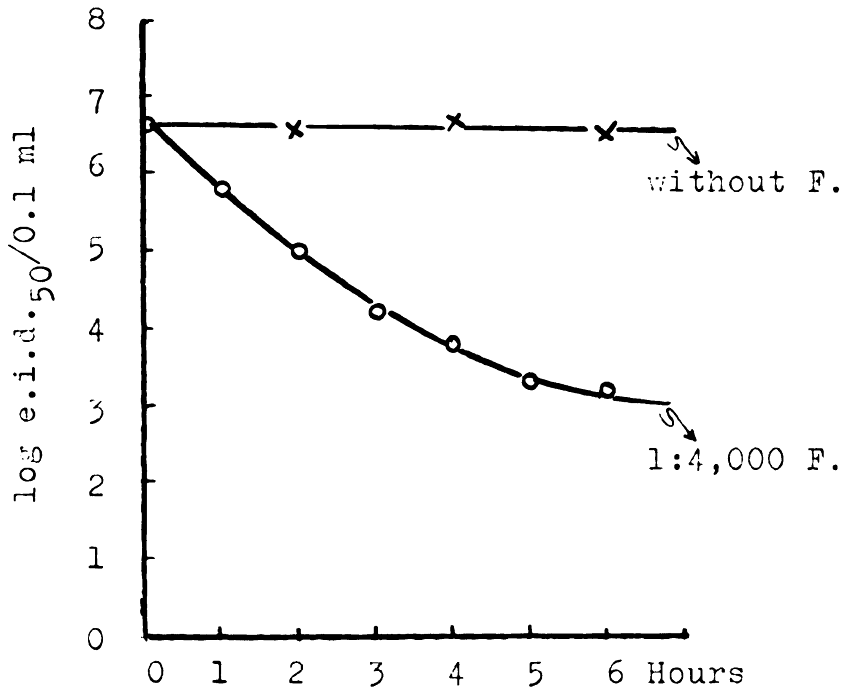


FIGURE 25

INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS, 19-12 (0_{14}), BY FORMALIN (F) AT pH 7.5 AND 37 C.



DISCUSSION

THERMAL INACTIVATION

The successful isolation of O phase of IBV by the differential inactivation of the thermolabile D phase from an O-D population led to the first and major step in substantiating the assumption, earlier proposed on the basis of bimodal thermal inactivation rates, that IBV in early egg-passage exists in two phases.

When isolating O phase, it was found that with certain strains a considerably longer time was required to inactivate D phase than would be anticipated on the basis of the time obtained by extrapolation. This may be considered to be due to two possible reasons. (1) Consideration must be given to the fact that at the base line, one infectious dose of virus may be present when calculations are made on logrithmic basis since 10^0 equals 1. All e.i.d.$_{50}$ calculations were made on the basis of 0.1 ml inoculum. If one e.i.d.$_{50}$ were present in this volume, then, theoretically, 20 e.i.d.$_{50}$ would be contained in the 2 ml samples employed for determinations of thermal inactivation. It would, therefore, be possible that purely by chance distribution, the sampling from the 2 ml of virus would contain one or more infectious doses of D phase virus which could manifest itself through multiplication and infectivity for the embryo. (2) The extent of selective inactivation of thermolabile D phase may be possibly considered to depend upon the ratio of O and D phases (O/D). There seems to be some experimental support for this point of view. From the data in tables 3 and 4, there appears to be a direct relation between O/D and the time required for inactivation of D phase. Strains 19-12 and 17-35 had the highest O/D and O phase was isolated on the basis of the time determined by extrapolation. With strains 3-12 and A-3-1, which had the lowest O/D, more time was required than that obtained by extrapolation.

The inability to isolate O phase from the O-D mixture of 40-26, under the conditions of the study, does not exclude the possibility that longer periods of heating would have completely inactivated D phase and thus permit the isolation of O phase.

The direct relationship of the increase of the reaction-rate constant of O phase with egg passage is an interesting part of this study. An increase in the reaction-rate constant reflects a direct increase in thermolability, which means that the O phase becomes increasingly susceptible to heat with serial egg-passage. This behavior is in agreement with the report by Hofstad[58] that there seems to be a tendency for IBV to become increasingly susceptible to heat with increased egg-passage. The question arises as to why the entire C phase population becomes uniformly more thermolabile at a constant rate with the entire population being affected at the same time. In contrast, the initial modification of C to D is a progressive process where only a portion of the viral population is affected as reflected by the increasing bimodal type of thermal inactivation with serial egg-passage.

This situation may possibly be explained on the basis that dilutions of the order of 10^{-5} of the C phase virus were employed as inoculum for serial egg-passages. The titer of the virus usually ranged from 10^{6} to $10^{6.5}$ before each egg-passage. Therefore, approximately 10 to 70 e.i.d.$_{50}$ were present in the inoculum. With such a small number of viral particles as compared to about 2×10^{7} cells in chorioallantoic membrane,[9, 91] the virus may undergo repeated multiplication cycles. In so doing, it is probable that all particles are uniformly subjected to the modifying effect of the chicken embryo cells, and as a result an homogeneous population is obtained. However, if undiluted virus is inoculated, about 10^{6} to 7×10^{6} infective doses will be present. In such a case, because of an overwhelming infection with a rapid utilization of all cells, or possibly multiple infection, all virus particles may not be uniformly influenced by the modifying environment of the embryo cells. As a result of this possibility, and probably others, only a part of the population is affected. Findings of somewhat similar nature with influenza virus have been reported by Von Magnus.[93] If influenza virus is passaged undiluted in embryos, a large number of non-infectious particles or "incomplete virus" are produced. If the virus is diluted 10^{-2} or higher for inoculum, standard or homogeneous virus with respect to infectivity is produced. The formation of a high yield of incomplete virus is considered to be due to multiple infection of cells.[60]

From the above findings, it appears that the decreasing thermolability of O phase particles by egg-passage indicates that this process may not be carried on for an unlimited number of passages. Nevertheless, by this technique an homogeneous population of the O phase may be maintained and provide a possible means of studying the rate of modification of IBV in egg-passage.

The study of the mixing of O and D phases in different proportions emphasizes that the bimodal inactivation is characteristic of the heterogeneity of the virus. Particles of at least two thermal sensitivities are represented, each of which is differentially inactivated according to a first-order reaction.

The fact that O phase particles are antigenic and pathogenic for chickens is in agreement with the findings that antigenicity and infectivity of IBV for chickens are directly associated.[25, 27, 57] The D phase particles are known to be non-pathogenic and non-antigenic. The parental O-D population of the O phases tested also possessed antigenicity and infectivity because the O phase was present in a concentration sufficient to initiate infection and to produce antibodies. Since this study was not planned for detailed analyses of the antigenic properties of IBV, the data do not permit quantitative evaluation of antigenicity of the O phases and of the respective O-D populations.

Similarities of the growth rates of O phase and of the O-D population in early egg-passage are in accord with the finding that differences in the growth rates of IBV are apparent only when early egg passage and high egg passage rates are compared. This is because the change from O to D is a gradual process and requires many egg-passages. In early egg-passage, the virus is still in the process of adaptation and the growth characteristics have not been so modified as to differ from O phase in this respect. Consideration must be given to the fact that not all the properties of a virus can be expected to be modified at a similar rate. This will perhaps depend upon the order of their susceptibility to the modifying effect of the host cells and also to the sensitivity of the technique for detection of such changes.

The properties of O and D phases of IBV as shown by this study were characterized primarily on the basis of differential thermal sensitivities. Since there is evidence that the progressive change from O to D is associated with egg-passage, the differential thermal sensitivities are considered to be only one of several properties that are involved. These properties have been described in detail in the review of literature but are presented in summary as follows:

TABLE 17

COMPARISON OF PROPERTIES OF IBV IN EARLY EGG-PASSAGE TO COMPLETELY EGG-ADAPTED IBV

IBV IN EARLY EGG-PASSAGE, O PHASE PREDOMINATING	COMPLETELY EGG-ADAPTED IBV, D PHASE ONLY
1. Virulent and antigenic for chickens.	Non-pathogenic and non-antigenic for chickens.
2. Produces characteristic curling and stunting of embryos. Mortality is low and not a constant finding.	Kills all embryos within 24 to 36 hours with no apparent characteristic gross lesions.
3. Trypsin-modified virus shows hemagglutinating activity.	Trypsin-modified virus shows little or no hemagglutinating activity.
4. Polylysine inhibits the infectivity for embryos.	Polylysine has little effect.

The above and similar properties are probably linked in one way or another with the nucleic acid and protein of all animal viruses. Pollard[77] stated that infectivity and the mechanism of host adaptation are the properties of nucleic acid. The fact that the antigenic properties of a virus reside chiefly in the protein coat is universally accepted. The inhibitory effect of poly-lysine is considered to be due to its combination with the acidic groups of the protein of the virus.[47] The hemagglutinating activity of IBV may be a function of the protein as evidenced by the necessity for trypsinization to unmask reactive sites on the surface of the virus.[14, 71] Thermal inactivation of a virus is considered to be due to two effects of heat: (1) Alteration of the basic structure of the virus particle due to differential expansion of its various parts, resulting in the breakage of hydrogen bonds and the change of the spatial relationship that is necessary to keep the integrity of nucleic acid within the protein coat.

(2) Progressive inactivation with the most sensitive part being affected first leading ultimately to complete denaturation.[77]

In summary, these properties strongly suggest the possibility of changes occurring in IBV as the result of the environmental influence of propagation in chicken embryos. The changes in the character of IBV from O to D phases may not be considered to be due to genetic variations for the following reasons which are based on thermal inactivation studies: (1) The D phase of the virus exists as the majority of the population as early as the 7th or 8th egg-passage. This indicates that the change from O to D with each egg-passage is far more frequent than would be expected if it were a genetically determined variation.

(2) The isolated O phase propagated by serial egg-passage becomes increasingly heat sensitive, indicating a progressive change to D. In addition, the O phase of 41-8 at the 10th egg-passage had reverted to the characteristic of an O-D population as determined by thermal inactivation.

The hypothesis that modification or heterogeneity was due to a protective coating comprised of extraneous material which the virus may have derived from the disintegrated cells of the embryo was not considered probable. If this were the case, the D phase should have been more thermostabile than the O phase, since the coating would protect the D phase against heat. Trypsinized O and D phases do not show the same hemagglutinating activity, which indicates that a protective coating does not exist and that the reactive sites on the two phases are different. As suggested by Gard[43] "this type of heterogeneity would be considered a chance phenomenon, because it is difficult to conceive of any mechanism by which the distribution of coating material would be regulated to such an extent as to provide a virus population of a constant make up from one experiment to another and from type to type."

FORMALIN INACTIVATION

The kinetics of formalin inactivation of 3-21, 19-21, 40-26, and 41-18, as found in this study, is similar to that described by Wesselen et al.,[96] for poliomyelitis virus and by Wesselen and Dinter[95] for foot-and-mouth disease virus.

Thermal and formalin inactivation of IBV appear to have certain features in common. Strains of IBV in early egg-passage containing O and D phases were inactivated by heat according to a bimodal rate. Inactivation by 1:2,000 and 1:4,000 formalin was curvilinear. The Beaudette egg-adapted strain containing D phase only, was inactivated exponentially by heat and 1:4,000 formalin. However, there are some differential features. Unlike thermal inactivation, formalin inactivation of the 14th egg-passage of O phases of strains 19-12 and 3-12, was not exponential. Nevertheless, inactivation of O phase by formalin was more exponential and prolonged than that of the respective O-D population. This would indicate some differences in the sensitivities of the virus with regard to formalin.

Considering these features and also the alteration of many properties of the virus as a result of egg-passage as discussed previously, it seems possible to explain the curvilinear behavior of inactivation of non-completely egg-adapted strains of IBV on the basis of heterogeneity of the virus. The Beaudette strain is considered to be homogeneous D phase and is distinguishable from other strains by many properties. Since there is strong evidence of modification of virus involving certain chemical groups of its protein and nucleic acid components, it is not unreasonable to consider the modification of the groups capable of reacting with formaldehyde. Therefore, there will be a mixture of particles with different sensitivities with regard to formalin.

SUMMARY

1. Inactivation of 6 strains of IBV at 56 C., was bimodal and consisted of two consecutive first-order reactions which were considered to be due to a thermolabile Derivative (D) phase and a thermostabile Original (O) phase. Each phase was inactivated according to a first-order reaction.
2. The O phases isolated from O-D populations of 5 of 6 strains by differential inactivation of D phase at 56 C. were inactivated exponentially at 56 C., at a rate similar to that for O phase in the respective O-D populations.
3. The O phases maintained through thirteen serial egg-passages using the limiting dilution technique decreased in thermal sensitivity with increasing egg-passage.
4. The bimodal inactivation at 56 C., exhibited by the O-D populations, was reproduced by mixing isolated O phase with D phase virus (Beaudette egg-adapted strain) in certain proportions by volume.
5. The isolated O phases were antigenic and infective for chickens.
6. The growth rates of isolated O phase were similar to those of O-D populations of IBV in early egg-passage.

The O phase multiplied in the presence of heat-inactivated D phase with no evidence of interference.

7. The change from O to D was considered to be due to the influence of the chicken embryo culture medium in serial egg-passage and not to genetic variation or mutation.

8. Inactivation of IBV by 1:2,000 and 1:4,000 formalin at 37 C., was curvilinear. The Beaudette egg-adapted strain was inactivated exponentially by 1:4,000 formalin. The O phases were resistant to formalin and their inactivation was less curvilinear than that of the respective O-D populations. Due to these features in common with thermal inactivation, the curvilinear inactivation rates of IBV were considered to be due to heterogeneity resulting from serial egg-passage.

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