STUDIES ON THE MODIFICATION, BY VARIOUS AGENTS, OF THE HEAT SENSITIVITY OF POLIOVIRUS

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ABSTRACT

The decrease in heat sensitivity of Poliovirus, Type I, after incubation with cystine, has been studied. Characteristics of the inactivation by heat have also been studied. The results have led to an hypothesis which, while in no way completely satisfactory, provides a useful picture by which the data can be discussed, and also serves to indicate the areas to be clarified by future experiments. This hypothesis proposes the existence of two reactivity classes of viral sulfhydryl groups: -SH groups of the first class are oxidized by oxygen and by iodosobenzoic acid, probably to disulfides: -SH groups of the second class are so situated as to be unable to form disulfide bonds readily, and thus cannot be oxidized by oxygen, but can be oxidized to $-SO_2H$ or $-SO_3H$ by iodosobenzoic acid.

The inactivation is postulated to consist of at least two steps: oxidation of sulfhydryl group(s), followed by denaturation of the viral protein. The existence of a denaturation step is suggested by the calculation of an Arrhenius constant for aerobic inactivation of 76,000 calories/mole. Evidence is presented to indicate that, at least in the case of oxidation by iodosobenzoate, the oxidation has a sensitizing effect; i.e., it increases the probability for the denaturation to occur.

The main requirement for stabilization appears to be the formation of a disulfide bond between a half cystine molecule and the viral -SH group. No compound has been found to produce stabilization as complete as L-cystine. The results suggest that the other compounds tested do not react with the viral -SH groups, and therefore one cannot decide whether formation of disulfide bonds with a stabilizing compound is in itself sufficient for stabilization. This does indicate, however, that the -SH groups must occur in an extremely stereo-specific environment.

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INTRODUCTION

The central phenomenon to be discussed in this work is the decrease in heat sensitivity of poliovirus after incubation with the disulfide compound cystine. The heat stabilizing effect of cysteine (-SH) on various biological materials is known, but in most instances this is understood to be due to the reducing power of the sulfhydryl group of the cysteine and can be duplicated with various other sulfhydryl compounds (e.g. sodium thioglycolate). The stabilizing influence of cysteine upon various animal viruses has been studied by several workers. Mueller, in 1928, showed that the presence of cysteine greatly decreases the inactivation at 37°C of the chicken tumor I of Rous(1). Zinsser and Tang, in 1929, demonstrated the same effect with Herpes Simplex Virus (2). This type of stabilizing effect requires the presence of a high concentration of the -SH compound at the time of heating, the stabilizing effect being lost upon dilution. It will become evident in the discussion which follows that this is not the mechanism of the phenomenon studied in the present work.

The only report suggesting a phenomenon similar to the one studied here is that of Labzoffsky, 1946, who studied the effect of various reducing agents on Eastern Equine Encephalomyelitis (3). This virus was found to have a greatly increased resistance to inactivation at 37° C when in the presence of as little as 0.005 mM cysteine. Since thioglycolate gave no protection, it is likely that the protection by cysteine was not based upon the reducing activity of this compound.

The basic features of the cystine stabilization reaction with Poliovirus have been studied in some detail by Pohjanpelto (4). The results of this worker were generally confirmed in the course of the present work. These characteristics may be summarized as follows:

1. The effect is not genetic. The progeny of stabilized virus has the same heat sensitivity as non-stabilized virus.

2. The stabilization is not reversible by dilution at 37° C, either at pH 7 or pH 4. A true chemical change has therefore occurred.

3. An increase in the temperature or the cystine concentration increases the rate of stabilization. Treatment with 0.2 mM cystine at 37° C and pH 7.4 produces maximal stabilization in about four to six hours.

4. The stabilization is pH dependent. The virus particles are stabilized most rapidly at pH's greater than 7. The rate of stabilization decreases as the pH is lowered from 7 to 4.

5. The stabilization appears to be all or none. It was found, from a study of the kinetics of inactivation, that a virus population only partially stabilized is essentially a mixture of completely stabilized or completely sensitive particles. A very small fraction of particles, if any, exhibits a heat sensitivity intermediate between stabilized and sensitive virus.

6. The stabilization may be prevented by para-chloromercuribenzoic acid (PCMB), a compound which reacts rather selectively with -SH groups.

7. Stabilization is destroyed by treatment with an excess of the sulfhydryl reducing agent, sodium thioglycolate.

Pohjanpelto also studied the inactivation of the virus at 50° C. These studies showed that inactivation was O_2 dependent, and that it could be inhibited by an excess of sodium thioglycolate. In addition, a phenotypically resistant fraction of virus was observed in all stocks in the absence of cystine. This resistant fraction was shown to retain its resistance in the presence of an excess of sodium thioglycolate, and therefore was considered not to be due to stabilization by cystine during the intracellular development of the virus.

The general conclusion from these results was that the usual heat inactivation of the virus at 50° C is due to the oxidation of -SH group(s); that cystine reacts with free sulfhydryl group(s) in the virus

coat protein, and thus stabilizes this protein against modification by heat. From kinetic data it was estimated that there are six to ten such -SH groups which must be stabilized.

The present work has been done in an attempt to understand more fully both the nature of the union between cystine and the virus protein, and the nature of the inactivation process.

MATERIALS AND METHODS

Phosphate Buffered Saline (PBS) - This pH 7.4 saline buffer was used in the preparation of the standard virus stocks, and also, in a few cases, for dilutions of virus prior to heat inactivation. One liter of PBS contains: 8 gm NaCl, 0.2 gm KCl, 0.1 gm $CaCl_2$, 0.1 gm $MgCl_2 \cdot 6\,H_2O$, 1.15 gm Na_2HPO_4, and 0.2 gm KH_2PO_4.

Versenate Buffer - This buffer of pH 7.4 was used in several experiments in which inhibition of -SH oxidation by O_2 was desired. One liter contains: 0.4 gm disodium versenate, 8 gm NaCl, 0.2 gm KCl, 1.15 gm Na_2HPO_4, and 0.2 gm KH_2PO_4.

Nutrient Overlay Medium - This medium is used mixed with an equal volume of a 1.8% aqueous solution of agar to make the nutrient agar overlay used in the plaque assay technique, and therefore is prepared with twice the normal concentration of all constituents. One liter contains: 2 gm Bovine albumen, 2 gm yeast extract, 2 gm glucose, 5.5 gm $NaHCO_3$, 14.35 gm NaCl, 0.8 gm KCl, 0.4 gm $CaCl_2$, 0.4 gm $MgSO_4$, 0.25 gm $NaHPO_4$, 0.003% phenol red, 40,000 units mycostatin, 10^6 units penicillin, 0.2 gm streptomycin.

Lactalbumin Hydrolysate Medium - This medium is used in the preparation of monkey kidney cell cultures. One liter contains: 5 gm lactalbumin hydrolysate, 0.0015% phenol red, 2.2 gm $NaHCO_3$, 7.2 gm NaCl, 0.4 KCl, 500,000 units penicillin, 0.1 gm streptomycin, and 20,000 units mycostatin. The medium is sterilized by filtration through a Seitz filter.

Monkey Kidney Monolayers - Petridishes of monkey kidney cell monolayers are prepared as follows: Kidneys are removed from the animal, diced with a scalpel, and then trypsinized to disperse the cells. After being washed with lactalbumin medium, the trypsinized cells are suspended in a lactalbumin hydrolysate medium containing 7% calf serum, and 5 ml of this suspension is then put into each petri-dish. The number of cells on each dish is such that a complete monolayer suitable for infectivity assays is present six days after preparation of the plates.

Infectivity Assay - In the experiments to be reported, infective Poliovirus particles are counted as plaque forming units on monkey kidney cell monolayers, the technique being essentially the same as that introduced by Dulbecco and Vogt(5). A 0.2 ml sample of an appropriate dilution of the virus suspension is allowed to adsorb at 37° C for 40 minutes, after which is added 5 ml of an agar overlay consisting

of 2.5 ml agar (1.8% in distilled H_2O) and 2.5 ml of the nutrient overlay medium. The plates thus prepared are incubated at 37° C in a 5% carbon dioxide atmosphere, and the plaques counted at 2, 2-1/2, and 3-1/2 days. Infective titers of virus samples are expressed as plaque forming units per milliliter.

Virus Stocks - The virus first used in this work was line R 68 of strain Brunhilde. This is by definition a genetically heat sensitive line. Various mutants considerably more resistant to heat inactivation have been isolated from it. During the course of the present work this line was purified by three successive plaque isolations, and a master stock made from the third isolate. The medium used to prepare this stock was Eagle's medium with monkey serum. This master stock was frozen, and subsequently used for the preparation of fresh stocks of virus for each experiment. In order to simplify the system as much as possible, these "experimental" stocks were grown in phosphate buffered saline medium in which glutamine (0.031%) was the only organic constituent. The principal chemical variable was then the material released by the cells as a result of virus infection.

The experimental stocks were prepared as follows: Monkey kidney cell monolayers were washed twice with phosphate buffered saline; 0.2 ml of virus stock were adsorbed for two hours, the plates

washed five times to remove the serum containing medium of the master stock, and finally overlaid with 5 ml phosphate buffered saline plus 0.03% glutamine. The virus was harvested after 20 to 24 hours, when most of the cells had detached from the glass, and centrifuged at 2000 rpm for 15 minutes to remove cell debris. A preliminary assay was then made, while the stock was stored at 4° C until the results of the assay were available. Stocks regularly reached a titer 1 to 2 x 10^8 pfu per ml. Most experiments were carried out 3 to 4 days after harvest of the stock. In control experiments, no loss of virus infectivity could be detected after several weeks at 4° C.

Stabilization Procedure - L-cystine (Calif. Corp. for Biol. Research) was dissolved in 0.2 ml of 1.5N HCL, made up to 1.0 or 1.5 mM with 0.1 molar phosphate buffer, and the final pH adjusted to about 7.1 with 1N NaOH. Any dilutions were made in 0.1 molar phosphate buffer at pH 7.4.

Before treatment with cystine, the virus stock was usually diluted with an equal volume of 0.1 molar phosphate buffer at pH 7.4. Equal volumes of the virus dilution and of cystine at the proper concentration were then mixed and placed at 37° C for the required time.

Treatment with any other compounds tested for their ability to produce stabilization was done in essentially the same way.

It should be mentioned that some difficulty was experienced in attempting to obtain maximal stabilization of all virus particles. Although complete stabilization (as shown in Fig. 1) was occasionally achieved, most often only about 30% to 60% of the particles appeared to be stabilized.

Inactivation Procedure - For routine testing of the extent of stabilization of a given sample of virus, 1 ml of appropriately diluted virus suspension in a 13 mm test tube was placed into the 50° C water bath for either 5 or 20 minutes; the 5 minute test was used most frequently. At the time the inactivation test was begun, an identical sample was placed into an ice bath to serve as the unheated (0 minute) sample. Inactivation was stopped by removing the entire tube into an ice bath. For more critical experiments, this procedure was modified according to the demands of the situation. The most common modification was to introduce 0.2 ml virus suspension into 1.8 ml buffer preheated to 50° C. Tubes with loosely fitting metal caps were used to permit circulation of air during the heating. The water in the bath was stirred throughout the inactivations to maintain the temperature between 49.8° C and 50.1° C.

Virus survival after heating (V/V_o) is measured as the ratio of the infective titer remaining after the heating (V), to the titer of the unheated sample (V_o) which was otherwise treated in an identical way.

A virus sample is considered to be stabilized if its survival after a certain heat treatment is greater than that of the control virus exposed to the same heat treatment. Stabilization is considered to be blocked if the survival after heating of a virus sample exposed to a stabilizing treatment is significantly lower than that of control virus exposed to the same stabilizing treatment. In the routine inactivation tests consisting of heating at 50° C for 5 minutes, the non-stabilized virus had a survival of 1 to 5×10^{-4}, while the completely stabilized virus had a survival of 6 to 8×10^{-1}.

Inactivation curves of stabilized and non-stabilized virus in 0.1 molar phosphate buffer at pH 7.4 are shown in figure 1.

EXPERIMENTAL RESULTS

A. NATURE OF STABILIZATION

A study of the nature of the heat stabilization produced by cystine includes a consideration of several related problems. First, one must consider the mechanism of the reaction between virus and cystine: With what chemical grouping(s) does cystine react? Does cystine remain bound to the virus? Is the structure of the virus altered by the reaction with cystine? Secondly, one must attempt to understand why this reaction results in a virus particle with increased resistance to heat inactivation: Is the chemical group with which cystine reacts essential to virus infectivity? What reaction occurring at 50° C is prevented by prior treatment with cystine?

It is convenient to visualize these problems on the background of hypothetical models for the stabilization. According to one model, the chemical group reacting with cystine is also the heat sensitive group; modification of this sensitive group by heat would lower the activation energy for denaturation of the protein coat; prior reaction with cystine would prevent the modification caused by heat. According to a second model, the sensitive group is required for infectivity,

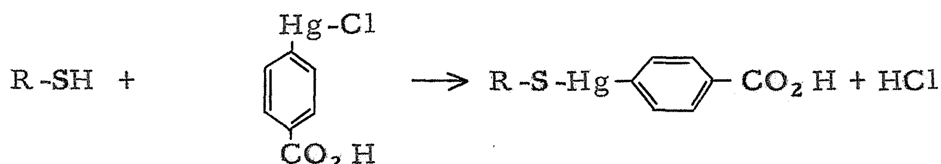
and thus the modification of this group by heat directly causes inactivation of the virus. Under either model, to understand the process of stabilization, the mechanism of inactivation must also be understood. The process of inactivation will be discussed in a later section of this thesis.

Two methods of approach have been used to obtain most of the information on the nature of stabilization. One is the use of compounds which prevent stabilization; the other involves the testing of various compounds similar to cystine in order to define the requirements for stabilization.

I. STUDY OF COMPOUNDS PREVENTING STABILIZATION

Parachloromercuribenzoic Acid (PCMB)

PCMB is considered to be one of the most specific and most reactive -SH blocking reagents. It is not, however, absolutely specific; in evaluating the results obtained with this reagent, the possibility of addition of PCMB to an unknown group other than -SH must always be considered (6, 7). PCMB is especially useful in that it reacts with single -SH groups, unlike most sulfhydryl oxidizing agents which require the proper steric configuration of two -SH groups. The reaction may be written as:



The R-SH complexed with the mercurial can be replaced by another sulfhydryl compound (R'-SH) if the R'-SH has a greater affinity for mercury than R-SH, or if its concentration is in sufficient excess over that of R-SH. The situation is one of competition between the two sulfhydryl compounds for the mercury. Therefore, if the PCMB becomes bound to an -SH group in a functional protein, any modification of activity caused by the binding should be reversible in the presence of an excess of added -SH compound (such as cysteine or sodium thioglycolate). If the modification of activity results from some other mechanism, reversal is unlikely. The reversal of the functional modification caused by PCMB then furnishes strong confirmatory

evidence that an -SH group is the functional group affected by the reagent. For these reasons it was of importance to extend the original observation of Pohjanpelto (4), who simply demonstrated that PCMB can prevent stabilization.

In interpreting the following data, one must remember that blockage of only one -SH group (out of six to ten) by PCMB may be sufficient to render the virus incapable of being stabilized. On the other hand, to reverse the effect of PCMB, all -SH groups must be freed.

Two experiments may be reported to present the major findings obtained with PCMB.

I. The first experiment demonstrates two points: a) PCMB prevents subsequent stabilization by cystine. b) Virus already stabilized by cystine does not lose its stability in the presence of PCMB. The experiment was done by adding PCMB to a poliovirus-cystine mixture after a fraction of virus had already been stabilized; the fraction of stabilized virus was then determined as a function of time, and compared with that observed in a control mixture to which PCMB was not added. The PCMB was added after stabilization had begun because a lag period usually occurs after mixing poliovirus and cystine, before an increase in stabilized particles is detectable. If PCMB were added at time zero along with the cystine, no increase in stabilized particles would be seen, and therefore no observations could be made either on

the rate of PCMB action, or on its effect upon previously stabilized virus particles.

Virus and cystine were mixed at time 0 to give a final cystine concentration of 0.2 mM, and placed into the 37° C bath. At various times, 0.2 ml samples were removed and diluted into 1.8 ml phosphate buffer pre-equilibrated at 50° C; after 20 minutes at this temperature the samples were chilled, and assayed for active virus to determine the extent of stabilization at the given time. At 1-1/2 hours a sample was taken and mixed with a solution containing PCMB and cystine, giving a final PCMB concentration of 0.05 mM, and maintaining the cystine concentration at 0.2 mM. At various subsequent times the virus survival after heating twenty minutes at 50° C was determined for the PCMB containing mixture as well as for the original control mixture.

The fractions of virus surviving after the twenty minute heating are plotted in figure 2. These fractions are not equal to the fraction of stabilized virus; but it is known from Pohjanpelto's work that they are approximately one half of this fraction. In the present work the inactivation rate of stabilized virus was different from that observed by Pohjanpelto, and consequently one cannot assume that this same ratio applies, although it cannot be very different. Since this relationship was not determined in the present work, the twenty minute survivals, rather than an estimated fraction of stabilized virus, have been used. It can be assumed, however, that the twenty minute survival

does bear some fairly constant proportional relationship to the fraction of stabilized virus, and the serves as a useful measure of the relative change in the fraction of stabilized virus.

The experiments show that in the sample treated with PCMB, the blocking of stabilization is complete between twelve and thirty minutes after addition of the PCMB. Since the survival of the PCMB-treated sample at eight hours is not lower than the survival at two hours, it is probable that PCMB does not cause any reversal of stabilization.

II. The second experiment demonstrates that the PCMB-caused inhibition of stabilization by cystine can be removed by treating the blocked virus with an excess of thioglycolic acid (TG). Cysteine, unlike TG, does not remove the PCMB inhibition, but it does react with free PCMB. This property was utilized in a parallel control experiment which determined the extent of blocking by PCMB at the time of TG addition. A second control independently demonstrated that the cysteine added to the first control was sufficient to remove all free PCMB from further reaction with the virus.

0.2 ml of 0.5 mM PCMB was added to 1.8 ml of virus suspension and placed at 37° C for thirty minutes. At this time 0.5 ml of the mixture was diluted with 1.5 ml of cold versene, to inhibit oxidation of the TG; crystalline sodium thioglycolate (TG) was then added to give a final TG concentration of 0.1 molar. After thirty minutes at 37° C, a 200 fold dilution was made in 1.5 mM cystine solution, and this mixture was incubated at 37° C overnight (line 3 of table 1).

Before the initial addition to TG at 30 minutes, a control sample was removed and treated with 1/3 mM cysteine to react with all unreacted PCMB (line 1 of the table). The effectiveness of the cysteine in reacting with all unreacted PCMB was determined in another control experiment by adding cysteine to the PCMB just before addition of virus (line 2 of the table). These two controls were then mixed with cystine and incubated overnight at 37° C. The heat stability of these samples was tested by heating for five minutes at 50° C. The survivals (V/V_o) are given in table 1.

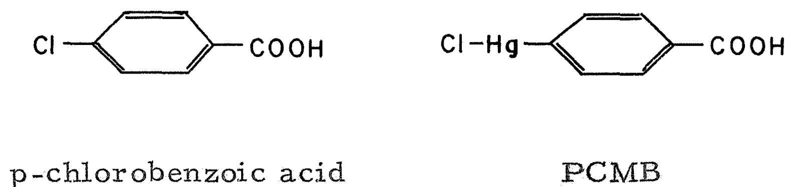
Table 1

		0' (V_o)	5' at 50° C	V/V_o
#1	(Virus + PCMB) 30 min + cysteine + cystine	8×10^7	8×10^4	10^{-3}
#2	[(PCMB + cysteine) + virus] 30 min + cystine	8×10^7	7×10^7	9×10^{-1}
#3	(Virus + PCMB) 30 min + TG + cystine	1×10^7	1×10^7	1.

In line #1 it is seen that the thirty minute PCMB treatment was sufficient to produce almost complete blocking (i.e., prevention of stabilization). Line #2 shows that the cysteine used in #1 can react with all free PCMB, since complete stabilization was obtained. A comparison of the survival of line #3 with that of line #1 demon-

strates that the TG treatment enabled the PCMB-blocked virus to be completely stabilized. Thus the effect of PCMB is completely reversed by the sulfhydryl compound, TG.

To check whether the formation of a mercury to sulfur linkage is responsible for the observed results, rather than some non-specific effect due to the benzoic acid configuration of PCMB, the effect of p-chlorobenzoic acid was tested.



p-chlorobenzoic acid PCMB

No inhibitory effect was noted when this compound was used at ten times the concentration normally used with PCMB.

These results with PCMB support the hypothesis that free -SH groups in the virus are essential for the stabilization by cystine.

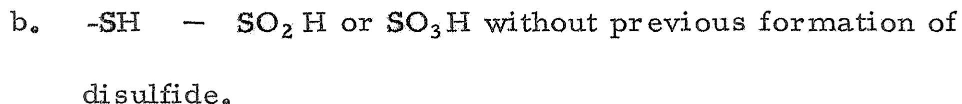
In relation to the question of the essentiality of these -SH groups for infectivity, it may be noted that PCMB does not inactivate poliovirus, even after nine hours at 37° C. This result is not a conclusive proof that the -SH groups are not essential, however, since the PCMB-virus complex is reversible in the presence of other -SH groups, and could be dissociated on or within the host cell.

Iodosobenzoic Acid

Although the PCMB results strongly suggest that free -SH groups are required for the stabilization reaction, it seemed important to obtain confirmatory evidence with additional reagents known to block -SH groups by mechanisms different from that of PCMB. One of the most useful of such other reagents has been o-iodosobenzoic acid, an oxidizing agent which is relatively specific for oxidation of -SH groups (6).

Hellerman et al. (8, 9) studied the reactivity of IB with various amino acids at pH 7 and room temperature, and found that it reacted only with sulfhydryl compounds. These were rapidly oxidized to the disulfide stage if no more than a slight excess of IB were used. With a large excess of IB, cysteine was oxidized beyond the disulfide stage. Cystine was not oxidized at neutral pH under these conditions, but in very acid media, cystine, as well as methionine, was oxidized. The authors suggest that at pH 7, two competing reactions occur:

a. 2 -SH — -SS-



b. -SH — SO_2H or SO_3H without previous formation of disulfide.

With equivalent amounts of -SH and IB, reaction a) predominates; with an excess of IB, reaction b) becomes more important. In reacting with proteins IB does not necessarily react with all the -SH groups, unless the protein is first denatured. It would also be expected

that IB will not oxidize two -SH groups to disulfide unless they occupy the proper spatial relationship.

In the present work, two effects of IB have been noted: 1) Prevention of the ability of poliovirus to be stabilized by cystine (blocking reaction), and 2) Inactivation of the virus. The blocking reaction occurs at room temperature or lower, and is not accompanied by significant virus inactivation. At 37° C virus inactivation occurs. Both of these reactions were studied in some detail. The inactivation reaction is discussed in a later section.

The basic procedure for studying the blocking reaction was the following: Equal volumes of virus and IB at the appropriate concentrations were mixed and incubated either at room temperature or at 17.5° C for the required time. At given times cysteine, prepared in versene immediately before use, was added in about 10 to 20 fold excess over the IB to stop the reaction by reducing all unreacted IB. Use of cysteine was possible because reversal of blocking by IB was never achieved with cysteine alone. After a short period at room temperature to allow the reduction to occur, cystine was added and the mixture then incubated at 37° C for several hours to permit stabilization. The fraction of stabilized virus was determined by heating at 50° C afterdilution into an equal volume of phosphate buffer. The pH at all times was maintained between 7.0 and 7.4, except in those experiments in which pH was an experimental variable.

a. <u>Concentration range.</u>

0.05 mM IB was found to block stabilization completely after 15 to 20 minutes at 27° C (room temperature). No effect was seen with 0.005 mM IB after 20 minutes at 27° C. Since the virus preparations contain non-viral protein which reacts with some IB, the absolute value of the effective concentration of the reagent does not have any theoretical significance for the process under study. An IB concentration of 0.05 mM has been used throughout this work.

b. <u>Rate of reaction at 17.5° C.</u>

The general characteristics of the blocking reactions by IB are shown best in the curves given in figure 3. These describe the inactivation at 50° C of virus samples treated for various periods of time with 0.05 mM IB at 17.5° C, followed by maximum stabilization with cystine. The "0" minute IB sample was done by reducing IB with cysteine immediately before mixing it with the virus suspension. The survival of the virus sample exposed 30 minutes to IB is higher than the survival of the control, untreated virus; this is an atypical result, not characteristic of IB-treated virus. In most other such experiments, the survival of completely blocked virus was identical to that of the control virus.

What is perhaps most striking about these curves is the appearance of very obviously intermediate rates of inactivation,

indicating that fairly large fractions of the virus population exhibit a heat sensitivity intermediate between fully stable and fully sensitive virus. This is in contrast to the results of Pohjanpelto on the inactivation of poliovirus after stabilization with various concentrations of cystine in the absence of IB (4). The survival curves of Pohjanpelto had suggested that virus particles having attained an intermediate degree of stabilization were only slightly more stable than non-stabilized particles; furthermore, the partially stabilized particles appeared to comprise only a small fraction of the population, the great majority of particles being either completely stable or completely sensitive. The absence of a whole range of intermediate degrees of stability is unexpected, since inactivation of any heat sensitive site can result in virus inactivation, as judged from the single-hit character of the inactivation curves. One would expect that the rate of inactivation should decrease as more of these sites are protected by cystine.

In attempting to interpret these IB data, two approaches are possible. If one accepts as experimental fact the absence of intermediate degrees of stabilization by cystine, these IB results on prevention of stabilization must be explained in some way other than simply blocking of -SH groups. If, on the other hand, the occurrence of intermediate degrees of stability is accepted on the basis of this IB experiment, one must explain why these are not observed during

the course of normal stabilization by cystine. This latter approach requires assumptions which are felt to be unjustifiable, while the former approach requires more reasonable assumptions, and has in addition led to an hypothesis which was proved to be useful in describing other data obtained in this work. This hypothesis is based upon the assumption that the various -SH groups of a virus particle have different reactivities; this will be referred to as the hypothesis of heterogeneity in the reactivity of -SH groups. This concept of heterogeneity will be utilized as the basis of discussion of many of the results still to be reported.

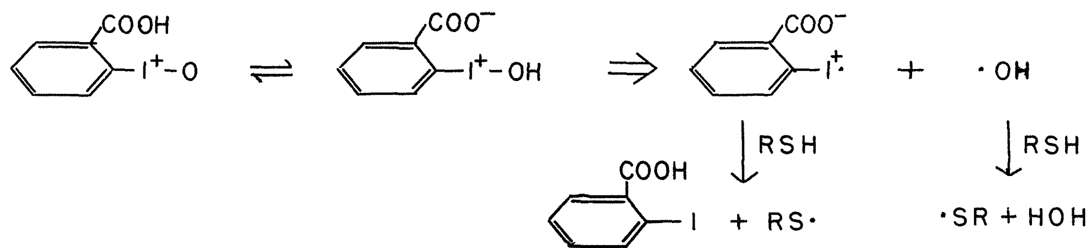
Assuming the heterogeneity of -SH reactivity, the preceding IB data may be described as follows: The IB treated, cystine treated virus is fully stabilized in the sense that the cystine has protected the groups which are sensitive to oxidation at 50° C, but the virus particles have been made more sensitive to heat inactivation by IB reaction at sites other than those which are oxidized by oxygen at 50° C. This hypothesis assumes that there exist (at least) two classes of viral -SH groups with the following properties: 1) -SH groups of the first class: they are oxidized (to an unknown oxidation product) by oxygen at 50° C, and by IB at 17.5° C mostly to disulfide. It is this class of -SH groups which must react with cystine to stabilize virus particles against the 50° C, aerobic inactivation. In the IB experiments, the addition of cysteine to reduce unreacted IB also reduces the disulfide

bonds formed by IB, restoring the original -SH groups; the subsequently added cystine will react normally with them. 2) -SH groups of the second class: they are not oxidized by atmospheric oxygen at 50° C, but are oxidized by IB at 17.5° C, possibly to $-SO_2H$ or $-SO_3H$. This oxidation is not reversible by cysteine. As more of these $-SO_3H$ groups accumulate on any virus particle, the virus becomes more sensitive to heat inactivation. The main difference between -SH groups of the two classes may depend upon steric factors. The groups of the first class may be sterically related to one another such that disulfide bonds are produced after mild IB action; those of the second class may not have a neighboring -SH group, with the result that disulfides cannot be formed, and oxidation to a higher oxidation state therefore occurs.

Such an hypothesis implies that oxidation of the -SH groups of the second class is not in itself responsible for the loss of virus infectivity, but that it increases the probability for the inactivation reaction (e.g. denaturation) to occur, this probability being related to the number of groups oxidized. This hypothesis also requires that the maximal rate of inactivation at 50° C is obtained when only one -SH group of the first class has been oxidized.

c. Effect of pH on the Blocking Reaction.

An important question is whether one could show that in our case the IB does indeed react with -SH groups. Some supporting evidence might be obtained by studying the pH dependence of the IB effect, since Pohjanpelto has shown the stabilization by cystine to be strongly pH dependent, maximal stabilization being attained at pH's greater than 7. In addition, aerobic oxidation of simple mercaptans has generally been found to be base catalysed (10). The situation in the oxidation of -SH groups by IB is somewhat complicated by the fact that the oxidation could go via a non-pH dependent free radical mechanism:



The important step in this case is the formation of the sulfur radical by abstraction of H· by either of the two radicals formed from the IB. Two RS· radicals can combine to form the disulfide, or, under conditions of excess IB, the R-S· might undergo further oxidation, perhaps by combination with ·OH. Abstraction of H· from R-SH by a radical is a well known reaction in the radical polymerization of styrene or

butadiene, where mercaptans limit the size of the polymer finally produced by yielding H• to the reactive radical end of the polymer chain, thereby terminating the chain (11).

The results obtained show a marked pH effect at 17.5° C, and a much smaller effect at room temperature (27° C). It is possible that this difference is due to the ionic reaction having a temperature coefficient different from that of the free radical reaction.

The experiment was carried out as follows: Equal volumes of virus and 0.1 mM IB, both in acetate buffer at pH 4.6, were mixed and placed, in one experiment at 17.5° C for 8 minutes, and in another experiment at 27° C for 35 minutes. At this time phosphate buffer at pH 7.4 was added, followed by cysteine to reduce unreacted IB. Cystine was then added, and the mixture placed at 37° C. The final pH of this mixture was about 7.0. After 8-1/2 hours at 37° C the virus was diluted with an equal volume of pH 7.4 phosphate buffer and heated at 50° C to test its stability.

The resulting inactivation curve for the experiment done at 17.5° C and pH 4.6 is shown in figure 3, where it may be compared with inactivation curves of virus treated with IB at pH 7.4 at 17.5° C. It is seen that there was nearly complete inhibition of the blocking reaction when it was carried out at pH 4.6 and 17.5° C. In the experiment at 27° C, however, the blocking was complete after the 35 minute

treatment (which is about twice as long as the time required to achieve complete blocking at pH 7.4 at this temperature). Thus the rate of the blocking could not have been greatly depressed at 27° C. The pH effect at 17.5° C indicates that the blocking reaction is centered at reactive groups having a dissociation constant compatible with that of -SH groups, but that the effect of pH is diminished at higher temperatures, perhaps because the higher temperature enables the oxidation to proceed by the free radical mechanism which does not require ionization of -SH groups.

d. Reversal of blocking reaction.

An experiment was done to test whether it is plausible that the prevention of stabilization caused by IB results exclusively from oxidation of -SH to -SS-. If this were the reaction an excess of added -SH compound should reduce the -SS- and permit complete stabilization to occur. If the oxidation goes beyond the disulfide, reduction by -SH is not possible. It should be emphasized, however, that the interpretation of the data to be presented is influenced by one's picture of the mechanism of "blocking."

Treatment with 10 mM cysteine was tried first. On subsequent exposure to cystine, no stabilization was obtained. Sodium thioglycolate was then used. Virus was treated at 17.5° C or at room temperature with 0.05 mM IB long enough to give complete blocking; thioglycolate (TG) was then added to give a final concentration of

0.1 molar. After 30 minutes at room temperature (to minimize virus inactivation before all free IB is reduced) and 20 minutes at 37° C, the mixture was diluted 1/200 into 1.5 mM cystine, and incubated 6-1/2 hours at 37° C. The heat resistance of this mixture was tested by heating for 5 minutes at 50° C, without dilution. An unheated sample was assayed also, since the TG treatment occasionally caused inactivation of the virus. Because of the lack of effect with cyst<u>e</u>ine, 0.05 mM cyst<u>e</u>ine was added to a parallel sample in place of TG, to determine the extent of blocking at the time of TG addition. The results of four such experiments are given in table 2.

Table 2

1 Exper. No.	2 Orig. stock titer	3 Titer after IB+TG+cystine (= V_o)	4 Titer of col. 3 after 5' at 50°C (= V)	5 Survival = V/V_o	6 Survival of control in which cysteine replaced TG
1	3.3×10^{7}	1.2×10^{7}	2×10^{6}	1.7×10^{-1}	not done
2	9×10^{7}	1×10^{8}	1×10^{6}	1×10^{-2}	1×10^{-3}
3	2×10^{8}	8×10^{7}	8×10^{4}	1×10^{-3}	5×10^{-4}
4	1.6×10^{8}	8×10^{7}	4.4×10^{4}	5.5×10^{-4}	5×10^{-4}

The IB treatment of number 3 was done at 17.5° C, while the rest were done at room temperature. Although experiment number 1 demonstrates the most significant effect of TG, no control was done to

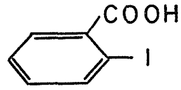
be sure that complete prevention of stabilization had been achieved. Number 2 is the only one of the four that demonstrates significant stabilization above that of the control.

The cause of the erratic results is unknown. They may be due to one variable which was not evaluated, i.e., the amount of non-viral organic material in the virus stock. Sulfhydryl groups in this material would be expected to reduce IB as efficiently as virus sulfhydryl groups. Thus the amount of IB available to virus -SH groups might vary according to the amount of non-viral -SH present. This could be an important factor, since in the presence of a large excess of IB, the -SH groups of the first class might be oxidized to $-SO_3H$, while in the case of limiting effective IB concentration, -SH groups of the second class might not be oxidized at all. In spite of this variability, some reversal of blocking was nevertheless obtained in experiment number 2. The data thus do not exclude the possibility that IB reaction with the virus can result in the formation of some disulfide bonds which prevent stabilization by cystine.

The results obtained can be accounted for by modifying slightly the hypothesis on heterogeneity of reactivity of -SH groups already presented to account for the prevention of stabilization by IB. That hypothesis as presented would predict that no stabilization is possible after IB treatment, whether TG is used or not, since the

inactivation at 50°C, of virus treated with cystine following IB treatment, is postulated to be a result of sensitization to heat by oxidation of -SH groups of the second class to $-SO_3H$ groups, which cannot be reduced by excess sulfhydryl compounds. The fact that some removal of blocking was obtained with TG suggests that the oxidation properties of the two postulated classes of sulfhydryl groups could better be characterized as follows: Sulfhydryl groups of the first class have a high probability of being oxidized to disulfides, but under some conditions, or with a constant low probability, are oxidized to $-SO_3H$; sulfhydryl groups of the second class are most frequently oxidized to $-SO_3H$ groups, but there is a very low probability of their being oxidized to disulfides. If disulfides were occasionally produced between -SH groups of the second class, and these were reduced by TG, but not by cyst<u>e</u>ine, one would observe greater virus survival after TG treatment than after cyst<u>e</u>ine treatment. If this explanation were correct, a complete inactivation curve of TG-treated virus would exhibit an intermediate inactivation rate. On the other hand, a curve with an unchanged initial slope, but with an increased fraction of completely stable virus, would cast considerable doubt upon the hypothesis as presented.

e. o-Iodobenzoic acid.



To test whether the blocking reaction by IB might result from some property other than the oxidizing activity of IB, virus was treated for one hour with 0.1 mM o-iodobenzoic acid at 37° C. Virus thus treated was completely stabilized by cystine, eliminating the possibility that some effect other than the oxidizing activity of IB was responsible for the prevention of stabilization.

Iodine.

Iodine, when used under proper conditions, is a reasonably selective oxidizing agent of -SH groups (12, 13, 14). It can, however, easily oxidize -SH beyond the disulfide stage, and conditions (especially I_2 concentration) must be controlled carefully to prevent this. Since in the present work the quantity of iodine needed to oxidize all -SH only as far as -SS- was not known, the oxidation state of the viral -SH groups following iodine treatment cannot be known either. The experiments carried out with iodine demonstrated that iodine treatment can prevent stabilization, although the blockage is not as complete as that obtained with IB. Perhaps the conditions used were not optimal; further work would therefore be required to clarify this point entirely.

The reactions of iodine which bear relation to the present work may be summarized as follows:

a. At 0° C, in IN KI, at pH 6.8, and with only a small excess of I_2, the principal reaction is 2 RSH → R-SS-R.

b. Oxidation may proceed beyond the disulfide stage in a large excess of I_2, or at low pH (the experiment reported in the literature was done at pH 3.2), or at an elevated temperature (experiment from literature used 37° C for two hours).

c. Iodination of tryptophan may also occur at low pH (pH 3.2), and tyrosine may be iodinated by reaction at higher temperatures (37° C, 2 hours). The presence of IN KI prevents the iodination of tyrosine even in a large excess of iodine.

Although the results reported in the literature varied somewhat depending upon the protein being studied, the conditions given in a) were found to be safest for specific oxidation of -SH groups.

Preliminary experiments showed that 1 mM I_2 at 37° C completely inactivated the virus, while 0.05 mM at 37° C had little, if any, effect on its activity. At 0° C, 1 mM I_2 was shown to prevent cystine stabilization to some extent, but inactivation occurring during the stabilization complicated the results. In further experiments, virus was treated for 30 minutes at 0° C with 0.5 mM I_2 in the presence of IN KI. At the end of this time, cysteine was added to reduce the

unreacted I_2 , and after 13 minutes in ice, a small sample of the mixture was diluted 1/40 into pH 7.4 phosphate buffered saline in ice. Following this, the virus was treated with 0.4 mM cystine for 8-1/2 hours at 37° C, then diluted with an equal volume of pH 7.4 phosphate buffered saline. One aliquot was placed into ice while the rest was heated at 50° C for 5 minutes. The results are given in table 3.

Table 3

	pfu/ml
a) titer of untreated stock virus	1.0×10^8
b) titer of virus from a) after 30 minutes I_2 at 0° C	7.5×10^7
c) titer of virus from b) after 8-1/2 hours cystine treatment at 37°C (= V_o)	3.2×10^7
d) titer of virus from c) after 5 minutes at 50° C (=V)	3.0×10^5

survival: $(V/V_o) = 9.4 \times 10^{-3}$

There was relatively little inactivation during the iodine treatment under these conditions. Cystine stabilization was inhibited only partially, but sufficiently to demonstrate the occurrence of the phenomenon. The use of a single 50° C five minute test point for determining heat stability subjects these data to the same limitations as those discussed in connection with the IB experiments. Here, also,

it would be important to determine the rate of inactivation; this, however, depends upon the availability of a complete inactivation curve.

Ferricyanide ion.

Ferricyanide ion is a mild oxidizing agent which is fairly specific for -SH groups when used at pH 6.8 for a short period of time (15). It may, however, fail to oxidize some -SH groups which are oxidized by iodosobenzoate. Ferricyanide ion oxidizes tyrosine, tryptophan, and cystine at increasing rate with increasing temperature, time, and concentration of ferricyanide. The major factor influencing these side reactions is the pH, however, the reactions being of little significance at neutral pH (the experiment described in the literature quotes pH 9.6 as the pH at which these oxidations are important). This reagent at 37° C and pH 6.8 for ten minutes gives essentially no reaction other than 2 -SH — -SS-. In the case of some proteins, oxidation of -SH groups by ferricyanide requires the presence of 0.004 mM Cu^{++} (16).

In the present experiments, three methods of testing for a reaction of the virus with ferricyanide were used: 1) The ability of the treated virus to be stabilized by cystine was tested. 2) The capacity of the treated virus to react with PCMB was tested. If the ferricyanide had in fact oxidized the class one -SH groups to -SS-, stabilization by

cystine might still be possible by a disulfide exchange reaction between the new viral disulfide and the cystine. PCMB could not prevent this stabilization since it would involve no free -SH groups. 3) The heat stability of the ferricyanide treated virus itself was tested, since at the time these experiments were done, it was thought possible that the formation of intraviral disulfide bonds might result in heat stable virus particles.

Poliovirus (pH 7.2) was mixed with an equal volume of a suitable concentration of ferricyanide (pH 6.6) and the mixture placed at 37° C for ten minutes. 0.004 mM Cu^{++} was present in this mixture. Different samples of this mixture were then treated as follows:

a) diluted 1/10 into pH 7.4 phosphate buffered saline and heated 5 minutes at 50° C (test #3)

b) diluted 1/5 into pH 7.4 phosphate buffered saline and treated with 0.4 mM cystine (test #1)

c) diluted 1/5 into pH 7.4 phosphate buffered saline and treated with 0.4 mM cystine plus 0.05 mM PCMB (test #2)

After 4-1/2 hours at 37° C, b) and c) were diluted with an equal volume of pH 7.4 phosphate buffered saline, and the heat stability of the virus was tested by heating for five minutes at 50° C.

Over the concentration range 0.004 mM to 50 mM there was no effect of any kind produced by ferricyanide. The oxidation reaction

resulting in blocking of stabilization thus seems to require the more strenuous oxidizing effect of iodosobenzoate or iodine.

II. STUDY OF CHEMICAL REQUIREMENTS FOR STABILIZATION

Stabilization with cysteine.

The present work has confirmed Pohjanpelto's observation (4) that cysteine stabilizes in the presence of air. It is, however, very likely that this is due to the oxidation of cysteine to cystine before stabilization occurs, since cysteine in solution is very rapidly oxidized to cystine at neutral pH. The question of whether poliovirus can be stabilized by cysteine - in the reduced form - is of great interest for understanding the chemical mechanism of stabilization. To answer this question, stabilization should be carried out under conditions preventing the oxidation of cysteine. Such an experiment is easy to perform; however, it raises the following difficult problem. It is very likely that the stabilization process involves the formation of a disulfide bond between -SH group(s) of the virus and the half cystine molecule. Thus, if reduced cysteine is able to stabilize, its reaction in stabilization and its reaction to form cystine would be chemically identical oxidations requiring an oxidizing agent, such as atmospheric oxygen. Consequently, conditions preventing formation of cystine should also prevent stabilization by reduced cysteine. The experiment under discussion still has significance, however, for if stabilization with cysteine were found to occur under conditions preventing its oxidation, one would have to question seriously the

correctness of the hypothesis that stabilization involves the formation of disulfide bonds. These same conditions should not prevent stabilization by cystine, however, since the hypothesis does not require any net oxidation of -SH:

(Virus)-SH + R-SS-R ⟶ V -SS-R + R-SH

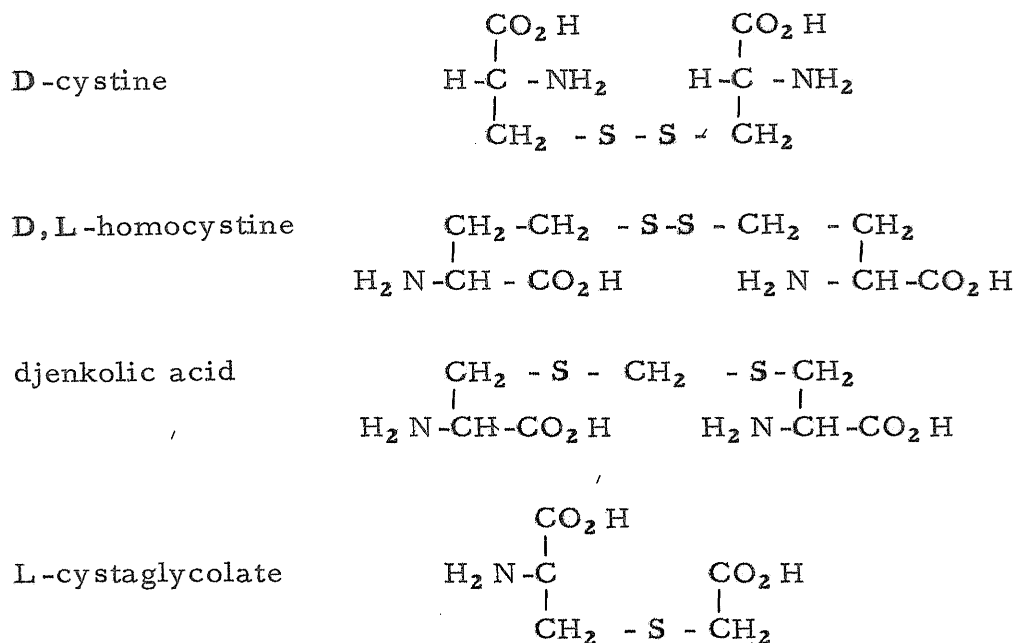
The experiment was carried out as follows: 1 ml of virus, 4 ml of versene buffer, (used to inhibit oxidation of the cysteine), and 0.05 ml of 1N NaOH were placed in the main section of a Thunberg tube. 0.628 mg of cysteine hydrochloride were placed in the cap, and the tube sealed. The tube was evacuated, filled with nitrogen washed with alkaline pyrogallol, and evacuated a second time. The cysteine was then dissolved and the tube placed into the 37° C bath. At the required time, the tube was opened, and its contents were diluted 1/20 into pH 7.4 phosphate buffered saline; 1.2 ml of the diluted material was heated at 50° C for 5 minutes, and assayed, along with a non-heated control, for active virus. The result was clear: Whether treated 2-3/4 or 6 hours with cysteine, the survival of the virus after heating was no higher than that of non-treated virus. A control using 0.8 mM cystine in place of cysteine, but using otherwise identical conditions, gave complete stabilization in 2-1/2 hours, demonstrating that cystine can stabilize equally well under aerobic or anaerobic conditions.

Other Chemical Requirements.

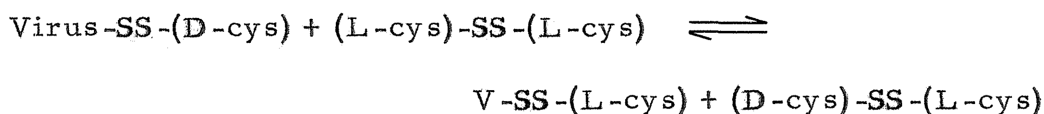
Although only stabilization produced by L-cystine has thus far been considered, it is through a study of compounds other than L-cystine that additional insight into the nature of stabilization might be gained. L-cystine is the normal metabolite, but the process of stabilization may have no relation to the metabolic requirements of virus multiplication. The strict requirement for L-cystine may be related to the fact that, in nature, this isomer is the one most readily available. Pohjanpelto has shown that D-cystine and D,L-homocystine do not produce stabilization. An additional selection of sulfur-containing compounds has been tested in the present study. These compounds were chosen primarily because of their chemical similarity to cystine.

Solutions of the compounds were prepared at approximately 1 to 2 mM, mixed with the virus, and incubated at 37° C from 6 to 8 hours or overnight. The mixture was diluted 1/10 before the heating test. Unheated samples were always taken at the same time and assayed for virus infectivity, since treatment with some of the compounds resulted in virus inactivation.

Compounds definitely producing no stabilizing effect were:



It seemed possible that D-cystine or homocystine might react with the virus, but having the "wrong configuration," could not stabilize it. Two methods were used to test this possibility. First, if the "wrong" compound were in fact bound to the active center, it might prevent stabilization by L-cystine. It was found, however, that a 6 hour pretreatment with 0.4 mM D-cystine or with 0.4 mM D,L-homocystine did not prevent stabilization of the virus by 0.4 mM L-cystine. Since disulfide exchange reactions such as:

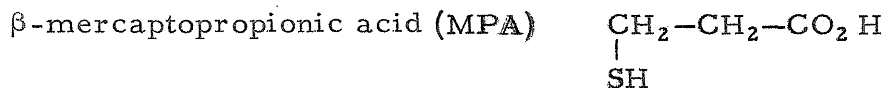


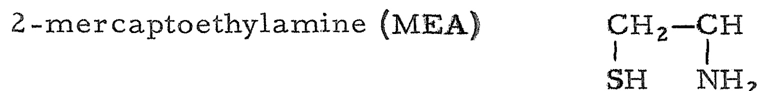
$$\text{Virus-SS-(D-cys)} + \text{(L-cys)-SS-(L-cys)} \rightleftharpoons \text{V-SS-(L-cys)} + \text{(D-cys)-SS-(L-cys)}$$

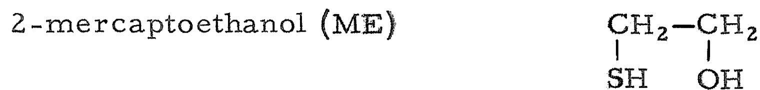
might allow cystine to stabilize normally in spite of D-cystine or homocystine being bound, a second approach was tried. If the vital -SH groups were bound to either of these compounds, they could not

react with PCMB; thus addition of this reagent after treatment of the virus with D-cystine or homocystine should not be able to prevent stabilization by L-cystine. When this experiment was done, 0.005 mM PCMB was found to prevent stabilization just as completely as in the normal case. There is thus no evidence that either D-cystine or D,L-homocystine react in any way with the reactive -SH groups of the virus.

Various other compounds tested have given variable results in the stabilization tests. As a consequence of this variability, no definitive conclusions concerning the chemical requirements for stabilization can be made; the variability might be eliminated by further experiments. On the basis of the available data, it is only possible at present to make preliminary hypotheses about the chemical requirements for stabilization. The compounds in this group are all thiols:







Virus samples treated overnight at 37° C with either 2 mM MPA or 2 mM MEA have exhibited, after five minutes at 50° C, survivals significantly higher (about ten fold) than the untreated controls. PA appears to have an even smaller effect, if it is significant, and ME had no effect at all on the survival.

The possibility was considered that, in the stoppered tubes used at first in these experiments, the concentration of sulfhydryl groups might establish a reducing environment sufficient to inhibit stabilization. Consequently, in one experiment the solution of the compound to be tested was bubbled with air several times during a six hour incubation at 37° C (to enable oxidation to disulfide to occur), the virus then added and this mixture also bubbled with air occasionally during the treatment. The tubes were covered with loosely fitting metal caps to permit circulation of air. In spite of the increased opportunity for oxidation, stabilization was not increased beyond that previously observed with each compound.

One of the first questions of interest to be explored in the case of MPA and MEA was whether the higher survival after heating was due to a decrease of the rate of inactivation or to an increase in the proportion of fully stabilized particles. This is of importance because the formation of particles as stable to 50° C as those treated with cystine would imply that the only requirement for stabilization is the formation of disulfide bonds. If this were true, one would

expect that, given the proper conditions, the entire population of virus particles would be stabilized. If the rate of inactivation were changed, however, two alternative explanations would be possible:
1) The compound reacts with all the virus -SH groups, but the stabilization is not as effective as with cystine due to the differences in the part of the molecule not bound to the virus sulfur, and which permit additional interaction between the bound compound and the virus protein - for example, an electrostatic bond between the carboxyl group of cystine and a positive charge on the protein.
2) Depending on the nature of the chemical groups surrounding them, the virus -SH groups vary in their reactivity to cystine analogues; thus, only some -SH groups would react with any of the thiol compounds tested, but all groups would react with cystine. These alternatives can be distinguished experimentally by determining whether cystine can increase the stability of these partially stabilized particles, and, in the positive case, whether PCMB, added before cystine, prevents the increase in stability. If this experiment were entirely positive, it would show that cystine is reacting with residual free -SH groups, and alternative number 2) would be supported. If cystine did not increase the stability, or if PCMB could not prevent the increase, one would conclude that all the free -SH groups of the virus reacted with the test compound (alternative number 1). If

alternative number 2) were to be indicated by the results, the use of MPA along with MEA might have an additive effect.

A few preliminary experiments have been done along the lines indicated above. The results must not be considered conclusive. A 50° C inactivation curve was done with MPA-treated virus. This curve indicates that the inactivation rate of the entire population has been changed. Virus treated with either MPA or MEA appears to be completely stabilizable when it is subsequently treated with cystine. Exposure to PCMB before cystine prevents this additional stabilization. The MEA plus MPA experiment was not done.

From these results one can form the tentative conclusion that MPA and MEA do not react with all the sensitive -SH groups, presumably because they lack the necessary chemical qualifications for reaction at certain of these critical states. This conclusion implies that all the -SH critical sites are not identical, and that stabilization of a few critical sites can produce a measurable degree of stabilization.

CHARACTERISTICS OF THE INACTIVATION REACTION

To understand the phenomenon of stabilization it is essential to know what is prevented by this stabilization. The present investigations were expanded to include a study of the properties of heat inactivation. Pohjanpelto's work had shown that the exclusion of oxygen, or the presence of an excess of reducing agent during heating, resulted in an inhibition of the inactivation at 50° C. An oxidative reaction is therefore involved at some point in the inactivation process. Since -SH groups are oxidized fairly easily, the simplest tentative hypothesis for a mechanism of inactivation is that the -SH groups with which cystine reacts in stabilization are the same groups which are oxidized in the inactivation process. The work to be discussed here supports this idea and suggests some additional hypotheses about the inactivation process.

It should be pointed out that no detailed chemical studies of the mechanism of mercaptan oxidation have been carried out to date. This makes any interpretation of the experiments to be reported somewhat uncertain. It is generally accepted that aerobic oxidation of -SH groups to oxidation states higher than the disulfide is very unlikely, but, in proteins, the postulated sulfur free radical produced in the oxidation process might find some other group with which to react.

The reactivity of sulfur radicals is not very great, however, and such radicals would probably be able to react only with very reactive aromatic nuclei (e.g. tyrosine) in addition to other sulfhydryl groups. Because of the uncertainties involved, it is probably best, at present, to make no assumptions with regard to the chemical nature of the oxidation reaction which occurs in the aerobic inactivation of polio-virus at 50° C.

I. INACTIVATION UNDER CONDITION OF REDUCED OXYGEN TENSION

Experiments on virus inactivation in the absence of oxygen were done to extend Pohjanpelto's basic observation that inactivation is decreased under anaerobic conditions. Since complete inactivation curves were not published, it was not possible to know whether the decreased inactivation was due to a change in the rate of inactivation, or whether some fraction of the virus population had been completely protected. To clarify this point, the following experiment was carried out:

Virus was diluted either 1/10 into versenate buffer (experimental) or 1/10 into pH 7.4 phosphate buffered saline (control). Versenate buffer was used to chelate any metal ions present which might catalyze the oxidation of -SH. The virus in versene was distributed into several tubes, and bubbled for four hours with nitrogen washed in alkaline pyrogallol. The tubes were then sealed, and placed into the 50° C bath. The control was heated in the normal way in tubes with loosely fitting metal caps. Individual tubes were removed into an ice bath at appropriate times. The results, given in figure 4, show conclusively that although reducing the concentration of O_2 does inhibit the inactivation, it does so not by reducing the rate of inactivation of the entire population, but rather by increasing the proportion of

maximally resistant particles. That is, the inactivation curve of the control reaches a very shallow slope after ten to twenty minutes heating, at a survival of about 5×10^{-5}, while the N_2 -versene virus reaches a plateau at a survival of about 1×10^{-2}. The variability in the survival values found for the N_2 -versene curve probably results from the variable extent of elimination of O_2 from tube to tube. This makes it impossible to compare the slope of the inactivation curve of this "resistant" virus fraction with that of the resistant fraction appearing in the aerobic inactivation curves. When one performs the inactivation in the presence of 0.1 molar thioglycolate, the survival is considerably greater (about 3×10^{-1} at five minutes). It is likely that the lower survival obtained in the anaerobic experiment is due to incomplete removal of oxygen.

The lack of change in the inactivation rate was somewhat surprising. However, in the light of the experiments with iodosobenzoic acid inactivation to be reported later, this observation finds a possible explanation. The IB work suggests that inactivation is at least a two step reaction, with the oxidation being the first step. If we remember that oxidation at a single sensitive site is sufficient for inactivation, and we assume that the oxidation is not the rate limiting process in inactivation, the absence of a change in inactivation rate can be explained. Under anaerobic conditions the oxygen is limiting, and

is perhaps used up before all virus particles have had at least one -SH group oxidized. In the population there would then be one fraction consisting of oxidized, inactivable particles, and another fraction composed of non-oxidized, non-inactivable particles.

A considerable number of experiments were done to determine whether the inactivation of stabilized virus was also dependent upon oxygen. Comparisons were made between samples bubbled with nitrogen and others bubbled with air during the heating. It was subsequently found that the bubbling itself has some variable inactivating activity, and thus no conclusions could be drawn from this work. Experiments have not been done using Thunberg anaerobic conditions, which would eliminate the need for bubbling. It is interesting to note that observations of surface inactivation by bubbling have also been made with some bacterial viruses (17). In the present work, as well as in the work with bacterial viruses, the presence of proteins in the medium was found to protect the virus from this type of inactivation.

II. EFFECT OF pH

The effect of pH on the stabilization reaction has already been reported by Pohjanpelto (4) and was not studied here. A study of the inactivation rate as a function of pH during heating was undertaken, considering that if the inactivation proceeds through the same reactive groups as the stabilization, it might exhibit a similar pH dependence. On the other hand, the results of experiments on the inactivation by IB (to be presented in a later part of this thesis) have suggested that the effect of low pH is not upon the oxidation of the -SH groups, but rather upon a subsequent step in the inactivation. If this result were also to apply to aerobic inactivation, as in the experiment being considered here, inhibition by low pH would not necessarily imply the participation of sulfhydryl groups.

Buffers were prepared according to Gomori (18), and were the following:

Na_2HPO_4	$-NaH_2PO_4$	-pH 7.51, 6.90, 6.03
citric acid	$-Na_2HPO_4$	-pH 6.14, 4.76
acetic acid-sodium acetate		-pH 4.63

Overlapping buffer ranges were chosen so that the effect of the same pH could be tested with two different buffers; any influence on inactivation rate by any of the ions themselves could thus be detected. The buffers were generally used at 0.1 molar, which was quite sufficient

to maintain the pH when the virus sample was diluted 1/5 to 1/10 into the buffer. Immediately after heating, the sample was diluted 1/5 into 0.1 molar pH 7.4 phosphate buffer, which brought the pH between 7.0 and 7.4, and enabled the sample to be plated undiluted when necessary.

45° C was chosen as the inactivation temperature in this case, since the inactivation at 50° C was too fast for the inactivation curves to be determined conveniently. The rates of inactivation were not calculated due to the complex nature of the curves; a qualitative comparison of the curves is sufficient to yield the desired information about pH dependence of inactivation.

Heating was begun by introducing 0.2 ml of virus stock (diluted 1/2 into PBS) into 1.8 ml of the appropriate buffer at 50° C. The entire tube was removed to the ice bath to end heating. Figure 5 shows the results.

It is seen in this figure that lowering the pH markedly slows the rate of inactivation at 45° C. The greatest change occurs between pH 7 and pH 6. The curves obtained below pH 6 are not significantly different in this experiment. The fact that the rate of inactivation decreases sharply below pH 7 agrees very well with the observation of Pohjanpelto that the stabilization is inhibited markedly as the pH falls below pH 7. This similarity supports the hypothesis that the groups reacting with cystine are also directly involved in the inactivation reaction.

III. INACTIVATION BY IODOSOBENZOATE

It was observed that incubation in IB at 37° C caused inactivation of the virus at a rate much faster than in the absence of the reagent. This phenomenon has been studied in some detail, and the results to be presented will attempt to show that the inactivation produced by IB is in many respects chemically similar to the normal 50° C heat inactivation. In spite of the fact that IB is specific for -SH groups when reacting at low temperature, one cannot assume that the inactivation of the virus at 37° C, which is much slower than the reaction of IB with -SH groups, is due to an action of IB upon these groups. It was therefore essential to demonstrate that the chemical groups attacked by IB in this inactivation were in fact the heat sensitive -SH groups. The experiments carried out have reached the point where one can draw several tentative conclusions about the reactions resulting in virus inactivation. A definitive scheme of reactions, however, will have to await further experiments.

The first experiments were carried out as follows: The virus stock was diluted 1/5 into 0.1 molar pH 7.4 phosphate buffer, mixed with an equal volume of 1 mM IB, and immediately placed into the 37° C bath. To stop the IB reaction, samples were diluted 1/20 into phosphate buffer in ice at appropriate times (including 0 minutes). Control experiments demonstrated that there is negligible inactivation

of the virus when the reaction mixture is diluted 1/20 into cold buffer and kept at ice bath temperature. The results of such an experiment are shown in figure 6, curve #1. The slope of the curve was found to be quite variable from one experiment to another, possibly due to the presence of variable amounts of cellular debris with which IB may react. This variability requires that any comparisons of rates of inactivation be made in the same experiment. It should be noted that, although they will be discussed separately, all the curves given in figure 6 were obtained in the same experiment, and thus the differences in rate are meaningful.

a. Relationship of IB inactivation and 50° C inactivation.

An important question which arose immediately was whether this inactivation could be considered to be analogous to the normal 50° C inactivation. That is, whether at 37° C the IB carries out the same oxidation which occurs at 50° C in the absence of IB (but in the presence of oxygen). Curve 2 of figure 6 shows that cystine stabilized virus is almost completely protected from the IB inactivation. This would not be so if the IB inactivation reaction were centered at some other, non-cystine protected site. Therefore, all IB-sensitive sites can be protected by cystine.

The second line of evidence is somewhat less direct. It will be recalled that Pohjanpelto observed in all poliovirus stocks a fraction

(about 10^{-4}) of particles which is phenotypically resistant to heat inactivation. One explanation is that in this fraction the heat sensitive groups have in some way been protected. If the IB sensitive groups were different from the heat sensitive groups, survivors of IB inactivation would be a random sampling of the virus population with respect to these phenotypically protected or unprotected heat sensitive groups. Survivors of IB inactivation when tested at 50° C should therefore always contain the normal fraction of heat stable particles. On the other hand, if the heat sensitive and the IB sensitive groups were identical, heating at 50° C of IB survivors should always yield the same absolute number of surviving heat stable particles; the fraction of these particles among IB survivors would, of course, increase as the fraction surviving IB treatment decreases.

To test this, virus was treated with 0.05 mM IB at 37° C for 1-1/2, 3, and 4-1/2 hours. At these times, samples were diluted 1/20 into phosphate buffer, one portion of which was placed into ice, while the other was heated at 50° C for 5 minutes. Both portions were then assayed for active virus. The results are shown in table 4.

Table 4

1 Length of IB treatment	2 Titer of virus after IB treatment: (V_o)	3 5 minutes-50°C (V)	4 V/V_o
0 hours	2.9×10^7	7×10^3	2.5×10^{-4}
1-1/2 hours	3.0×10^6	5×10^3	1.7×10^{-3}
3 hours	7.7×10^5	4.6×10^3	6.0×10^{-3}
4-1/2 hours	2.4×10^5	4.9×10^3	2.0×10^{-2}

It is clear that the absolute number of particles in the stable fraction remains constant in spite of the hundred fold loss of infective virus due to IB inactivation. This result implies that IB inactivation at 37° C has at least one step in common with aerobic inactivation at 50° C, and is consistent with the hypothesis that both types of inactivation are centered at the same sensitive group.

b. Effect of pH.

An additional test for a sulfhydryl group reaction might be to determine whether low pH inhibited this inactivation by IB. It will be remembered that low pH inhibited the blocking reaction at 17.5° C, but not at 27° C. If the 37° C inactivation reaction is limited to these same sites, it should not be inhibited by low pH, but if additional -SH groups are oxidized in the inactivation, inhibition might be observed.

With this in mind, the following experiment was done. It should be noted, however, that the pH could have its effect on the (postulated) subsequent denaturation, and not on the oxidation reaction. Experiments to be reported in the following sections, in fact, are most easily understood if one assumes that the acidic conditions do inhibit the denaturation, and not the oxidation.

Virus and IB were first diluted into buffers at the appropriate pH (acetate-pH 4.6, phosphate-pH 6.9 and pH 7.5). The inactivation experiment was then carried out as already described. The results are given by curves 1, 3, and 4 in figure 6. It is seen that considerable inhibition of inactivation is produced by the lower pH, although there is still significant inactivation. This pH effect will be discussed in the next section, where additional data on the effect of pH are presented.

c. IB inactivation data supporting the hypothesis of heterogeneity in reactivity of virus -SH groups and of sensitization to heat by oxidation of a class of -SH groups.

The results from the work on the prevention of stabilization by treatment with IB at room temperature and at 17.5° C suggested the hypothesis that there exists a heterogeneity in the reactivity, and in the product of oxidation, of the virus -SH groups. One of the proposed classes would be oxidized to -SS-, while the other would be oxidized to

$-SO_2H$ or $-SO_3H$, the latter oxidation resulting in a concomitant increase in the probability of heat inactivation. Two modifications of the basic IB inactivation experiment yielded evidence in support of this hypothesis.

1) The first modification was to pretreat the virus with IB at room temperature before submitting it to the 37° C IB inactivation. This experiment was carried out to test for a different hypothesis which was being considered at the time the experiment was done. This alternative hypothesis was that every -SH group in the virus protein would be oxidized by IB at 17.5° C to -SS- only, with no accompanying increase in sensitivity to heat. This hypothesis predicts that a virus particle pretreated with IB at 17.5° C would not be inactivated by IB at 37° C, since all of its oxidized -SH groups would be disulfides, and IB does not attack disulfide bonds at pH 7.

The present hypothesis of heterogeneity in the reactivity of -SH groups predicts no such inhibitory effect of pretreatment. Under this hypothesis two situations could exist with respect to 37° C inactivation by IB: a) The treatment at room temperature could oxidize all oxidizable -SH groups; the inactivation at 37° C would then be entirely a result of the sensitization produced by these oxidations, since no additional -SH groups would be oxidized at 37° C. b) At 37° C additional -SH groups would be oxidized; the rate of inactivation at 37° C would

then be determined both by the previous oxidations and by those occurring at 37° C. If this were true, low pH might inhibit the inactivation resulting from additional oxidations, while for case a) no inhibition at low pH would be expected. Again one must consider, however, that if the effect of low pH were upon the denaturation, inactivation would be inhibited in either case.

A test of these possibilities was made by treating virus at room temperature for 35 minutes with 0.05 mM IB, then transferring it into 0.5 mM IB at 37° C at both pH 7.4 and pH 4.6. The inactivation at 37° C of this IB pretreated virus was identical with that of the non-pretreated virus at the same pH (curves #1 and #4 of figure 6 thus represent both pretreated and non-pretreated virus).

This result clearly excludes any model involving complete homogeneity in reactivity of all -SH groups, and supports the hypothesis of heterogeneity. One might have expected a faster inactivation rate at pH 7.4 for the pretreated virus than for the non-pretreated virus, since the former is presumably already partially (if not completely) sensitized. If one considers the relative rates of the two reactions at room temperature and at 37° C, however, this result is not surprising: the sensitization at room temperature is complete within 20 minutes, while it requires more than five hours to complete the inactivation at 37° C.

The result at pH 4.6 is more interesting. If one assumes that the effect of low pH is upon the oxidation reaction, the fact that the inactivation of pretreated and non-pretreated virus is identical suggests that the pretreatment at room temperature has had no sensitizing effect. On the other hand, if the pH effect were upon the denaturation, the effect of a sensitization might not be seen. Since this is a most important point, an experiment was done to test directly for a sensitizing effect of the room temperature IB treatment.

2) The second modification was to pretreat the virus with IB at 17.5° C or at room temperature, and then to dilute the mixture so as to reduce the residual IB concentration to a level at which no inactivation of non-pretreated virus took place at 37° C. This diluted mixture was then placed at 37° C, and the virus survival was determined after various times. This experiment provides a direct test for a sensitizing effect of the room temperature IB treatment.

Virus was diluted 1/5 in phosphate buffer, pH 7.4, then mixed with an equal volume of 0.1 mM IB and incubated at room temperature (28° C) for 32 minutes. This mixture was then diluted 1000 fold in phosphate buffer, and placed at 37° C. The results are shown in figure 6, curve 5, and should be compared with curve 1, which gives the inactivation of non-pretreated virus in 0.5 mM IB. The control of untreated virus diluted to the same extent, and heated at 37° C in the presence of 5×10^{-5} mM IB showed practically no inactivation.

It is clear that the pretreatment at 28° C resulted in a virus with increased sensitivity to inactivation at 37° C. Inactivation by IB can therefore be described as a sensitizing oxidation followed by a second, inactivating reaction, which has been made more probable by the prior oxidation. Evidence to be presented later in this thesis suggests that this second reaction is a denaturation.

It is this same experiment which suggests very strongly that the effect of low pH is not upon the -SH oxidation step, but instead is upon the subsequent denaturation. This conclusion is drawn for the following reasons: Since in this experiment the virus at pH 4.6 in 0.5 mM IB, and that diluted in pH 7.4 phosphate buffer were both removed from the same pretreatment mixture after approximately the same length of treatment, one is safe in assuming that they should be sensitized almost identically. Assuming that they are identically sensitized, and that the dilution into pH 7.4 phosphate buffer is sufficient to prevent any additional action of IB, and that low pH likewise prevents any further oxidation by IB, and has no other effect, the inactivation rates at 37° C of the two samples should be identical. If the low pH permitted slow, additional oxidations in 0.5 mM IB, the inactivation rate might be faster than that of the pH 7.4 diluted virus; but in no case could the diluted virus have a faster inactivation rate than the pH 4.6 virus, unless the pH effect were on some step of the inactivation process subsequent to oxidation by IB.

IV. EFFECT OF MOLARITY

During the course of experiments using virus sedimented in a cesium chloride density gradient, it was discovered that the presence of a high concentration of CsCl resulted in protection of the virus against heat inactivation. This phenomenon has been investigated in some detail. The method of testing stability in high molarity media was to add 0.1 ml virus to 1.9 ml salt solution. The salt solutions were prepared at about twice the concentration finally used; before addition of the virus, this was diluted into an equal volume of 0.1 molar phosphate buffer, so that the final pH was close to 7.4.

The effective concentration range was studied first. The results are shown in figure 7. It is seen that a concentration of about 1 molar provides the maximal protection. The inactivation curve in 1 or 3 molar CsCl at 50° C shows that approximately 70% of the particles are probably as sensitive to heat as in phosphate buffer, whereas the remainder is stable. The stable fraction varied between 30% to 60% from one experiment to the next. The fact that the maximum stabilization does not protect 100% of the population suggests that the high salt concentration increases the fraction of "completely" stable particles, and does not alter the rate of inactivation of the unstable particles. A complete inactivation curve at some intermediate CsCl concentration (e.g. 0.33 molar) will be required to prove this.

To learn something of the generality of the phenomenon, the effect of 4 molar KCl was tested. This provided approximately the same protection as did CsCl.

To test whether this phenomenon was due to the ionic strength of the medium, virus inactivation was carried out in solutions of the non-ionic substances glucose and sucrose, at a concentration of 1.5 molar. Both of these sugars protected the virus to the same extent as 1 molar CsCl, demonstrating that the effect was not an ionic phenomenon.

An important point to determine was whether the virus was permanently stabilized by treatment with 3 molar CsCl, or whether the stability was lost when the virus was diluted out of the high molar environment. To test this, the following experiments were done:

1. Virus was treated for 10-1/2 hours at room temperature with 3.5 molar CsCl. This was then diluted 400 fold into phosphate buffer, and heated 5 minutes at 50° C. The survival was 4×10^{-4}, indicating no stabilization. Thus there had been no permanent change in the virus sensitivity to heat.

2. Virus was treated for 10-1/2 hours in 0.035 molar CsCl, and heated at that CsCl concentration. As expected from the results of #1, there was no stabilization.

3. Virus and CsCl were mixed to give a final CsCl concentration of 3.5 molar and heated immediately. The survival was 3×10^{-1}, indicating that no pretreatment was necessary for expression of the protective effect.

4. Glucose gave the same results.

One possibility was that the high molar environment might somehow decrease the reactivity of the reactive, heat-sensitive groups, which, by our hypothesis, would also be the groups reacting with cystine and PCMB. With this in mind, the effect of 3 molar CsCl and 1.5 molar sucrose on stabilization with cystine and blocking by PCMB were tested. The results, given in table 5, show some inhibition of the PCMB effect and a little inhibition of the cystine effect by high molar medium, indicating that there may be some change in reactivity of the -SH groups.

Table 5

	survival after 5 minutes at 50° C
cystine stabilization in 3.5 M CsCl	6.4×10^{-1}
PCMB treatment in 3.5 M CsCl	1.1×10^{-2}
cystine stabilization in 1.1 M sucrose	1×10^{-1}
PCMB treatment in 1.4 molar sucrose	1×10^{-3}
cystine stabilization in 0.1 molar buffer	8×10^{-1}
PCMB treatment in 0.1 molar buffer	1 to 4×10^{-4}

The phenomenon of protection by high molarity was utilized in an attempt to demonstrate the occurrence of a sensitizing reaction (analogous to that occuring with IB) as one of the steps in the inactivation process at 50° C. This might be expected to occur if the high molarity inhibited the second step only. To test this, virus was heated for one hour in 1 molar CsCl at 50° C. It was then diluted 1/10 into phosphate buffer, and heated at 37° C for five hours. There was no detectable loss of virus titer in this time, indicating that a high salt concentration does not permit a permanent sensitization reaction to occur at 50° C.

Preliminary experiments on the effect of 3 M CsCl on various IB reactions were done in an attempt to define more clearly the mode of action of high molarity media. These have indicated that both the inactivation at 37° C and the "blocking" reaction at 17.5° C are inhibited.

These data permit several interpretations. The high molarity medium can be considered as a dehydrating agent. Loss of water molecules from the virus protein could result in modification of the steric relationships within the protein, possibly modifying the position of the -SH groups relative to each other and to other groups, thus altering their reactivity, and perhaps also increasing or decreasing the likelihood of intraviral disulfide formation.

If, as proposed, a denaturation is the final process which results in loss of infectivity, the dehydration effect might also influence this step of the inactivation. It has been proposed (19) that heat denaturation of proteins requires that water molecules be able to flow between the peptide chains as they begin to unfold, presumably to prevent return to the stable configuration. Thus one might expect the virus to be more stable in a medium which tends to remove water molecules from the virus protein. This effect, coupled with the altered reactivity of the -SH groups, could account for all observations of inhibition of inactivation by high molarity media.

V. EFFECT OF TEMPERATURE ON RATE OF INACTIVATION

The preceding data have suggested that heat inactivation of poliovirus consists of at least two reactions. The first reaction is presumably an oxidation of -SH groups. Although none of these results indicated anything about the nature of the second reaction, it was thought that the step could be a denaturation. If this reaction were a denaturation, and if this denaturation were the rate-limiting reaction in the overall inactivation process, one would expect to obtain a high value for the activation energy of inactivation, as calculated from the Arrhenius equation. The integrated Arrhenius equation, expressing the relationship between reaction rate and the absolute temperature, can be written:

$$\ln k = \frac{-E}{R} \left(\frac{1}{T} \right) + \text{constant}$$

where R = gas constant, T = absolute temperature, E = Arrhenius constant = activation energy. The term "E", or Arrhenius constant, for an ordinary chemical reaction is generally less than 25,000 calories per mole, while that observed for protein denaturation is between 40,000 and 200,000 calories per mole. Throughout the course of this work, inactivation had been carried out at several different temperatures; the data from these various experiments were used to calculate the Arrhenius constant.

For the initial part of the survival curve, heat inactivation may be represented by the expression:

$$V/V_o = e^{-kt}$$

where V/V_o is the survival after the time "t" (in hours) at a given temperature, and k is the inactivation rate constant. The rate k was calculated by substituting values for V/V_o and t, taken from the initial part of the survival curves.

At 50° C one has the difficulty that the earliest point (3 minutes) is already at the minimum V/V_o. In this case, therefore, the calculated rate is a minimum value.

The inactivation curves at the various temperatures were done at different times throughout the course of this work, and consequently the methods used varied slightly. It is not believed that these variations had any important effect on the inactivation rates observed. In the curves at 50° C, 45° C, and 37° C the samples were stored in ice, and assayed after the last sample had been taken. The 17.5° C samples were frozen in phosphate buffered saline containing 25% monkey serum, and all finally assayed at the same time.

The curves used for these calculations are shown in figure 8, where each is plotted on an appropriate time scale. The rates calculated from the initial slope of each of these curves are:

50° C	-	138	/hour
45° C	-	39.4	/hour
37° C	-	0.251	/hour
17.5° C	-	0.00108	/hour

These values of k are plotted against 1/T on figure 9. From this plot one can calculate that E = 76,000 calories per mole. This value is compatible with the hypothesis that a denaturation is the rate limiting step in the inactivation process.

VI. NATURE OF THE REFRACTORY FRACTION

Throughout the course of this work, various exploratory experiments were carried out in an attempt to learn something of the nature of the phenotypically heat resistant fraction of virus. This stable fraction of virus is found in all stocks of heat sensitive virus, and results in a rapid decrease in inactivation rate when the survival reaches about 1 to 5×10^{-4} (see figure 1). This value varies somewhat from one stock to another. The virus particles in this fraction are not genetically heat resistant, since their progeny have the same heat inactivation characteristics as the original heat sensitive stock. (21) Pohjanpelto (4) showed that it was not due to stabilization by cystine during intracellular multiplication of the virus, since thioglycolate did not abolish its stability. The present experiments will be mentioned only briefly, as none of them yields important information on the point. The nature of this resistant fraction's stability remains obscure.

It was tested whether virus released very late in the growth cycle has a different fraction of stable particles. In one experiment, at 11-1/2 hours after infection, and in a second experiment at 16-3/4 hours after infection, the plates were washed twice with phosphate buffered saline, and fresh buffered saline plus glutamine were added. Virus was harvested at 23 hours after infection, when most of the

cells had detached from the glass. The survival after 5 minutes at 50° C was no different from a control stock prepared in the normal way.

To test whether the stable fraction was due to partial protection by the salt present in the medium, virus was heated at 45° C in a medium of molar concentration 1/5 that of the normal buffer. Although the inactivation seemed to be faster, the background fraction was the same as that of the control. The stable fraction thus is not due to protection by salt present in the medium.

It should be remarked that the inactivation rate of the stable fraction of sensitive, non-stabilized virus is similar to that of virus in 3 molar CsCl, to that of cystine stabilized virus, and to that of temperature resistant mutants of poliovirus (21). It is impossible, however, to conclude that these inactivation rates are identical. The similarity suggests that the mechanism of virus inactivation is similar in all these cases of increased stability, but that the inactivating reaction is centered at sites different from those involved in the inactivation of the majority of particles in heat sensitive, non-stabilized virus preparations.

DISCUSSION

During the course of this work a number of hypotheses were formulated, and as the work progressed, were discarded and substituted by new ones. The hypothesis around which the final part of the work developed is the heterogeneity hypothesis already presented. The results are far from demonstrating that this hypothesis is an accurate description of the facts. At present, however, it can account for many of the observations. It also serves as a useful scheme around which the complicated experimental results can conveniently be examined.

Before discussing this hypothesis, however, the evidence that sulfhydryl groups are, in fact, the chemically reactive groups participating in the reactions will be reviewed. The fact that reaction with cystine prevents inactivation is itself the best evidence that sulfhydryl groups are involved in the inactivation process, since cystine is considered to be the most specific of all reagents for oxidation of sulfhydryl groups (22). This does not exclude the possibility of a disulfide interchange reaction between cystine and viral disulfide bonds; that they are sulfhydryl groups is supported by the demonstration that a mercurial (PCMB) can prevent stabilization, and that this inhibition can be removed with an excess of a sulfhydryl compound.

Further support is given by the finding that stabilization can be prevented also by iodosobenzoate and iodine, when used under conditions promoting maximum specificity for oxidation of sulfhydryl groups. The fact that thioglycolic acid can remove the stabilization produced by cystine suggests in addition that the stabilization requires the formation, and maintenance, of disulfide bonds, as would be expected if stabilization occurred through an oxidation by cystine, as postulated. Because of these various types of evidence, it is felt that the central role of sulfhydryl groups in these reactions is rather well established.

One further problem should be considered before the hypothesis itself is discussed. Since much of the hypothesis has arisen from the results with iodosobenzoate, it is important to evaluate the relationship between this hypothesis and the aerobic inactivation at 50° C, in the absence of IB. The inactivation by IB at 37° C was shown to be composed of (at least) an initial, sensitizing reaction (presumably an oxidation) and a subsequent reaction resulting in the loss of infectivity. The aerobic, non-IB inactivation is known to require oxygen, and exhibits a temperature dependence characteristic of protein denaturation. The heterogeneity hypothesis describes the IB reactions preceding inactivation, but no experimental evidence is available to enable one to conclude that the aerobic inactivation at 50° C proceeds by way of the same reactions. It is fairly certain that an oxidation step is involved at 50° C, but there is no evidence for the formation

of a chemically stable, oxidized intermediate analogous to that formed after IB sensitization at 17.5° C. With respect to the second reaction, there are no data on the temperature dependence of the inactivation at 37° C after IB treatment, and consequently one cannot assume this to be a denaturation.

In the following discussion of the data in terms of the heterogeneity hypothesis, however, some transposition of conclusions from IB results to describe aerobic, 50° C results, and vice versa, will be done in order to present a uniform picture.

The main point of the heterogeneity hypothesis is that those -SH groups which affect the survival of the virus vary in the ease with which they are oxidized, and in the end product of the oxidation. These -SH groups are classified and characterized as follows:

Class 1) -a. oxidized by atmospheric oxygen at 50° C, this oxidation eventually resulting in loss of infectivity.

b. when all groups of a particle have reacted with cystine, the virus particle becomes stable to heat inactivation at 50°C.

c. oxidized by IB, at 17.5° C, to disulfides, which can in turn be reduced by cysteine.

Class 2) -a. not oxidized by atmospheric oxygen, thus do not participate in the aerobic inactivation at 50° C.

b. oxidized by IB at 17.5° C or room temperature to $-SO_2H$ or $-SO_3H$, resulting in an increase in sensitivity to heat inactivation (additional -SH groups of this class may be oxidized at 37° C, or by more concentrated IB solutions).

c. react with cystine, which prevents oxidation by IB.

The main reason for postulating two classes of sulfhydryl groups was the observation of intermediate rates of inactivation after cystine treatment following oxidation by IB at room temperature. Since intermediate degrees of stability are not found during normal stabilization, some description other than the blocking of a fraction of the sensitive -SH groups was necessary to explain the IB result. Postulation of the existence of two classes of -SH groups furnishes one such description. This picture is not unlike that found in many enzymes or other proteins that have been studied in some detail chemically. It is quite common in studies of protein -SH groups to find that not all these groups exhibit the same reactivity to various reagents: for example, some may react more slowly than others, and some only with more powerful reagents (see discussion on differential reactivity of -SH and -SS- groups in Boyer (7)). The picture is likely to be complicated further by variability in the reactions of the -SH groups of a given class; for instance, it is likely that -SH groups of

the first class may be oxidized beyond the disulfide under certain conditions. The relevance of these complications to the present work cannot be assessed on the basis of the available data.

Using this hypothesis as a basis, and remembering the problems involved in intermixing conclusions from IB and 50° C aerobic inactivation data, the various observed reactions may be described as follows (discussion of each point will follow):

A. Loss of infectivity at elevated temperatures results ultimately from a denaturation reaction. This denaturation is preceded by an oxidation involving sulfhydryl groups.

B. Virus treated with IB, followed by cystine, shows intermediate rates of inactivation at 50° C; these are attributed to a sensitization to heat caused by oxidation of -SH groups of the second class.

C. The rate of -SH group oxidation is faster, at any temperature studied, than the denaturation.

D. Stabilization by cystine consists of its reaction with free -SH groups, thereby preventing the sensitizing oxidation of these -SH groups.

Discussion of points A through D.

A.: Loss of infectivity at elevated temperatures is attributed, ultimately, to a denaturation reaction. The support for this view

comes mainly from the results obtained with iodosobenzoic acid, and from the determination of the Arrhenius constant. The IB data suggest that the inactivation is composed of at least two steps, an oxidation step and a subsequent reaction resulting in loss of infectivity; the oxidation alone does not appear to inactivate the virus. The Arrhenius constant for aerobic inactivation at 50° C is of the order of 75,000 calories per mole; this value supports the hypothesis that the rate limiting step in the inactivation is a denaturation of the viral protein.

The following mechanisms are proposed to suggest how oxidation of -SH groups could lead to protein denaturation. In the case of oxidation by IB, the formation of $-SO_2H$ or $-SO_3H$ groups results in a larger group than was originally present, and in the addition of a negative charge, for at pH 7.4 few of the native -SH groups are ionized. These changes might make the protein more sensitive to heat denaturation (see proposal of Lumry and Eyring discussed below).

The aerobic inactivation at 50° C presents a more difficult problem. Since oxidation of -SH groups by oxygen does not appear to proceed beyond the disulfide stage, one must consider the possibility that disulfide bond formation within the virus protein may result in a virus particle which is more easily denatured. One commonly

thinks of disulfide bonds as being stabilizing, rather than sensitizing. Although this may be true for disulfide bonds found in native protein, it seems plausible that unnatural bonds formed at a fairly high temperature might have the opposite effect by "stabilizing" a protein configuration in which hydrogen bonds have been weakened to a great extent.

Formation of such "unnatural" bonds, and subsequent decrease of stability of the protein molecule, have been proposed by Lumry and Eyring (23). They propose that random small configurational changes of the protein molecule could place sterically hindered -SH groups temporarily in a position permitting reaction. If they should react at that time, the molecule could not return to the more stable configuration. Accumulation of such events could alter the structure of the protein considerably, and could lead to denaturation.

It should be considered also whether some intermediate step in the oxidation of -SH groups by O_2 may lead to protein denaturation. The only plausible intermediate to consider is the sulfur radical. If the radical cannot react with another sulfur radical, or an -SH group, it is probably limited to reaction with reactive aromatic systems. No estimate of the importance of this type of reaction can be made.

B. Intermediate rates of inactivation after IB treatment:

This has already been discussed as being the principal reason for postulating the existence of two classes of -SH groups. Alternative explanations for the observation of particles with intermediate stability in terms of true blocking of stabilization sites would have to be quite speculative. One such explanation might be that in normal stabilization, reaction of the first cystine molecule is followed very rapidly by all the others required for stabilization. Thus one could never see the intermediate stabilities unless some sites were prevented from reacting with cystine.

The fact that IB appeared to sensitize virus to heat inactivation offered a more appealing explanation, however, and the sensitization hypothesis was adopted. The results of the efforts to remove the block to stabilization tend to make this hypothesis somewhat less plausible. A complete lack of reversal would agree with this hypothesis much better than the partially successful reversals actually obtained. Occasional apparent reversal might be obtained if several class 2) -SH groups were by chance oxidized to -SS-, instead of to $-SO_3H$. These could then be reduced by the TG, with the result that sensitivity to heat is decreased. As stated previously, although this is possible, no estimate of its probability can be made.

C. Rate of -SH group oxidation faster than denaturation:

This hypothesis is supported by the Arrhenius constant calculation, which suggests that a denaturation is the rate limiting step. It is especially useful in explaining two different observations. First, the lack of effect of decreased oxygen concentration on rate of inactivation is understandable if the rate limiting reaction were not the oxidation. Second, the IB inactivation at 37° C of IB pretreated and non-IB pretreated virus is identical, even though the pre-treatment has sensitized the virus to some extent. The simplest interpretation of this result is that the sensitizing reactions occur very rapidly at 37° C, with the result that a virus not previously exposed to IB is very soon identical with one which has been exposed. A test for this would be to determine very carefully the inactivation curves in IB at 37° C.

Since it has been proposed that the virus sensitivity to heat denaturation increases as more -SH groups are oxidized, one would expect a curve with continuously increasing slope. This would not be true, however, if the oxidation were so rapid that all -SH groups were oxidized in the first few minutes of treatment. The three point inactivation curves reported in the present work are not precise enough to decide this point.

D. Stabilization:

If reaction of the -SH groups to form a disulfide link with some added compound were sufficient to protect the -SH groups, compounds like mercaptopropionic acid and 2-mercaptoethylamine might have been expected to produce complete stabilization. These results would be consistent with the proposed mechanism of stabilization, however, if the -SH groups of class 1) were to vary in their ionic environment in such a way that all compounds are prevented from reacting with the -SH groups except those with the proper charge(s) in the proper steric configuration. The fact that these compounds do not produce complete stabilization would then imply nothing about what chemical reaction is required to protect the -SH group. On the other hand, it is the opinion of Boyer (7) that no data presently available demonstrate clearly that depression of -SH reactivity is due to the ionic environment of the -SH group. Most observed cases of depressed reactivity appear to be best explained on the basis of steric hindrance of the -SH groups by the position of the peptide chains. Boyer's principal reason for this opinion is that in general no difference is noted whether reagents used are anionic, cationic, or zwitterionic.

In the present case it is likely that it is not the ionic effect alone which prevents reaction. Steric factors undoubtedly also are

of importance. It should be remembered that D-cystine did not produce stabilization. This suggests that steric requirements for stabilization, as well as the ionic requirements, are very strict. If very exacting steric and ionic requirements must both be met, it would not be surprising to find that only L-cystine would be capable of producing maximal stabilization.

REFERENCES

1. Mueller, J. H., J. Exp. Med., 48: 343-349 (1928).

2. Zinsser, H. and F. Tang, J. Immunol., 17: 343-355 (1929).

3. Labzoffsky, N. A., Canadian J. Res., 24 (E): 119-133 (1946).

4. Pohjanpelto, P., Virology, 6: 472-487 (1958).

5. Dulbecco, R. and M. Vogt, J. Exp. Med., 99: 167-182 (1954).

6. Chinard, F. P. and L. Hellerman, Methods of Biochemical Analysis, 1: 1-26 (1954).

7. Boyer, P. D., The Enzymes (2nd edition), Vol. 1: 511-588 (1959).

8. Hellerman, L., F. P. Chinard and P. A. Ramsdell, J.A.C.S., 63: 2551-2553 (1941).

9. Hellerman, L., F. P. Chinard and V. R. Deitz, J.B.C., 147: 443-462 (1943).

10. Reid, E. E., Organic Chemistry of Bivalent Sulfur, Vol. 1: 118-120 (1958).

11. Snyder, H. R., J. M. Stewart, R. E. Allen, and R. J. Dearborn, J.A.C.S., 68: 1422-1428 (1946).

12. Anson, M. L., J. Gen. Physiol., 23: 321-331 (1940).

13. Anson, M. L. and W. M. Stanley, J. Gen. Physiol., 24: 679-690 (1941).

14. Anson, M. L., Adv. in Protein Chem., II: 361-386 (1945).

15. Anson, M. L., J. Gen. Physiol., 23: 247-261 (1939).

16. Anson, M. L., J. Gen. Physiol., 25: 355-367 (1942).

17. Adams, M. H., J. Gen. Physiol., 31: 417-431(1948).

18. Gomori, G., Methods in Enzymology, Vol. 1: 138-146 (1955).

19. Haurowitz, F., Chemistry and Biology of Proteins, Chapter 7, p. 129 (1950).

20. Anson, M. L., Symposium on Mechanism of Enzyme Action, in discussion of Kauzmann's paper, p. 116 (1954).

21. Dulbecco, R. and M. Vogt, Personal Communication.

22. Barron, E.S.G., Adv. in Enzymology, II: 201-266 (1951).

23. Lumry, R. and H. Eyring, J. Phys. Chem., 58: 110-120(1954).

FIGURE I

50°C Inactivation of Stabilized and Non-stabilized Poliovirus

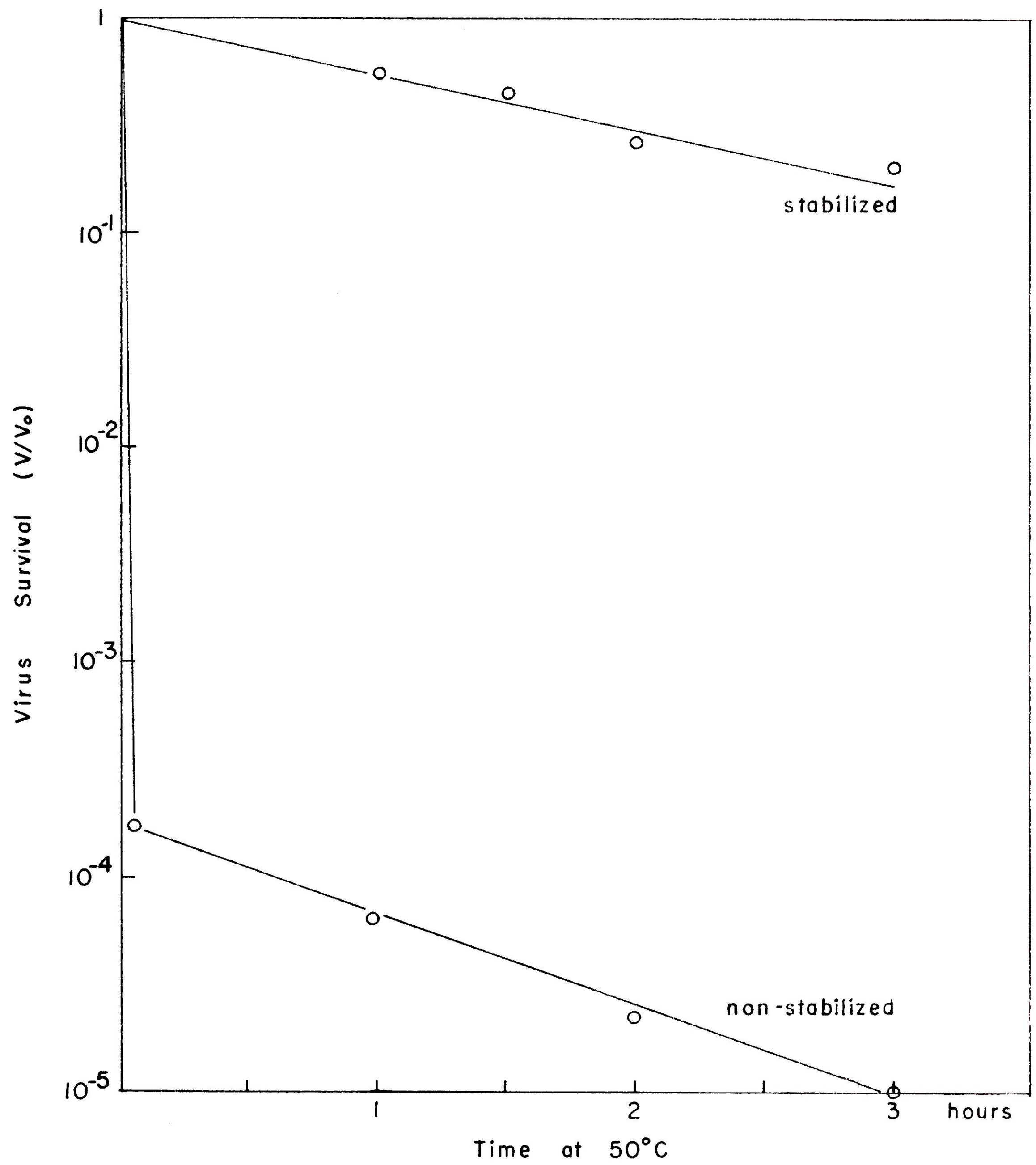


FIGURE 2

Inhibition of Cystine Stabilization by PCMB

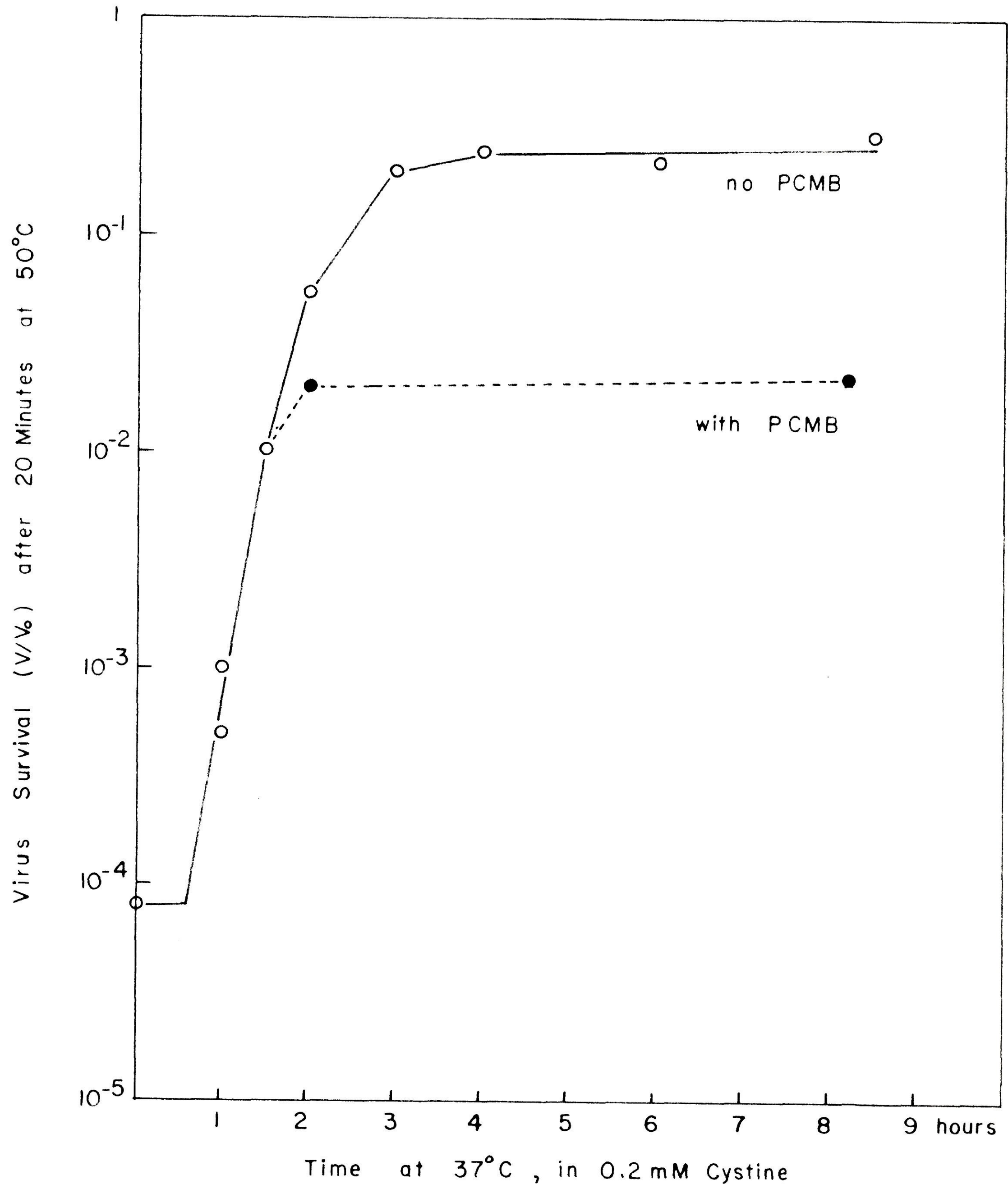


FIGURE 3

Inactivation Curves at 50°C
of Virus Exposed to Iodosobenzoate
for Various Times before Treatment with Cystine

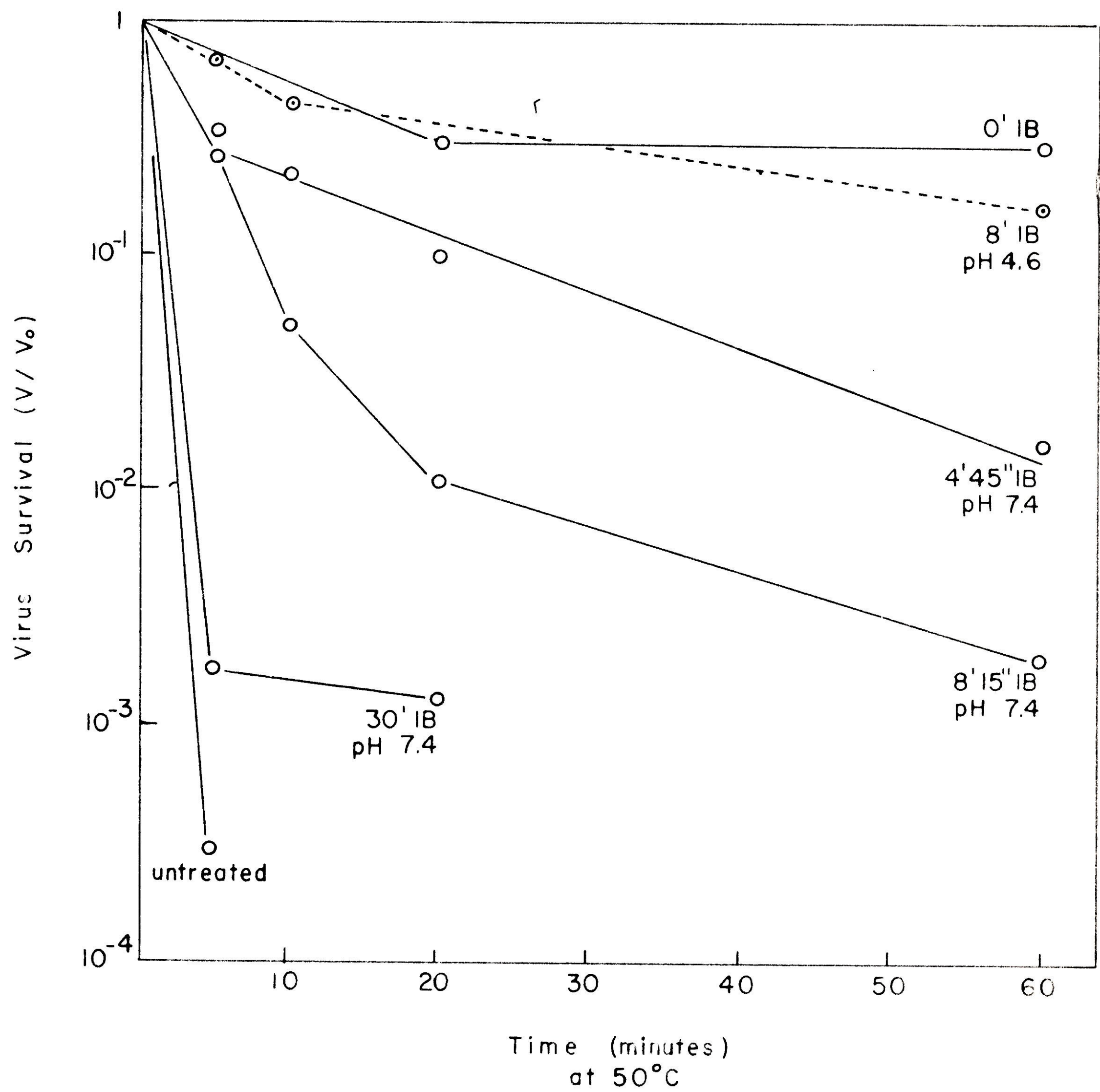


FIGURE 4

50°C Inactivation of Sensitive Virus in Air and in Nitrogen

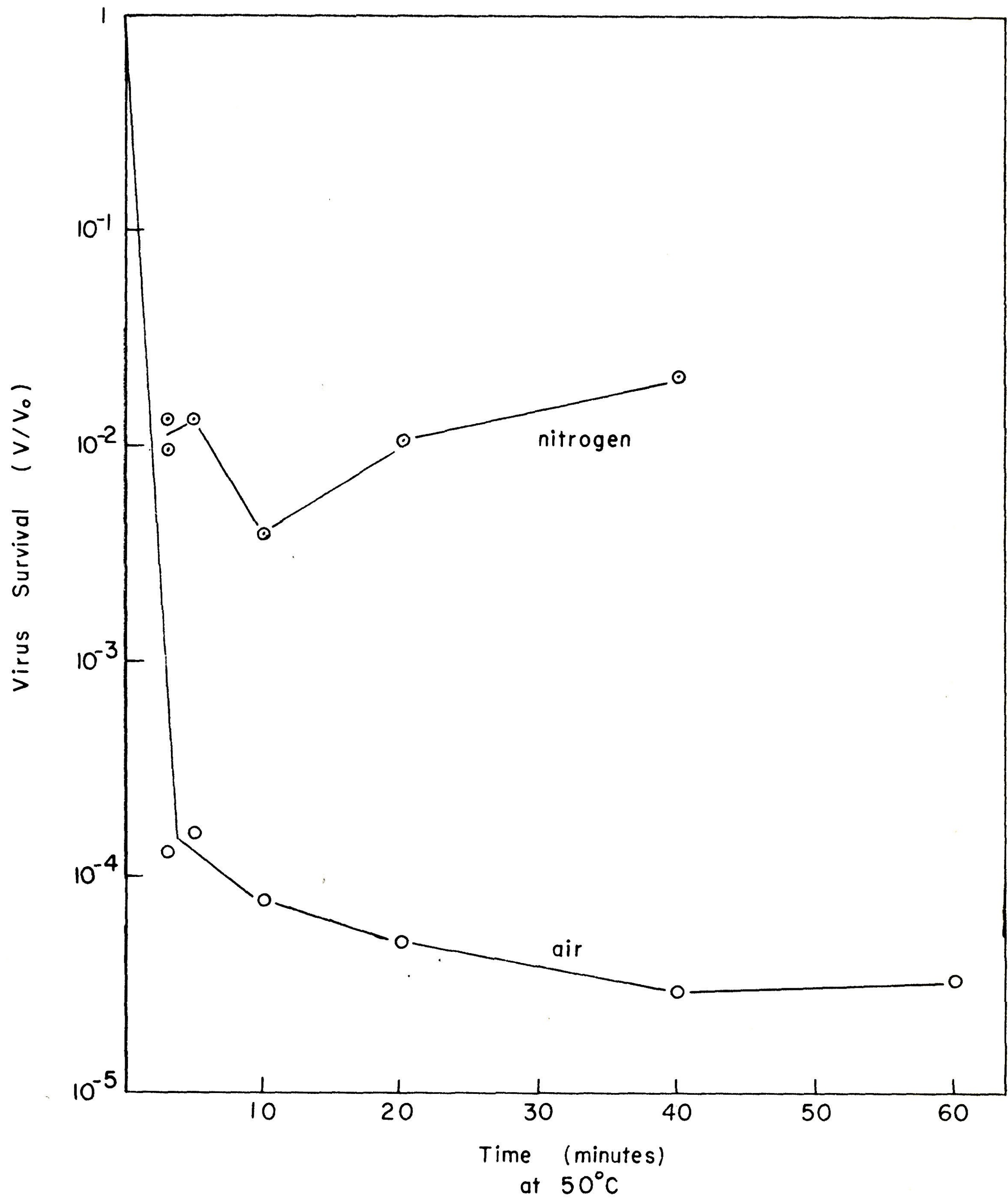


FIGURE 5

Inactivation Curves at 45°C

as a Function of pH

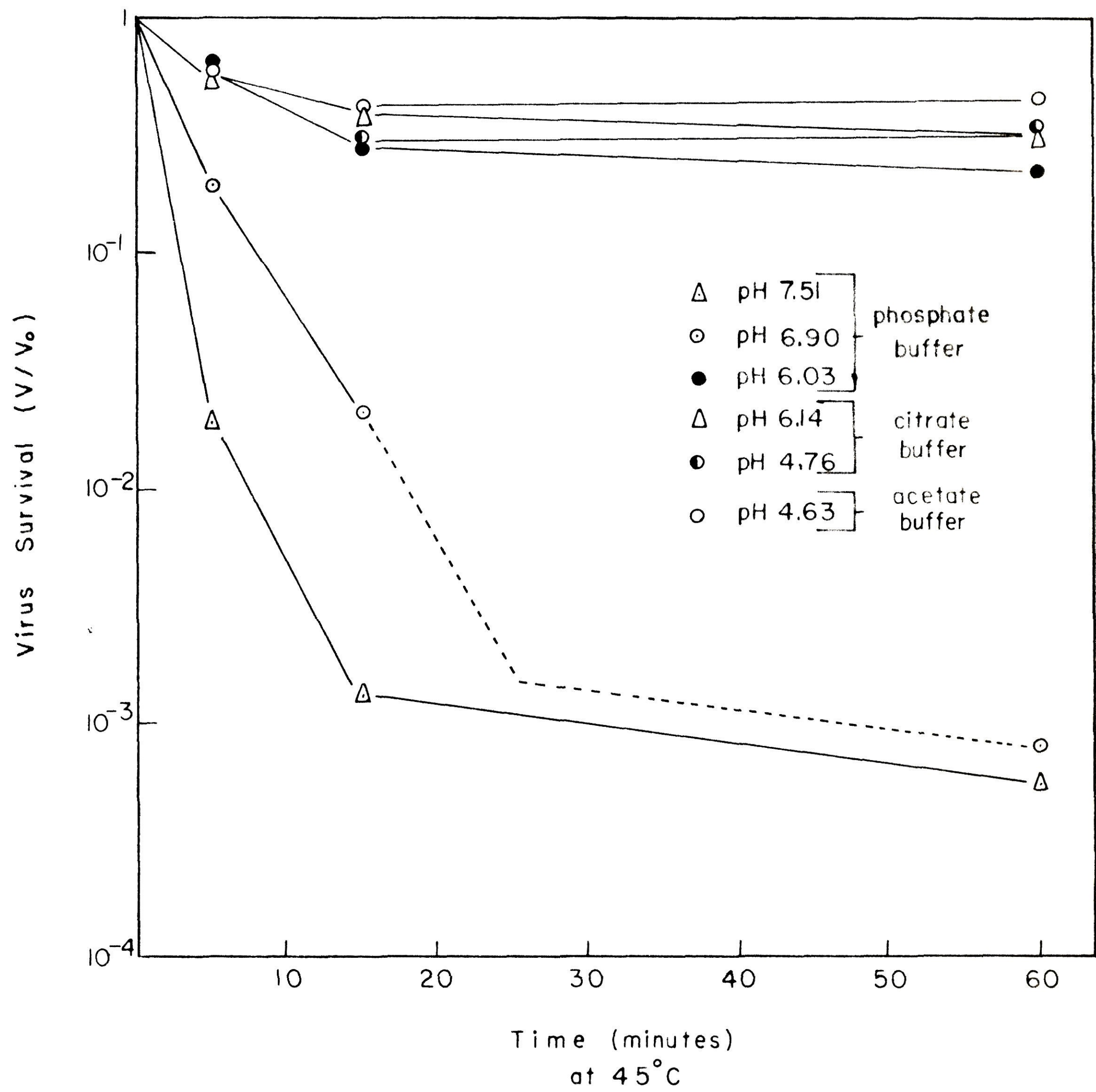


FIGURE 6

Inactivation by Iodosobenzoate at 37°C

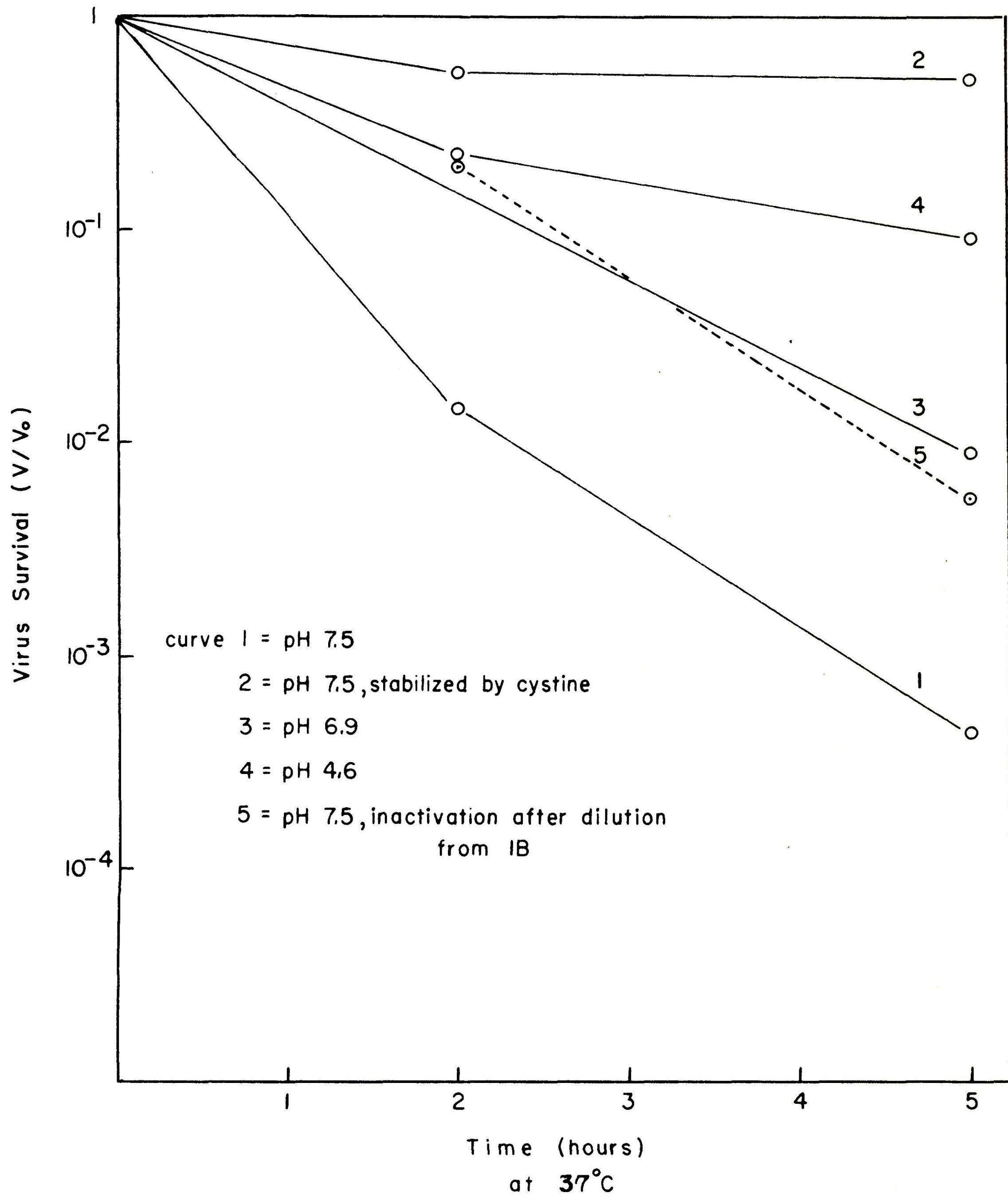


FIGURE 7

Inactivation, at 50°C, of Virus

in Solutions of High CsCl Concentration

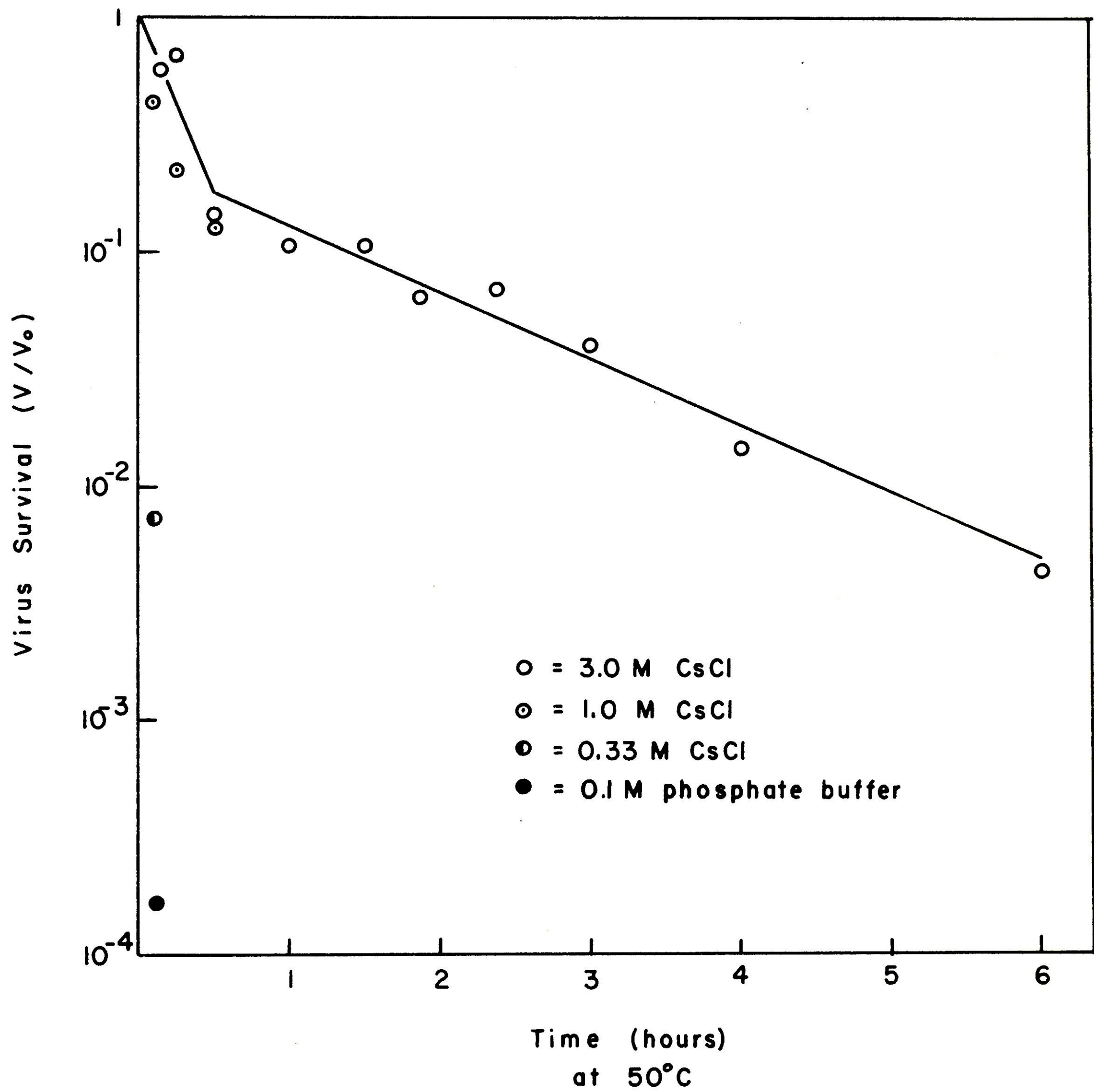
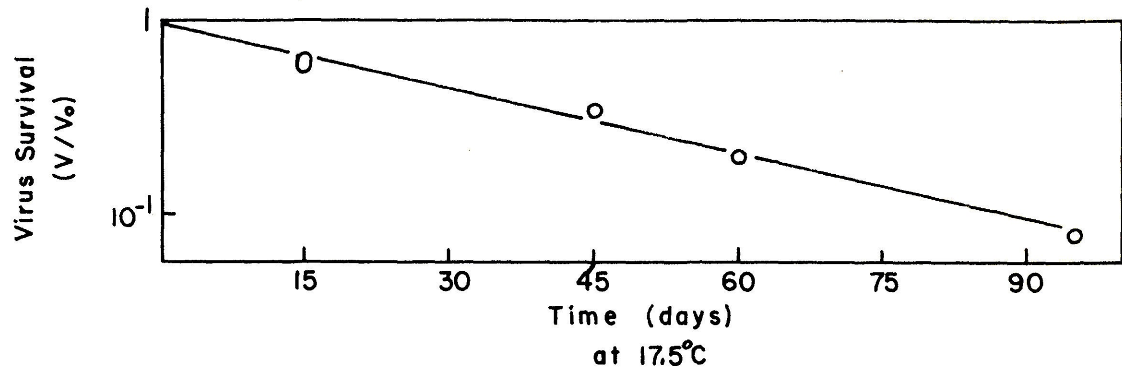
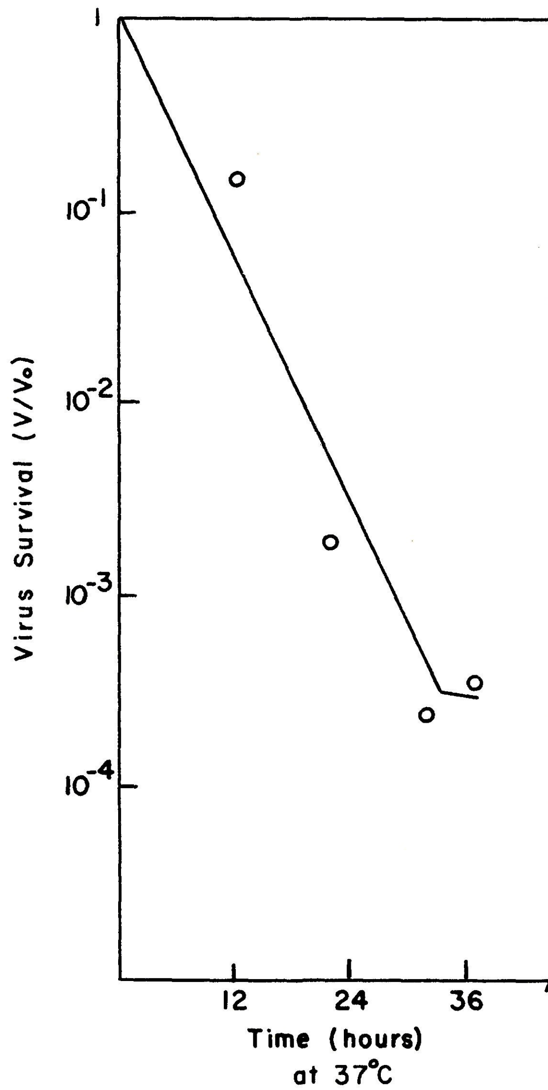


FIGURE 8

Inactivation Curves at Various Temperatures





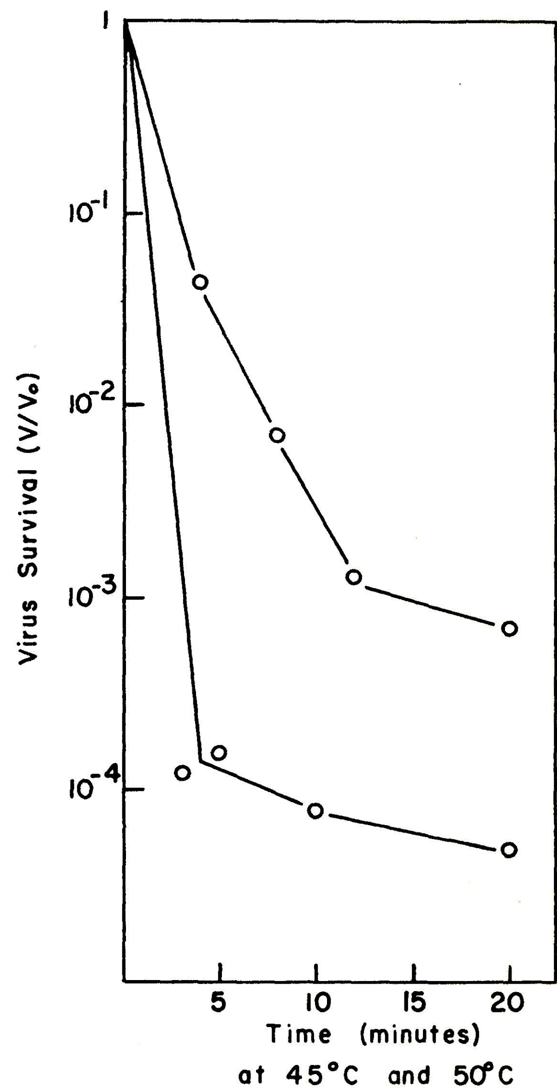


FIGURE 9

Arrhenius Plot of Inactivation Rate Constants Determined at Various Temperatures