INACTIVATION OF INFECTIOUS BRONCHITIS VIRUS INFECTIVITY BY PARA-CHLOROMERCURIBENZOATE

by

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INTRODUCTION

Proteins possess an abundance of functional groups. One of these is the sulfhydryl (SH) group to which much of the biological activity of enzymes has been attributed. When treated with SH reagents this group can be altered with the effect that the catalytic properties of the enzyme are vastly changed.

The same principles of protein chemistry apply to viruses due to their high protein content. By exposing certain viruses to SH reagents, it has been possible to alter the protein structure of the viruses and change their biological activity. The viral response is indicative of the presence of SH groups in the viral protein.

The main objective of this study was to determine the effect of the SH reagent, para-chloromercuribenzoate (CMB), upon infectious bronchitis virus (IBV) as an indication of the possible presence of SH groups in the viral protein coat.

LITERATURE REVIEW

Infectious Bronchitis Virus

Infectious bronchitis virus (IBV) is the causative agent of an acute, contagious, respiratory disease of chickens. According to electron microscopy, it is approximately spherical with a diameter of 60 to 135 mu, and bears bulbous projections or "spikes" on its surface (Berry *et al.*, 1964; Nazerian, 1960; Reagan *et al.*, 1950; Reagan and Brueckner, 1952).

The virus can be readily propagated in embryonating chicken eggs. The Beaudette chicken embryo-adapted culture of IBV can also be cultivated in several types of cell culture. Some of those in which the virus will multiply without evidence of cytopathic effects (CPE) are chicken embryo liver and heart (Fahey and Crawley, 1956), whole embryo (Mahonty and Chang, 1963), isolated chorioallantoic membrane (Ferguson, 1958; Ozawa, 1959), and de-embryonated chicken eggs (Buthala, 1956). Others in which the virus will multiply with the production of CPE are whole chicken embryo (Mallmann and Cunningham, 1963), adult chicken kidney (Kawamura *et al.*, 1961), and chicken embryo kidney (Chomiak *et al.*, 1958; Mahonty and Chang, 1963; Spring, 1960; Wright and Sagik, 1958).

The virus forms plaques in monolayers of chicken embryo kidney cells overlaid with agar. These plaques may be used for assay of viral infectivity (Wright and Sagik, 1958; Cunningham and Spring, unpublished data).

Sulfhydryl Groups in Protein Chemistry

The sulfhydryl (SH) or mercapto group has perhaps received more attention than any other reactive group of the enzyme protein molecule due to its apparent importance in protein structure and activity (Boyer, 1959). Another term frequently encountered is "thio", a prefix denoting compounds in which sulfur replaces oxygen. This prefix is often expanded to "thiol," and usually refers to a complete SH-containing compound rather than to the SH group itself. Thiols, particularly aliphatic derivatives, are also known as mercaptans.

Chinard and Hellerman (1954) have emphasized that many enzymes must retain at least a fraction of their substituent SH groups in the reduced form for normal catalytic activity. Examples of this are urease and triosephosphate, succinic, and alcohol dehydrogenases.

Two other enzymes require at least one intact SH group each to be active (Cecil and McPhee, 1959). Papain contains one reactive SH group which is essential for activity but the exact mechanism of its activity is unknown. Glyceraldehyde-3-phosphate dehydrogenase contains two to three reactive SH groups which, through an S-acyl mechanism, are involved in enzyme function. In the case of both enzymes, the

remaining SH groups were somewhat less reactive with para-chloromercuribenzoate (CMB), the reagent used. The precise function of these SH groups is unknown.

Oxidation of SH groups results in the formation of disulfide groups (SS), a reaction which has a marked effect on enzyme activity. Such interconversions of enzyme groups in tissues may be an important biological control mechanism (Boyer, 1959). Rather than playing a direct role in the biological action of the protein, the majority of native SS groups seem merely to form part of the covalent structure of the molecule. Consequently, their primary function seems to be to aid in the maintenance of molecular structure (Cecil and McPhee, 1959).

The willingness of SH groups to react with other functional groups makes them more significant from the standpoint of biological activity than the SS groups. With the exception of hydrogen ion associations, the SH group has greater reactivity toward covalent bond formation than any other group (Boyer, 1959).

Proteins have been experimentally exposed to three types of SH reagent: (1) oxidizing, such as iodine and ferricyanide; (2) alkylating, such as iodoacetate and iodoacetamide; and (3) mercaptide-forming, such as CMB. Both oxidizing and alkylating reagents react as rapidly, but not as specifically, with SH groups as do the mercaptide-forming compounds (Chinard and Hellerman, 1954).

Organic and inorganic mercurials have a high affinity for anions or groups, specifically SH groups, with an available pair of electrons (Boyer, 1959). For biological studies, aromatic derivatives are generally preferred over the aliphatics because they are more soluble and less cytotoxic (Cecil, 1963). The mercaptide-forming mercurials fulfill most of the requirements of an ideal protein reagent in that they (1) have high specificity, (2) react stoichiometrically under gentle conditions, and (3) can readily be removed and the original SH group replaced (Fraenkel-Conrat, 1957). Another advantage is that mercury reacts essentially with one SH group per molecule when linked through one covalent bond to an organic moiety (Boyer, 1959; Chinard and Hellerman, 1954).

Hellerman et al (1943) introduced CMB as a specific reagent for thiol enzymes. Boyer (1959) stated that of 134 reports on enzyme inhibition by SH group reagents, 101 involved the use of CMB. The following groups: imidazole, indole, guanidyl, amide, amine, phenol, carboxyl, disulfide and alcohol were negative to the action of CMB (Fraenkel-Conrat, 1947). The reagent is not difficult to use since many reactions can be performed near neutrality and at room temperature. Such conditions are considered to be non-denaturing for most enzymes (Fraenkel-Conrat, 1957).

Sulfhydryl groups in proteins are classified as reactive or unreactive. The reactive groups respond to

SH reagents as do simple thiols. There is little or no response by the unreactive groups in the native protein. After denaturation, however, unreactive groups react normally to SH reagents (Cecil, 1963). Such groups have been classified as "masked", "inaccessible," or "sluggish" (Fraenkel-Conrat, 1957; Fraenkel-Conrat, 1959; Cecil and McPhee, 1959).

Cecil (1963) stated that it seems reasonable to assume that these "masked" groups are involved in covalent bonding of some nature. This may not be the case since the type of covalent bond suggested would have to be one which is broken by protein denaturation, thereby rendering the unreactive groups fully reactive. Such treatment includes exposure to urea, guanidine, detergents, or changes of pH, none of which would be expected to cleave a covalent bond.

The phenomenon of increased reactivity accompanying protein denaturation has been mentioned by Boyer (1959) and Cecil and McPhee (1959), although it has never been fully explained. It does, however, seem to be a reflection of the unique structure of the native protein, and seemingly indicates that chemical reactivity is sterically blocked by the protein structure. Protein denaturation results in structural changes whereupon the steric hindrance no longer exists.

By causing structural changes in enzyme protein or by removing an SH that plays a primary role in catalysis, a

chemical reaction involving CMB may alter enzyme function. Boyer (1959) considers that there are basically three types of enzyme with respect to reaction with CMB: (1) enzymes whose SH groups react rapidly with loss of activity; (2) those whose SH groups react slowly with gradual loss of activity; and (3) those with SH groups that react rapidly without loss of activity but which subsequently lose activity as more "sluggish" groups react.

It is possible, in the case of many enzymes, to reverse the effect of CMB through the addition of a slight excess of a thiol compound. Chinard and Hellerman (1954) illustrated the reaction as follows:

En-S-HgR - R'SH -------En-SH - R'S-HgR

Cysteine and reduced glutathione are the mercapto substances of choice (Boyer, 1959; Chinard and Hellerman, 1954; Hellerman et al, 1943). This reversal of inactivation by low molecular weight thiols may be due to: (1) structural changes brought about by rupturing an S-S bond; (2) reconstruction of an SH group possessing primary enzymic function; or (3) removal of a heavy metal inhibitor such as mercury (Boyer, 1959). Hellerman et al (1943) reported that the activity subsequent to cysteine reactivation frequently seemed to exceed the initial activity but no explanation was offered.

Another widely used method of demonstrating reactive groups in proteins is the titration of SH groups with CMB using nitroprusside as an indicator (Boyer, 1959; Chinard

and Hellerman, 1954; Fraenkel-Conrat, 1957; Hellerman et al., 1943). This procedure has been modified with good results by first adding an excess of standard CMB, then back-titrating with cysteine to produce a positive nitroprusside test (Chinard and Hellerman, 1954). Boyer (1954) has employed spectrophotometric techniques in SH determination. The interaction of CMB with SH groups leads to higher readings at 255 mu (Boyer and Segal, 1953).

Bennett (1948, 1951) reported that mercury orange, known commonly as RSR or red sulfhydryl reagent, is a specific stain for thiol in tissues, and may be used to locate SH groups in regions of tissue previously not known to contain them as retinal rods and nerve cell bodies.

Dithizone reacts with CMB, and may be used in the colorimetric determination of the reagent. Fridovich and Handler (1957) noted a linear decrease in the absorption of dithizone at 6250 Å as the amount of mercurial in the system increased.

Sulfhydryl (SH) Groups in Virus Protein

The SH group plays a major role in viral stability and infectivity. One of the most consistent demonstrations of the essential nature of the virus SH group is inactivation of infectivity using appropriate cell cultures as the indicator system. Many sulfhydryl reagents have been used but CMB is most frequently chosen.

Choppin and Philipson (1961) studied the inactivation of enterovirus infectivity by CMB. The rate of inactivation

depended upon the ionic environment. Inactivation was rapid im most cases. With ECHO 7 and 9 and Coxsackie B3 viruses, there was a 50% inactivation within one to two minutes.

Allison et al (1962) concluded that most viruses, as well as the pleuropneumonia and psittacosis-lymphogranuloma groups of organisms, are adversely affected when exposed to CMB and iodoacetamide. Some arboviruses and enteroviruses are resistant.

According to Allison and Valentine (1960b), there was no detectable change in the rate of attachment of CMB-treated fowl plague virus to host cells but viral infectivity was reduced. The same pertains to other myxoviruses (Knight and Stanley, 1944; Philipson and Choppin, 1960; Philipson and Choppin, 1961).

Allison (1962) investigated the effects of CMB, iodoacetamide and $HgCl_2$ on partially purified vaccinia and fowl plague viruses. Infectivity was considerably reduced. The treated virus not only attached to the host cells but may have penetrated them. Intracellular multiplication of the virus apparently did not occur.

Oxidized 2,3-dimercaptopropanol (BAL) inactivated poliovirus types 1, 2 and 3, as well as ECHO 7 (Philipson and Choppin, 1962). A mixed disulfide may have been formed between the virus SH groups and BAL.

Philipson and Choppin (1962) noted that the hemagglutinating activity of ECHO 7 was inactivated by BAL. This

supported two earlier reports (Buckland, 1960; Philipson and Choppin, 1960) that enterovirus hemagglutination (HA) could be prevented by CMB and other similar reagents. Inactivation of viral HA was due to the inability of CMB-treated virus to adsorb to erythrocytes (Philipson and Choppin, 1960). These results indicate a reaction between the reagent and SH groups on the virus particle, and further suggest that viral SH groups are involved in the attachment of enteroviruses to erythrocytes. Buckland (1960) observed that the HA activity of myxoviruses and arboviruses remained unaffected by CMB, and Philipson and Choppin (1960) confirmed this with the myxoviruses. All agreed that the SH reagents had no effect on erythrocyte receptors.

Incubation of poliovirus type 1 with L-cystine enhanced thermostability of the virus (Pohjanpelto, 1958). This phenomenon was attributed to an exchange between cystine and protein SH groups resulting in S-S bonds. If the virus were exposed to CMB prior to L-cystine, there was no enhancement of thermostability. The thiol reagent combined with the SH groups of virus protein, thereby making them inaccessible to cystine. Thermostability of the virus was not increased by CMB treatment alone.

In 1961, Hirst reported that in addition to cystine, several polysulfides such as Na_2S_2 and Na_2S_4 stabilized poliovirus type 1 to high temperatures.

Inactivation of influenza virus by various mercurials has been studied using mice and embryonating chicken eggs as

assay systems (Klein et al., 1948; Klein and Perez, 1948; Knight and Stanley, 1944; and Perez et al., 1949).

Reversible inactivation of Rous sarcoma virus in vivo by CMB has been reported by Schmidt (1957).

Reichmann and Hatt (1961) suggested that the action of CMB on the viral particle results in dissociation of virus into subunits. They postulated that substitution of SH groups with a bulky radical, such as mercuribenzoate, might interfere with subunit linkage sites, thereby resulting in particle degradation.

Kaper and Houwing (1962), using spectrophotometry (Boyer, 1954), determined that turnip yellow mosaic virus (MW 3.5 x 10^6) possesses 650 SH groups per molecule. Fridovich and Handler (1957) reported 5400 SH groups per molecule of potato virus X (MW 35 x 10^6), as measured by the dithizone method.

Reactivation of virus inactivated by SH reagents has occurred consistently subsequent to the addition of thiol compounds (Allison, 1962; Choppin and Philipson, 1961; Hirst, 1961; Klein et al., Klein and Perez, 1948; Perez et al., 1949; Philipson and Choppin, 1960; Philipson and Choppin, 1962). Such reversal of inactivation by added thiols is indicative of a previous reaction between SH groups and reagents whether in viral protein or in an isolated enzyme system.

MATERIALS AND METHODS

Virus

The Beaudette chicken embryo-adapted culture of IBV, designated as No. 42 in the Michigan State University repository, was used. The virus had previously been passed 111 times in chicken embryo kidney cells (CEKC) in a cultural medium consisting of Hank's balanced salt solution (BSS), lactalbumin hydrolysate (LAH), and bovine serum. For the experiments in the present study, medium 199 (General Biochemicals), supplemented as described below, was used.

Allantoic fluid from infected embryonating chicken eggs was used in one experiment for comparison with cell-cultured virus.

Virus was harvested 48 hours after inoculation of the cultural system, dispensed into vials, and stored at -62°C until used.

Cell Culture

Primary cell cultures were prepared from the kidneys of 18- to 19-day-old chicken embryos. The kidneys were removed from the embryos, minced, and washed in BSS (Cunningham, 1963). The tissues were then treated with 0.25% trypsin (1:250, Nutritional Biochemical Corporation) in BSS, pH 8.0 to 8.4, allowing approximately 10 ml of

enzyme solution per pair of kidneys. Trypsinization was carried out at 37°C for 40 minutes. To facilitate digestion, the flask was clamped in place above a magnetic stirrer, and a sterile Teflon bar was placed in the tissue-trypsin mixture.

Cells were washed free of trypsin and debris by centrifuging at 437g at 4°C for five minutes, decanting the supernatant, and resuspending the cells in fresh BSS. After the third cycle of centrifugation, the supernatant fluid was decanted, and the packed cells were resuspended in cultural medium to a concentration of 1 to 1.5 x 10^7 cells per milliliter.

The cultural medium consisted of medium 199, and 1% solutions each of vitamins, L-glutamine, and amino acids (General Biochemicals); 5% newborn calf serum (Grand Island Biological Company); 100 units penicillin G potassium, 0.1 mg dihydrostreptomycin sulfate, and 100 units Mycostatin, respectively, per ml (E. H. Squibb and Sons). The medium was adjusted to pH 7.0 with 10% saturated $NaHCO_3$ (Cunningham, 1963). Plastic petri dishes (Falcon Plastics), 60 x 15 mm, were seeded with 4 ml per dish of the cell-medium 199 suspension.

All cultures were incubated at 37°C in an atmosphere of 8% CO_2, two atmospheric changes per hour, and 85% relative humidity.

Buffer

The buffer system employed was "Sigma 121" tris (hydroxymethyl) aminomethane (Sigma Chemical Company), 0.01M, with no additional salts added. The pH was adjusted from 9.7 (initial) to 7.0 since the reagent used (CMB) gives optimum results at neutrality (Chinard and Hellerman, 1954). For adjusting the pH, 5N HCl was used so as not to extensively alter buffer molarity.

Reagents

p-Chloromercuribenzoate (CMB), Na salt and L-cysteine HCl monohydrate, A grade (Calbiochem respectively) were dissolved in 0.01M tris buffer, pH 7.0. The solutions were freshly prepared prior to each experiment.

Dialysis

Dialysis tubing (Visking Company), average diameter of 4.8 mu, was used for dialyzing the virus samples against 0.01M tris buffer, pH 7.0. Individual tubes were prepared in advance and soaked overnight in buffer. After virus samples had been poured into their respective dialysis bags, which were tied at one end, the open end of each bag was tied securely and a tag attached. The bags were then suspended in a large beaker containing buffer. To increase agitation, a Teflon bar was put in the buffer and the beaker was placed on a magnetic stirrer. The sample was dialyzed for 24 hours at 4°C, during which time the buffer was changed three or four times.

Filtration

All virus samples were filtered subsequent to dialysis using Type HA, 0.45 u pore size, 47 mm disc diameter Millipore filters.

Storage

Samples which were not immediately used following dialysis and filtration were dispensed in vials and stored at -62°C.

Plaque Assay

The cultural medium was decanted from 72-hour-old cell cultures. The monolayers were washed with 2 ml of BSS and inoculated with 0.5 ml of serial tenfold dilutions of virus with BSS as the diluent. Two to four cultures were used per dilution of virus. After incubation for 120 minutes, the inoculum was poured off but the cells were not washed. Agar medium overlay consisting of equal volumes of 1.8% Special agar-Noble (Difco Laboratories) and two times concentrated medium 199 was added, 4 ml to each monolayer. After the agar had hardened, the cultures were returned to the incubator. After three days, 0.5 ml of a 0.1% neutral red dye in phosphate-buffered saline solution was added to each culture. The cultures were incubated at 37°C without CO_2 for 15 to 30 minutes, and then for an hour at 4°C before the plaques were counted.

The amount of infective virus was expressed in plaque-forming units (PFU).

Experimental Procedures

The CMB solutions were equilibrated at the reaction temperature immediately prior to use. Virus was thawed and mixed with an equal volume of twice concentrated CMB solution in a flask. A control consisted of equal volumes of virus and tris buffer. In the single instance in which virus in allantoic fluid was used, the sample was thawed, centrifuged at 437g for 15 minutes at 4°C to remove cellular debris, and the supernatant fluid was used.

The flask was frequently shaken and, at appropriate intervals, samples of the mixture were removed. Each sample, including the control, was dialyzed, filtered, and assayed for infective virus.

To arrest CMB-inactivation at specific times, equal volumes of CMB-treated virus and L-cysteine were mixed. The final proportions of virus:CMB:L-cysteine were 1:1:2. These mixtures were held at room temperature (25°C) for varying periods of time, depending upon the experiment. Controls consisted of CMB-treated virus in tris buffer, untreated virus in tris, and untreated virus in L-cysteine.

The foregoing procedure was altered to establish the effect of L-cysteine upon virus previously untreated and then exposed to CMB. One part of untreated virus was mixed with two parts of L-cysteine to match the ratio of respective components in other experiments. The mixture was agitated at 25°C. Samples were removed at 15 and 30

minutes, pipetted into flasks containing a volume of CMB equal to that of the virus, then incubated at 25°C for 15 minutes. Controls consisted of untreated virus plus tris, and untreated virus plus L-cysteine.

For reactivation of CMB-treated virus after 15 minutes at 25°C, samples were added to flasks containing varying concentrations of L-cysteine. The proportions of virus:CMB: L-cysteine were the same as in previous experiments. Controls consisted of untreated virus plus tris, and virus plus CMB. After 15 minutes at 25°C, all samples were assayed for infective virus.

RESULTS

The concentrations of CMB and L-cysteine referred to are the final concentrations in the mixture.

Determination of Optimum Concentration of CMB for Inactivation of Infectivity

Inactivation of virus increased with the concentration of CMB during the one hour reaction time (Table 1, Figure 1). There was at least an 85% reduction of infectivity. Both the 10^{-3} and 10^{-2}M concentrations were adequate but, since an excess of reagent in the system would be of no advantage, the former concentration was selected. All subsequent experiments utilized $1x10^{-3}$M CMB.

Determination of Optimum Concentration of L-cysteine for Arresting CMB-inactivation

L-cysteine, $1x10^{-3}$M, did not arrest inactivation within 15 minutes at 25°C (Table 2, Figure 2). At $2x10^{-3}$M, inactivation was reversed. At $1.5x10^{-3}$M, L-cysteine halted inactivation but did not reverse it. During the subsequent 15 minutes to the termination of the experiment, there was only an insignificant decrease in viral infectivity. For subsequent studies, $1.5x10^{-3}$M L-cysteine was employed.

There was no noticeable decrease of infectivity of the untreated virus control.

The results of this experiment indicate that most of the CMB-inactivation process occurs within 15 minutes.

Table 1. Effect of CMB on IBV after One Hour at 25°C

Sample	Infectivity Titer (Pfu/ml)	Log V/V_o	% Decrease in Infectivity
Control (Virus + Tris)	8.5×10^5	0	0
Virus + CMB (1×10^{-2}M)	6.3×10^3	-2.1301	99.3
Virus + CMB (1×10^{-3}M)	1.2×10^4	-1.8502	98.5
Virus + CMB (1×10^{-4}M)	1.2×10^5	-0.8502	85.7

Figure 1. Effect of CMB on IBV after One Hour at 25°C

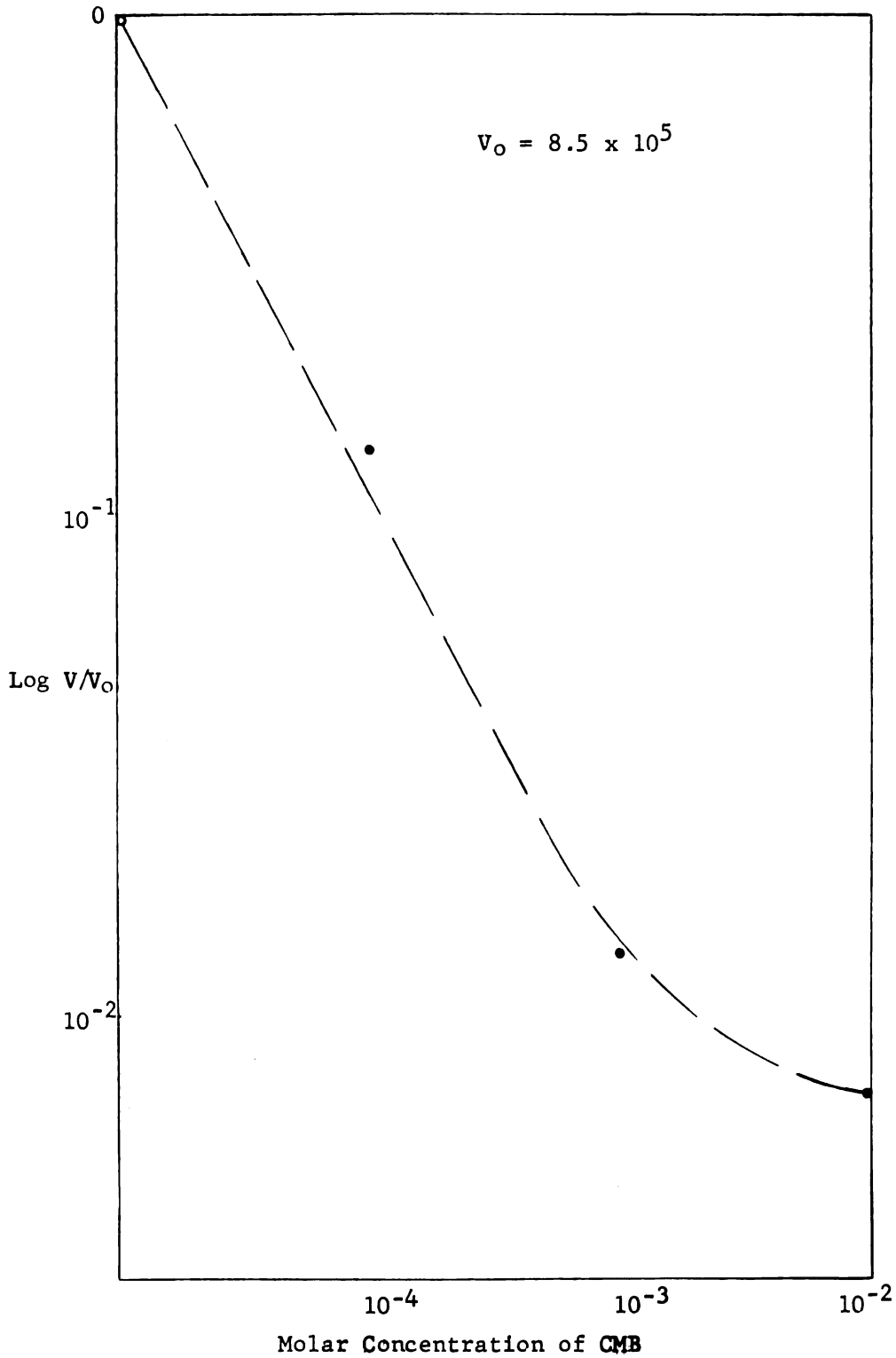
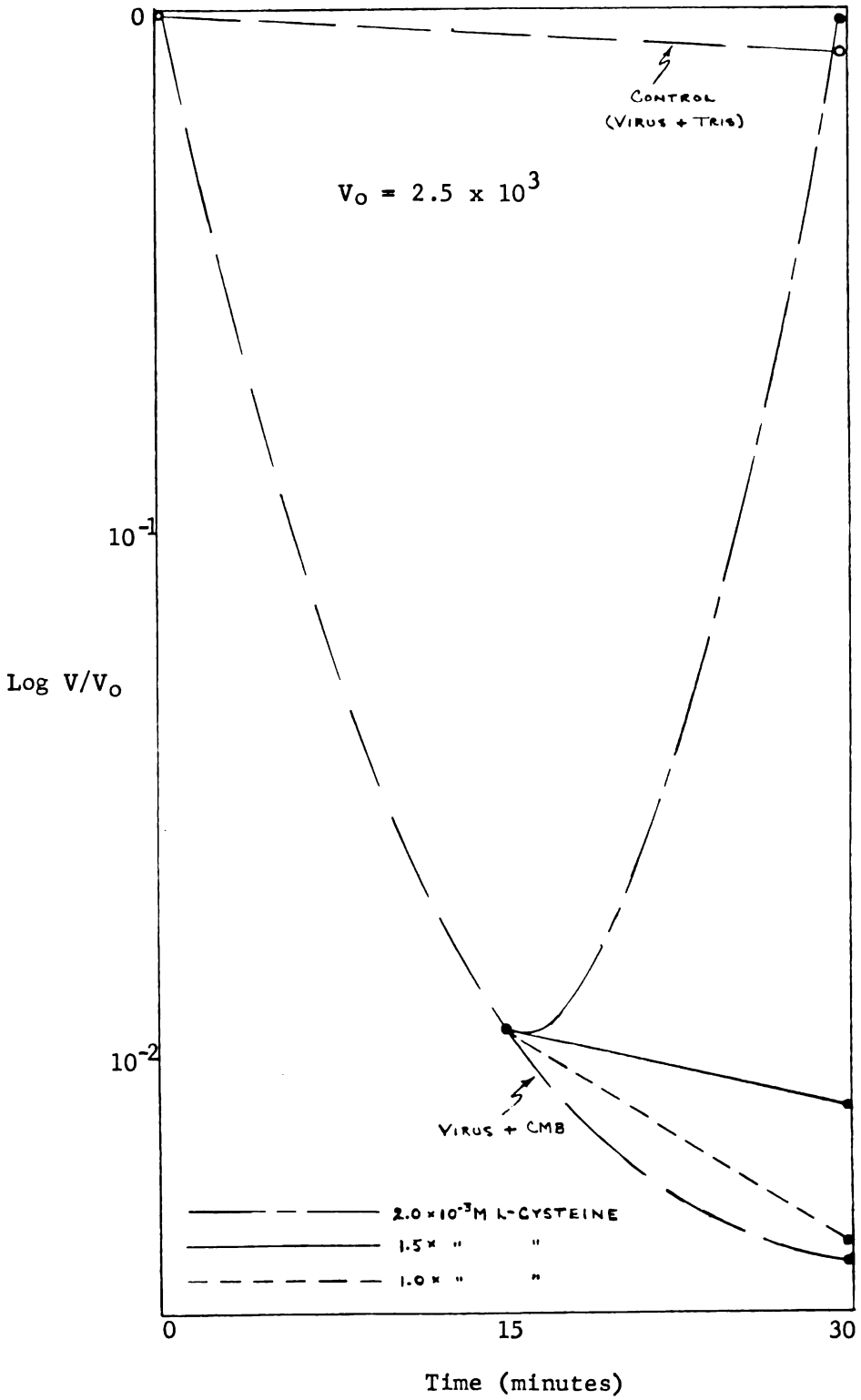


Table 2. Effect of Different Concentrations of L-cysteine in Arresting and/or Reversing Inactivation by CMB (1×10^{-3}M) at 25°C

Sample	Infectivity Titer (Pfu/ml)			Log V/V_o		
	0 min.	15 min.	30 min.	0 min.	15 min.	30 min.
Virus control (Virus + Tris)	2.5×10^3	---	2.1×10^3	0	---	-0.0757
CMB control (Virus + CMB)	2.5×10^3	2.8×10^1	1.0×10^1	0	-1.9507	-2.3979
Virus + CMB plus:						
1.0×10^{-3}M L-cysteine	---	---	1.1×10^1	---	---	-2.3565
1.5×10^{-3}M L-cysteine	---	---	2.0×10^1	---	---	-2.0969
2.0×10^{-3}M L-cysteine	---	---	2.4×10^3	---	---	-0.0177

Figure 2. Effect of Different Concentrations of L-Cysteine in Arresting and/or Reversing Inactivation by CMB (1×10^{-3}M) at 25°C



Kinetics of Inactivation of IBV by CMB at 25°C

Inactivation by CMB was halted at desired time intervals by the addition of L-cysteine. There was a 98% reduction in viral infectivity within ten minutes after treatment with the reagent (Table 3, Figure 3). A decrease in the rate of effect was noticeable at about 15 minutes and extended to the termination of the experiment. The virus was never completely inactivated.

The titer of the controls remained fairly constant throughout the experiment. At 60 minutes, there was a 78% greater reduction in infectivity in the treated sample than in the untreated sample. After 180 minutes, the untreated virus had lost only 40% of its infectivity.

The L-cysteine control indicated that the thiol compound had no deleterious effect on the untreated virus.

Kinetics of Inactivation of IBV by CMB at 4° and 37°C

The inactivation rate of the virus was directly related to temperature (Tables 4 and 5, Figure 4). The curves from the data at 4° and 37°C have been superimposed on those at 25°C.

The infectivity titer of the treated, 180 minute sample at 4°C was no lower than that of the 60 minute sample, offering another example of "tailing-off" after the initial reaction. Total inactivation during the three hour period was only a small fraction of that observed at the other two reaction temperatures. The untreated control (4°C) retained 74% infectivity after the three hour period.

Table 3. Inactivation of IBV by CMB ($1x10^{-3}M$) at 25°C

Sample	Infectivity Titer (Pfu/ml)	Log V/V_o	% Decrease in Infectivity
Untreated Controls: (Virus + Tris)			
0 minutes	2.5×10^5	0	0
60 "	2.0×10^5	-0.0969	21
180 "	1.5×10^5	-0.2218	40
(Virus + L-cysteine)			
60 minutes	2.1×10^5	-0.0757	16
Treated Virus: (Virus + CMB)			
0 minutes	2.5×10^5	0	0
5 "	4.5×10^4	-0.7447	82
10 "	5.1×10^3	-1.6903	98
15 "	8.5×10^3	-1.4685	96.6
20 "	3.2×10^3	-1.8928	98.7
25 "	1.8×10^3	-2.1426	99.3
30 "	1.7×10^3	-2.1675	99.32
45 "	1.2×10^3	-2.3187	99.44
60 "	1.1×10^3	-2.3565	99.56
90 "	3.8×10^2	-2.8181	99.85
120 "	2.3×10^2	-3.0362	99.91
180 "	3.0×10^1	-3.9208	99.99

Figure 3. Inactivation of IBV by CMB (1×10^{-3}M) at 25°C

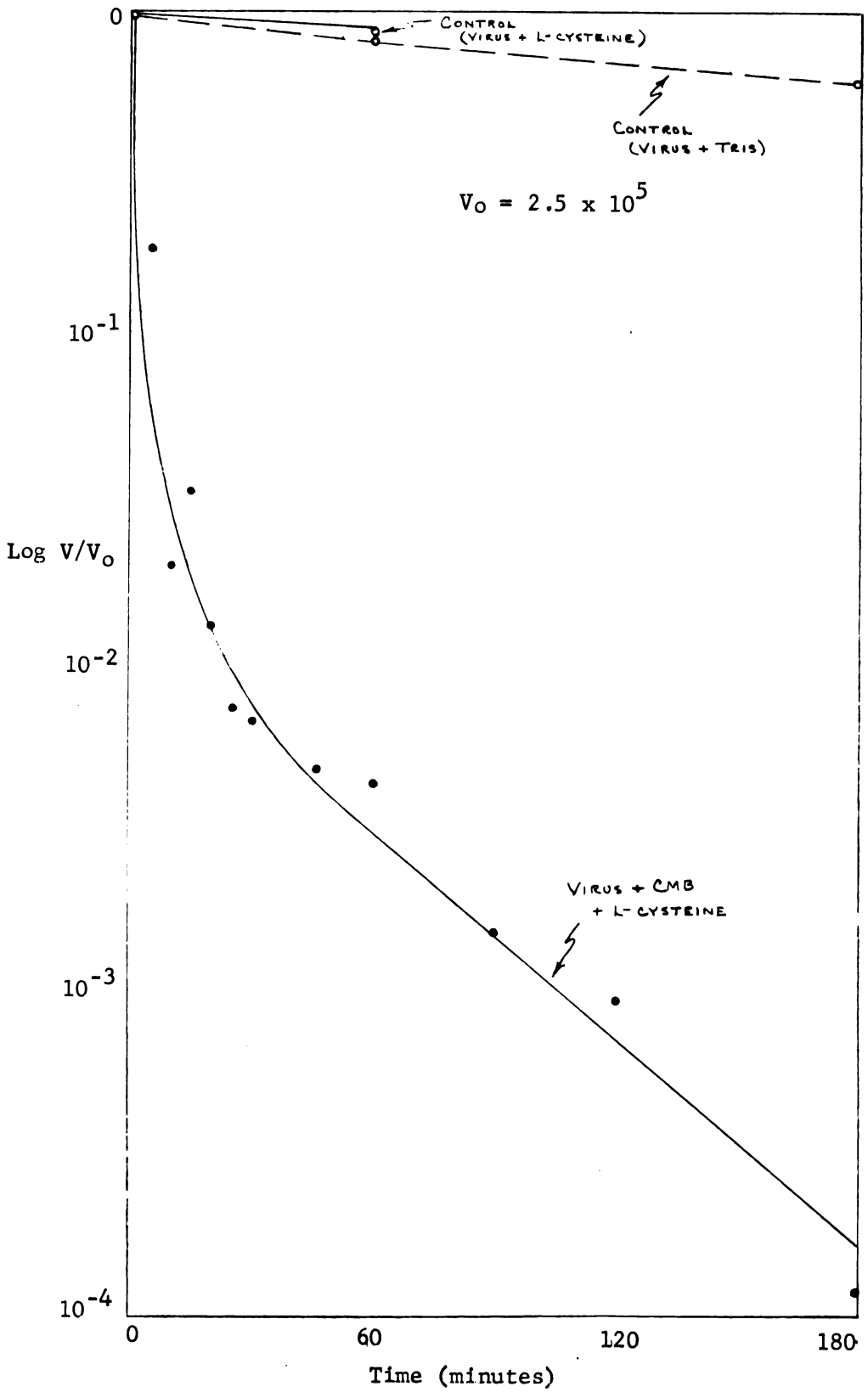


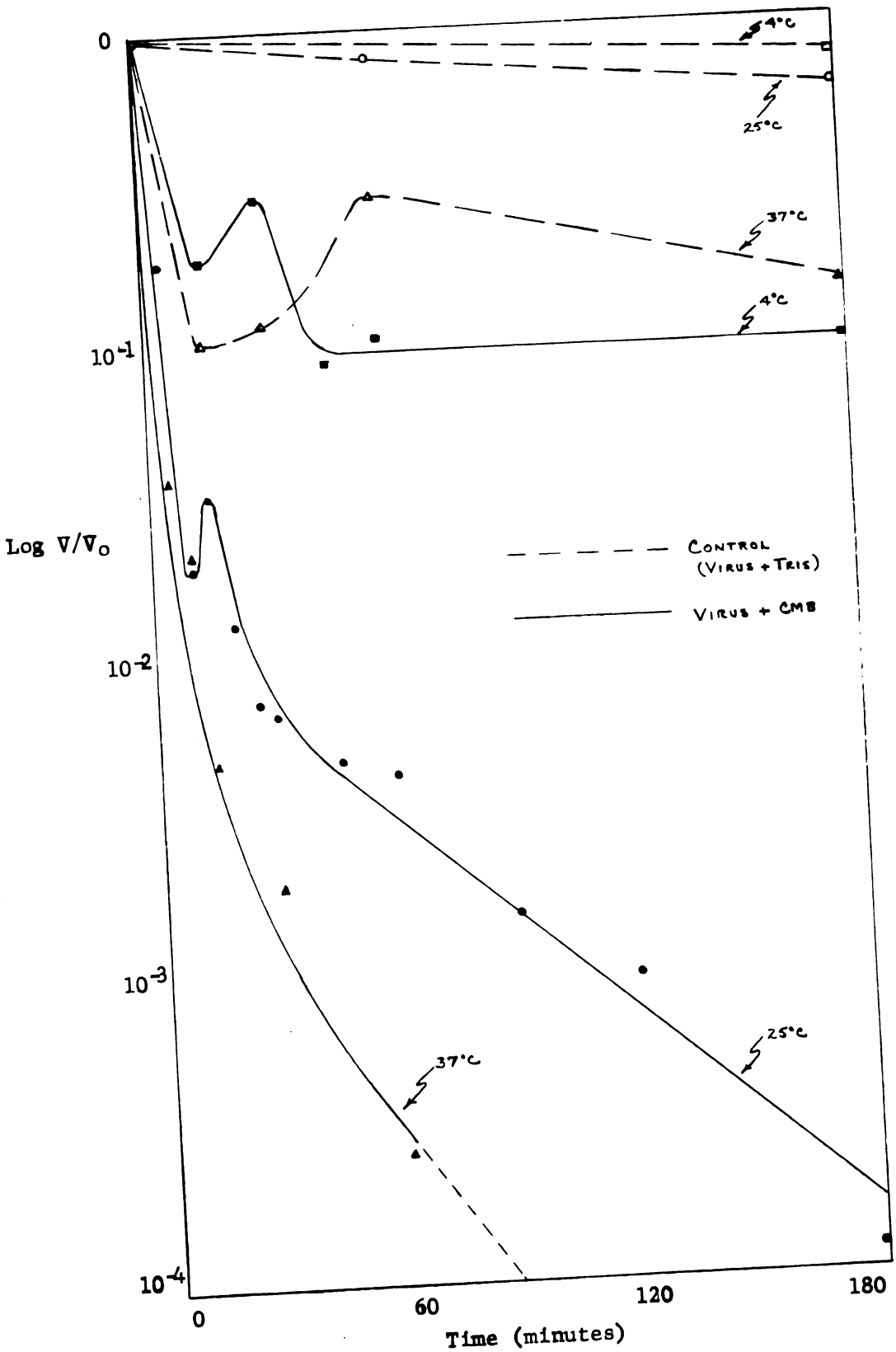
Table 4. Inactivation of IBV by CMB ($1x10^{-3}M$) at 4^{o} and $37^{o}C$

	Infectivity Titer (Pfu/ml)			
	Untreated (Virus + Tris)		Treated (Virus + CMB)	
Time Samples	$4^{o}C$	$37^{o}C$	$4^{o}C$	$37^{o}C$
0 minutes	2.5×10^{5}	2.5×10^{5}	2.5×10^{5}	2.5×10^{5}
5 "	---	---	---	9.6×10^{3}
10 "	---	---	---	5.4×10^{3}
15 "	---	2.6×10^{4}	4.8×10^{4}	1.2×10^{3}
30 "	---	3.0×10^{4}	7.6×10^{4}	4.9×10^{2}
45 "	---	---	2.2×10^{4}	---
60 "	---	7.3×10^{4}	2.6×10^{4}	6.9×10^{1}
180 "	1.9×10^{5}	3.5×10^{4}	2.4×10^{4}	none

Table 5. Inactivation of IBV by CMB ($1x10^{-3}M$) at 4^{o} and $37^{o}C$

Time Samples	Log V/V_o			
	Untreated (Virus + Tris)		Treated (Virus + CMB)	
	$4^{o}C$	$37^{o}C$	$4^{o}C$	$37^{o}C$
0 minutes	0	0	0	0
5 "	---	---	---	-1.4156
10 "	---	---	---	-1.6655
15 "	---	-0.9829	-0.7167	-2.3187
30 "	---	-0.9208	-0.5171	-2.7077
45 "	---	---	-1.0555	---
60 "	---	-0.5346	-0.9829	-3.5591
180 "	-0.1191	-0.8538	-1.0177	none

Figure 4. Inactivation of LBV by CMB (1×10^{-3} M) at 4°, 25°, and 37°C



At 37°C, the reaction reached completion sometime prior to the final sampling. At 15 minutes, 0.5% infectivity remained and, after one hour, 0.03% was detectable. Due to the elevated temperature, the "tailing-off" process also was accelerated.

The titer of the untreated control after three hours at 37°C represented 14% of the original infectivity.

Kinetics of Inactivation of Embryo-adapted IBV by CMB at 25°C

The CEKC-propagated IBV was used for all studies up to this point. The chicken embryo-cultured virus, later adapted to CEKC, was tested with CMB for comparison of susceptibility. Although the rates of inactivation were approximately the same for both strains, the embryo-cultured virus was not as susceptible to CMB as was the tissue-cultured virus (Table 6, Figure 5).

Effect of Exposing IBV to L-cysteine Prior to CMB Treatment at 25°C

To further determine the effect of L-cysteine, three different molar solutions of the thiol compound were mixed with untreated virus. After 15 and 30 minutes at 25°C, CMB was added.

None of the concentrations fully protected the virus from the effects of CMB (Tables 7 and 8, Figure 6). Inactivation, however, was considerably less than that observed with CMB alone. In contrast to previous results, there was a marked decrease of infectivity of the controls

Table 6. Inactivation of Embryo-adapted IBV by CMB ($1x10^{-3}M$) at $25^{o}C$

Sample	Infectivity Titer (Pfu/ml)	Log V/V_o	% Decrease in Infectivity
Untreated Controls: (Virus + Tris)			
0 minutes	2.5×10^5	0	0
60 "	2.2×10^5	-0.0555	12
180 "	1.6×10^5	-0.1938	36
Treated Virus: (Virus + CMB)			
0 minutes	2.5×10^5	0	0
15 "	2.0×10^4	-1.0969	92
30 "	8.1×10^3	-1.4894	96.8
60 "	3.6×10^3	-1.8416	98.6
180 "	6.0×10^1	-3.6197	99.98

Figure 5. Inactivation by CMB ($1x10^{-3}$M) at 25°C of IBV from Two Different Sources (CEKC and AF)

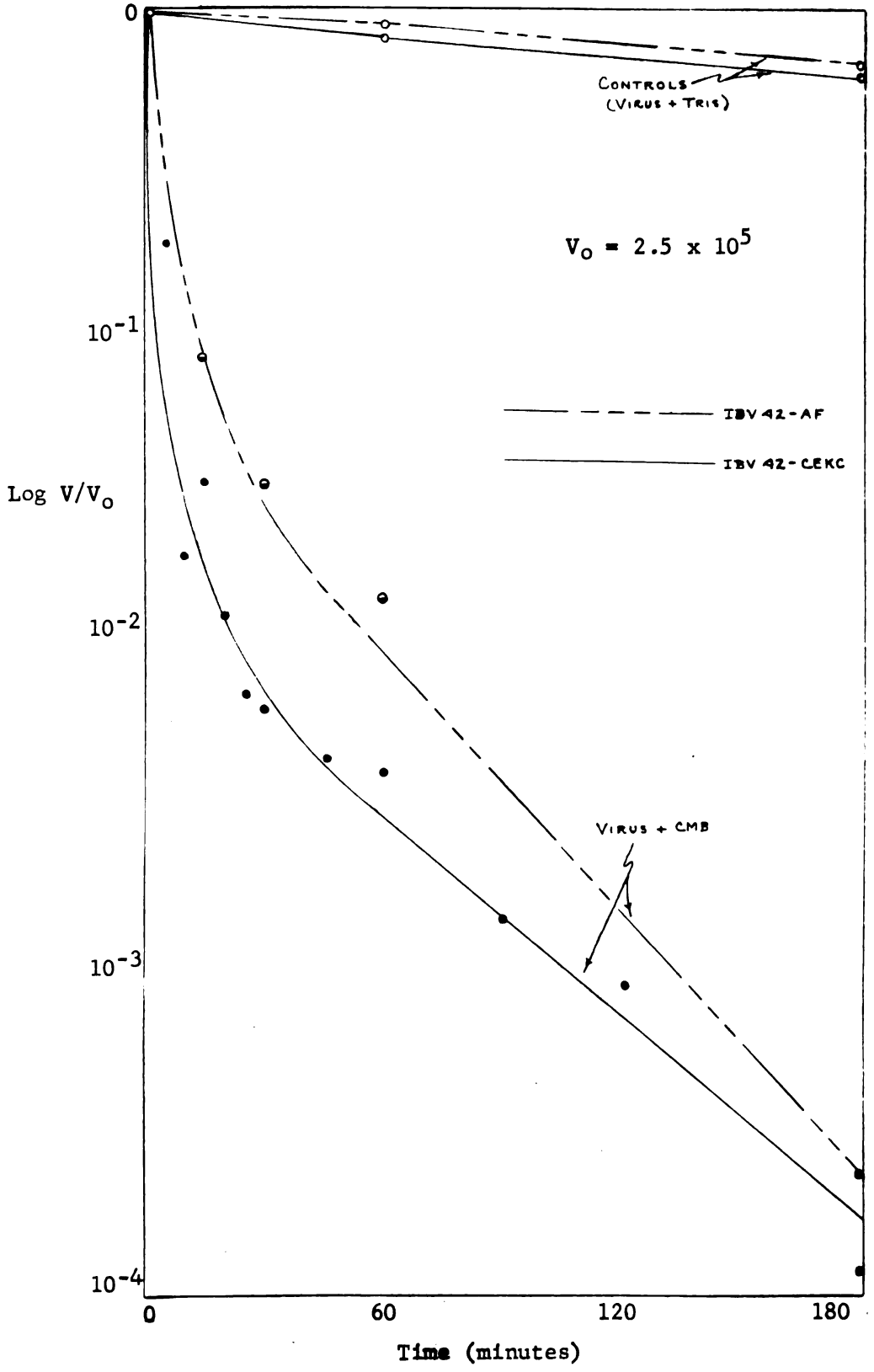


Table 7. Effect of Exposing IBV to Various Concentrations of L-Cysteine Prior to CMB ($1x10^{-3}M$) Treatment at 25°C

Untreated Controls (Virus + Tris)	Infectivity Titer (Pfu/ml)	% Decrease in Infectivity
0 minutes	2.5×10^5	0
45 "	6.0×10^4	76

* * * * * * * * * * * * *

Samples	Concentration of L-Cysteine					
	$1x10^{-3}M$		$1.5x10^{-3}M$		$2x10^{-3}M$	
	Pfu/ml	%	Pfu/ml	%	Pfu/ml	%
Virus + L-cysteine (15 min.) + CMB	1.6×10^4	93.6	2.2×10^4	91.2	1.3×10^5	46.4
Virus + L-cysteine (30 min.) + CMB	3.0×10^4	88.0	3.9×10^4	84.4	9.4×10^4	62.4
Virus + L-cysteine (30 min.) + Tris	4.9×10^4	80.4	5.9×10^4	76.4	2.5×10^5	0

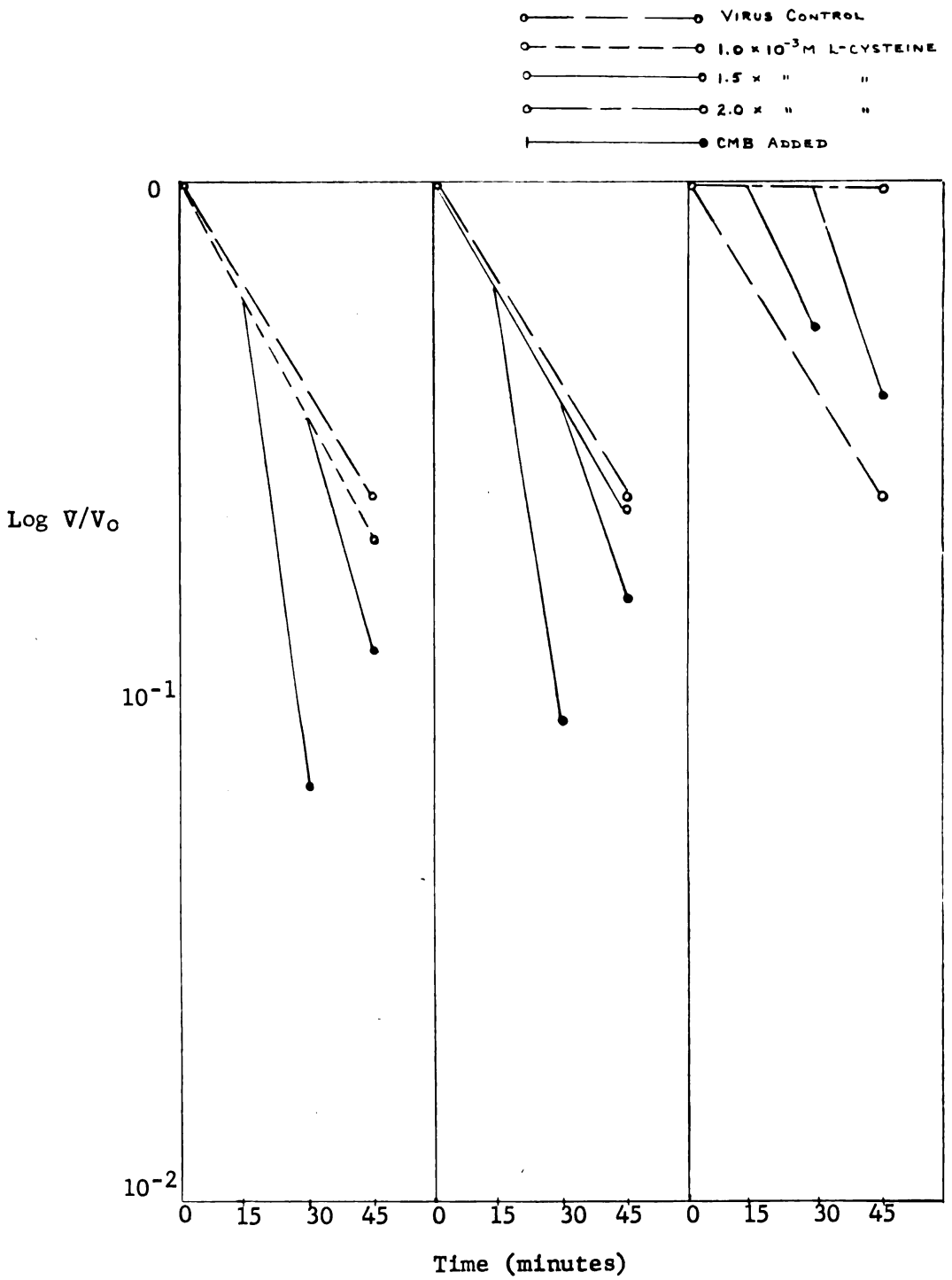
Table 8. Effect of Exposing IBV to Various Concentrations of L-Cysteine Prior to CMB ($1x10^{-3}M$) Treatment at 25°C

Untreated Controls (Virus + Tris)	Log V/V_1	% Decrease in Infectivity
0 minutes	0	0
45 "	-0.6197	76

* * * * * * * * * * *

Samples	Concentration of L-Cysteine					
	$1x10^{-3}M$		$1.5x10^{-3}M$		$2x10^{-3}M$	
	Log V/V_o	%	Log V/V_o	%	Log V/V_o	%
Virus + L-cysteine (15 min.) + CMB	-1.1938	93.6	-1.0555	91.2	-0.2840	46.4
Virus + L-cysteine (30 min.) + CMB	-0.9208	88.0	-0.8068	84.4	-0.4248	62.4
Virus + L-cysteine (30 min.) + Tris	-0.7077	80.4	-0.6270	76.4	0	0

Figure 6. Effect of Exposing IBV to Various Concentrations of L-Cysteine Prior to CMB ($1x10^{-3}$M) Treatment at 25°C



with one exception. When compared with these controls, it is seen that inactivation due to CMB was not extensive. The exceptional control consisted of virus and $2x10^{-3}M$ L-cysteine. This sample was stable to whatever environmental factors were responsible for decreasing the titers of the other controls. It also confirmed that L-cysteine, under these circumstances, did not inactivate the virus.

There was no consistent difference between a 15 and 30 minute exposure of virus to L-cysteine.

Reactivation of CMB-treated Virus

By adding $2x10^{-3}M$ L-cysteine to CMB-inactivated virus, it was possible to reactivate viral infectivity (Table 2, Figure 2). This indicated that previous inactivation was due to a reaction between CMB and viral protein SH groups.

DISCUSSION

The susceptibility of the CEKC- and embryo-adapted IBV 42 to CMB indicates that the reagent reacts with SH groups in the viral protein. The inactivation reaction with CMB appears to be first-order for the first ten minutes during which there was a 98% decrease of infectivity. At this point, there was a break in the curve and a "tailing effect" which extended to the termination of the experiment. A decrease such as this is quite common in virus survival curves (Hiatt, 1964). The initial slope may be abrupt but more often is gradual, as was the case here.

The problem arose as to whether the reaction was first-order followed by a "tailing effect", or second-order involving two populations of the virus. The former seemed to apply here because the curve was linear until 98% of the virus had been inactivated. Hiatt (1964) states that "thermal inactivation of viruses has often been observed to proceed as a first-order reaction until about 90% or more of the infective particles have succumbed." Had a heterogeneous population been involved, an earlier break in the curve would have been expected.

Since only three points were available for the first ten minutes, wherein the greatest inactivation occurred, no attempt was made to determine the velocity constant (k) or energy of activation (E).

One experiment (Figure 5) compared the inactivation rates of viruses from two different sources, CEKC and infected AF. The regression curves for inactivation of CEKC-cultured virus and viral-infected AF were relatively parallel, although the AF virus was somewhat less susceptible to the reagent. There are two possible explanations for this differential response: (1) The tissue culture (TC) virus, originally derived from AF virus, may have undergone a modification of its protein coat as a result of propagation in kidney cells. This implies the possible acquisition of more SH groups essential to infection than the "parent" strain possesses; (2) The AF virus, in the highly proteinaceous embryonic fluid, may not have been in as intimate contact with as many CMB molecules as was the TC virus. It is possible that much of the reagent united with SH groups in the AF proteins. The TC medium contains proteins, also, but perhaps not as many with SH groups as may be found in AF. It is possible that both alternatives are involved.

The regression curves for inactivation at 4°, 25° and 37°C were linear for initial inactivation of virus with two exceptions which occurred at 15 minutes at 25°C, and 30 minutes at 4°C. No explanation can be offered for this other than a possible disruption of viral clusters due to temperature effects and agitation of the reaction flasks. At 37°C, such a disaggregation may have occurred before the first sampling so was not reflected in the curve. The time

required for this phenomenon to occur was directly related to temperature in all cases.

The presence of CMB in the sample appeared to accelerate dispersal of viral clusters, as indicated by the delayed response of the control at 37°C. Reichmann and Hatt (1961) reported that CMB caused the virus particle to dissociate into subunits. Control samples at 4° and 25°C were removed late in the experiment and disruption, if it occurred, was not detected.

L-cysteine added to the reaction mixture arrested inactivation of the virus by CMB. However, none of the concentrations of L-cysteine employed completely protected the virus from inactivation by subsequent treatment with CMB. This indicates that even though excessive thiol compound was present, at least a few CMB molecules altered some virus particles. This seems logical, although Allison (1962) stated that SH reagents react with cysteine much more rapidly than with virus. On this basis, one might expect nearly 100% protection by the excess cysteine. The present results indicate that CMB is not necessarily so very selective in its choice of SH groups and will react almost as readily with SH groups of viral origin as with those in cysteine.

The question arose as to why the 2×10^{-3}M L-cysteine permitted some inactivation by CMB with no evidence of reactivation, when the same concentration had reversed

inactivation in an earlier experiment. It might be assumed that a certain amount of excess cysteine remained free in the reaction mixture following the interaction of CMB with virus and cysteine molecules. Such free cysteine might be capable of reversing whatever viral inactivation had occurred if given sufficient time. The samples for assay may have been removed too soon for reactivation to occur.

An attempt at reactivation using a slightly modified technique proved unsuccessful. Each sample was dialyzed immediately after CMB treatment, and again after exposure to L-cysteine but there was no significant reversal of inactivation. Perhaps some protective substance was lost during initial dialysis, thereby rendering the virus susceptible to the low pH of the cysteine solutions. Adjusting the cysteine solutions to pH 7.0 resulted in a limited renewal of infectivity but this was not interpreted as positive reactivation.

During the initial dialysis, more than the removal of excess CMB may have taken place. Certain ions or substances essential for reversal by L-cysteine might also have been removed; or possibly some factor was involved which made the virus-CMB union more stable.

In a previous experiment, reactivation occurred after only single dialysis, that following the addition of L-cysteine to the virus-CMB mixture. Essential ions or substances, if they exist, must have been present in that

mixture as well as in the mixture that was dialyzed twice. It could be assumed that such substances would have demonstrated the same tendency to dialyze out, and yet reactivation occurred. Perhaps their egression was sufficiently gradual to enable them to assist cysteine in reactivation prior to being dialyzed out with excess CMB and thiol compound.

Second dialysis had no deleterious effect on the virus, as both controls and inactivated samples produced anticipated titers.

The technique of ridding the sample of excess CMB prior to thiol exposure has been employed with success by others (Allison, 1962; Choppin and Philipson, 1961). A possible improvement was suggested by Fraenkel-Conrat (1957) involving single dialysis of CMB-inactivated virus against a cysteine solution. The author claimed that this reversed the reaction and simultaneously removed the SH reagent.

The virus was more stable in tris buffer than in cultural medium, and the incorporation of L-cysteine into the buffer further enhanced viral stability. Hirst (1961) and Pohjanpelto (1958) stabilized certain enteroviruses to heat by exposure to cystine. It is possible that a similar phenomenon occurs with IBV but this aspect of SH involvement was not explored in the present study.

SUMMARY

1. The Beaudette culture of infectious bronchitis virus (IBV 42) is susceptible to inactivation of infectivity by the sulfhydryl (SH) reagent, p-chloromercuribenzoate (CMB). This reaction indicates the presence of SH-containing components in the viral protein.
2. Both the embryo-adapted and chicken embryo kidney cell cultured IBV 42 responded to CMB treatment. Differences in reaction rates of the two samples suggest that minor changes in protein structure may have accompanied adaptation of the virus to tissue culture from the chicken embryo.
3. Inactivation is typical of a first-order reaction, and proceeds rapidly at 25° and 37°C. A "tailing effect" is noticeable at all three reaction temperatures (4°, 25° and 37°C) but is most obvious at 25°C.
4. The possibility exists of a disruption of viral clusters in the early minutes of experimentation. The presence of CMB may have an accelerating effect but the same phenomenon was observed in an untreated control. This implies that such a breakup of particles is due primarily to factors such as temperature, pH, and agitation of the reaction flask.

5. Addition of L-cysteine to a CMB-virus mixture arrests or reverses the inactivation reaction depending upon the concentration of cysteine used. Reactivation by a thiol compound indicates the nature of the preceding inactivation process, and confirms that SH groups were involved.
6. Exposure to L-cysteine prior to CMB treatment affords the virus a certain degree of protection from the inactivating properties of the reagent. L-cysteine, as used in these experiments, had no deleterious effects on the virus.
7. IBV 42, when suspended in tris buffer, 0.01M and pH 7.0, is extremely stable to temperatures up to 37°C. This stability appears to be enhanced by the presence of certain concentrations of L-cysteine.

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