THE EFFECT OF SONIC OSCILLATIONS UPON THE T - 3 BACTERIOPHAGE

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THESIS

THE EFFECT OF SONIC OSCILLATIONS UPON THE T-3 BACTERIOPHAGE

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TABLE OF CONTENTS

PAGE

INTRODUCTION..... 1

EXPERIMENTAL METHODS..... 7

Table I..... 10

Experiment I..... 14

Chart I..... 16

Chart IA..... 17

Chart IB..... 18

Graph I..... 19

Graph II..... 20

Experiment II..... 21

Chart II..... 23

Graph III..... 24

Experiment III..... 25

Chart III..... 28

Graph IV..... 29

Graph V..... 30

Experiment IV..... 31

Chart IV..... 33

Graph VI..... 34

Experiment V..... 35

Chart V..... 37

Graph VII..... 38

DISCUSSION..... 39

SUMMARY..... 44

BIBLIOGRAPHY..... 45

INTRODUCTION

INTRODUCTION

Takahashi and Christensen (1934) first investigated the effect of sonic oscillations upon virus. They subjected the tobacco mosaic virus to 9,000 cycle sonic oscillations and found that the virus lost its infectivity after two hours of treatment. Stanley (1934) confirmed these results and also showed that if the virus preparation was sealed in a vacuum while being treated, little or no inactivation occurred. Anderson, Boggs, and Winters (1948) first subjected the T bacteriophage to sonic oscillations. They found that the T bacteriophage were not all inactivated at the same rate. T-2, T-4, T-5, and T-6 were all rapidly inactivated at a logarithmic rate, losing infectivity after slightly more than ten minutes of sonic treatment. T-1 and T-7 were also inactivated at a logarithmic rate but still retained one per cent of their infectivity after sixty minutes of treatment. T-3 was the only T bacteriophage that was inactivated at a nonlogarithmic rate. After sixty minutes of sonic treatment T-3 completely lost infectivity.

The following experiments were undertaken to determine if an increase in the infectivity titer of the T-3 bacteriophage could be produced by sonic oscillations, and further to investigate the general effects of sonic oscillations upon the T-3 bacteriophage.

For related experiments, conducted in this laboratory, it was necessary to produce large quantities of high titer T-3 bacteriophage. By producing the virus on a solid synthetic medium, harvesting the lysate with distilled water, and centrifuging it to remove unlysed bacterial cells and bacterial cell

debris, an aqueous suspension of bacteriophage was obtained. Thus, quantities of high titer T-3 bacteriophage, having low nitrogen content, were available for sonic treatment. The main advantage of using T-3 for these experiments is that it is not readily inactivated by sonic oscillations and can be treated for a period of several minutes before a great loss of infectivity occurs.

T BACTERIOPHAGE

The T-3 bacteriophage is a prismatic particle measuring 50 mu in diameter in air dried preparations and 47 mu in freeze dried preparations. A process, the so called "tail", measuring 15 by 10 mu was observed in the freeze dried preparations. The presence of the "tail" on the particles in air dried preparations is doubtful (Williams and Fraser 1953). Measurements made on virus produced in this laboratory yielded the following results; 50 mu in diameter in air dried, shadowed preparations, and 38 mu in diameter in unshadowed preparations. Some of the bacteriophage particles in the air dried preparations showed the presence of a "tail". The T-3 and T-7 bacteriophage are both prismatic particles of equal size. The rest of the T bacteriophage are much larger. The even numbered bacteriophage, T-2, T-4, and T-6, are prismatic particles, 95 by 65 mu with a process, 100 by 25 mu. T-1 is prismatic with a diameter of 50 mu and a process, 150 by 10 mu. The T-5 bacteriophage is also prismatic being 65 mu in diameter and having a process, 150 by 10 mu. The T bacteriophage are distinguished by their infectivity for a common host bacterium, Escherichia coli strain B (Luria 1953).

THESIS

SONIC THEORY

A sound wave is composed of successive compressions and rarefactions and can be transmitted through any molecular medium.

Sonic oscillations can be produced in the laboratory by two methods. It is possible to excite high frequency sound waves, using the piezoelectric properties of a quartz crystal. Energies up to 100 watts at one megacycle per second or up to thirty megacycles with less energy are possible (Pollard 1953). The oscillator used in these experiments is a magnetostriction oscillator. It does not make use of a piezoelectric crystal. A laminated nickel bar is surrounded by a coil that produces a strong magnetic field when a rapidly alternating current is passed through it. To this bar is attached a metal disc which is caused to vibrate when the coil is energized. The vibration is due to the rapid change in length of the individual laminated nickel strips forming the bar. In this way sonic oscillations are produced.

Fry et al.(1950) stated that the following effects must be considered when exposing biological substances to sonic oscillations, changes in temperature and pressure, and cavitation with concomitant effects. Sonic oscillations also exhibit strong dispersive power, degassing effects, oxidizing effects and, above all, produce rapid acceleration of particles of liquid in the radiation field (Gregg 1950).

The temperature changes produced are of two types, perodic and monotonic. The perodic changes are so small, 0.1 to 1°C even in high intensity sonic fields, that they are of little biological

importance (Fry et al. 1950). Monotonic temperature change is produced as the result of the absorption of sonic energy by a tissue or cell. The magnitude of the temperature change is dependent on the structure of the tissue or cell. As yet no one has investigated the monotonic temperature change of particles of the size of bacteriophage. Therefore, it is impossible to estimate the importance of this type of temperature change on virus inactivation.

Pressures as great as ten atmospheres can be produced by ultra sonic oscillators. Pressure is produced as the sound waves travel through the medium. It should be noted that the pressure will swing from positive to negative as each wave peak passes a given point. Constant positive pressure per se has little effect on virus particles (Johnson et al. 1948).

Cavitation and its related effects are considered to be the major cause of the deleterious effects produced upon biological systems by sonic oscillations (Johnson 1929, Schmitt and Uhlemeyer 1930, Fry et al. 1950 and Gregg 1950). Cavitation is the production of hollows or cavities in a liquid, formed by great differences in pressure existing between the crest and trough of a sound wave. Any dissolved gasses in the liquid naturally will fill the hollow and hence form a bubble. Bubble formation occurs explosively and is probably accompanied by local heat and and electrical potentials due to frictional loss (Gregg 1950).

Pollard (1953) stated that by immersing powdered explosives in a liquid by which they are not wet and subjecting them to sonic oscillations it is possible to measurethe critical detonation energy. If this is related to the flash temperature of the

explosive the local temperature can be estimated. In a sonic field of twenty watts per cm^2 at one megacycle, temperatures up to 230°C were obtained.

Forces are also encountered in the formation of the gas cavity that are of such magnitude that they could cause cellular disruption (Harvey et al. 1944).

Both Johnson (1929) and Schmitt and Uhlemeyer (1930) hypothesized that cavitation, occurring on the surface of the cell, is responsible for the disruption of single celled organisms.

Stanley (1934) showed that if tobacco mosiac virus was sealed in a vacuum during sonic treatment almost no inactivation occurred. Cavitation will not occur in a medium sealed in a vacuum.

Increasing the viscosity of the medium will decrease cavitation since the cohesive property of the medium is increased (Gregg 1950).

THE RAYTHEON SONIC OSCILLATOR

The oscillator used in these experiments was a Model DF 101, 250 watt, ten kilocycle sonic oscillator manufactured by the Raytheon Corporation of Waltham, Mass. It is a magnetostriction type oscillator. It consists of two units, the first being the power supply, and second the treatment unit consisting of the chamber and attached laminated nickel bar. The nickel bar is inserted into the center of the magnetic coil that constitutes the bulk of the base of the treatment unit.

The intensity of the oscillations can be regulated by three controls,(1) match control, (2) tune control, and (3) power control.

The match control can be switched to two positions. It was found that the most satisfactory results were produced if it was left in the position arbitrarily designated as position one. The tune control could be adjusted so as to vary the amperage over a range of approximately one ampere when the power control was at its maximum setting. After the unit was once tuned the settings were left the same for all succeeding experiments.

Tuning the oscillator was accomplished, as recommemded by the manufacturer, by setting it to produce the maximum frying effect or frying sound. This was done by setting the power control to its maximum setting and then adjusting the tune and match controls. The maximum frying sound was produced between 1.0 and 1.2 amperes and was variable depending upon the operator.

From the results obtained during duplicate trials it was assumed that the instrument was producing results as accurate as the biclogical methods used in the experiments.

EXPERIMENTAL METHODS

THESIS

EXPERIMENTAL METHODS

Synthetic Medium

The T-3 bacteriophage used in these experiments was produced on "F" medium (Adams 1950). This medium supports the growth of E. coli in a satisfactory manner and modifications of it have been used for the growth of E. coli upon which bacteriophage were produced by Putnam et al. (1949) and Hook et al. (1946). This synthetic medium consists of 1.0 gm NH_4Cl, 0.1 gm $MgSO_4$, 1.5 gm KH_2PO_4, 3.5 gm $NaHPO_4$, 9.0 gm lactic acid and 15 gm agar (Difco)* in 1,000 ml of distilled water. The pH of the medium was adjusted approximately to pH 7 with NaOH.

Bacterial Stock Cultures

Stock cultures of E. coli were grown on nutrient agar slants containing 0.0004 per cent gentian violet. The inoculated slants were placed in the incubator for 18 hours. The cultures were then transferred to the refrigerator for storage. The growth was removed from the slants by placing 5.0 ml of nutrient broth (Difco) on the culture and shaking the tube.

Bacteriophage Stock Suspensions

Stock bacteriophage suspensions were pooled virus lots remaining from other experiments conducted in this laboratory. These pooled virus suspensions were filtered through HAtype Millipore filters**. The resulting pooled lysate was refrigerated.

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*Difco Laboratories Inc., Detroit 1, Michigan.
** Millipore Filter Corp., Watertown 72, Massachusetts.

Production of Bacteriophage

For the production of bacterial cells in early experiments, standard Petri dishes were used; later larger, 150 mm Petri dishes were employed. In the former, 20 ml of synthetic medium was used, and in the latter, 100 to 150 ml of medium gave satisfactory results. The plates were seeded with 0.5 or 1.0 ml of a nutrient broth suspension of an E. coli stock culture. The inoculum was spread over the surface with a triangular glass spreader. The plates were incubated from 8 to 18 hours before adding 0.5 ml of stock suspension of bacteriophage to each standard plate, and 1.5 ml of stock suspension to each large plate. The suspension of virus was mixed with the bacterial growth and spread over the surface of the agar using a glass spreader. The plates were then incubated for 18 to 24 hours at 37°C. The virus was harvested as follows: 5.0 ml and 20 ml, respectively, of sterile distilled water or buffer solution were placed on the agar surface. The surface was then thoroughly rubbed using a glass spreader to remove the virus and bacterial debris. The lysate was pipetted off and placed into 25 ml metal centrifuge tubes. The crude lysate was centrifuged at 9,000 r.p.m. for twenty minutes at 4.0°C in an International Model PR-1 multispeed refrigerated centrifuge to remove the unlysed cells and cellular debris.

The lysates used in these experiments were of two types: (1) processed lysates, which were produced as outlined above, and (2) unprocessed lysates, which were prepared by harvesting the virus from the surface of the plates by using buffers of

various hydrogen ion concentrations. Unprocessed lysates were not centrifuged prior to sonic treatment.

Buffer Solutions

Three types of buffer systems were used: (1) acetic acid-sodium acetate system for hydrogen ion concentrations in the pH range between four and five, (2) dibasic sodium phosphate-monobasic sodium phosphate system for pH 5.3 to pH eight and, (3) ammonia-ammonium chloride system for pH nine. (Table I)

All hydrogen ion concentration measurements were made with a Beckman Model H-2 pH Meter.*

Treatment of Lysates

The treatment chamber of the sonic oscillator was prepared by washing it with Heamo-sol** and then thoroughly flushing it with water. It was partially filled with seventy per cent ethyl alcohol, shaken thoroughly, and allowed to stand for twenty minutes. The chamber was then flushed with sterile distilled water. This was accomplished by filling the chamber and shaking vigorously, repeating the process three times. The chamber was then placed in position in the treatment unit and circulation of cooling water started.

The chamber was cooled by running cold tap water through the water jacket surrounding it. While the chamber was cooling, the cover was left ajar so that the chamber could dry. The chamber

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*Beckman Instrument Co., Fullerton, California.
**Meinecke and Co., Inc., New York, New York.

TABLE I

BUFFER SOLUTIONS*

pH				
4.1	35 ml M acetic acid	plus	10 ml M sodium acetate	
4.2	30 ml M acetic acid	plus	10 ml M sodium acetate	
4.6	10 ml M acetic acid	plus	10 ml M sodium acetate	
4.8	8 ml M acetic acid	plus	10 ml M sodium acetate	
5.0	5 ml M acetic acid	plus	10 ml M sodium acetate	
5.3	12 ml M/5 Na_2HPO_4	plus	46 ml M/5 NaH_2PO_4	
6.0	5 ml M/5 Na_2HPO_4	plus	35 ml M/5 NaH_2PO_4	
7.0	12.5 ml M/5 Na_2HPO_4	plus	12.5 ml M/5 NaH_2PO_4	
8.0	16.3 ml M/5 Na_2HPO_4	plus	1 ml M/5 NaH_2PO_4	
9.0	5 ml M NH_3 (NH_4OH)	plus	10 ml M NH_4Cl	

*Kabat and Mayer (1948)

was thoroughly cooled before the treatment was commenced. The temperature of the effluent was between 12° and 16°C. During sonic treatment the temperature of the effluent never increased more than 4°C.

The lysate was then placed in the chamber. In all cases except the first trial (Trial I, Chart I) of processed lysate, 10 to 20 ml of liquid lysate was used. In the first trial 50 ml was used. Ten to fifteen ml. of processed lysate was treated in each trial. Between 15 and 20 ml of unprocessed lysate was treated because of the necessity of withdrawing a number of 2 ml test samples.

In early experiments the oscillator was tuned before each trial. After several tests had been completed the instrument was left at the same amperage setting.

In testing processed lysates, 0.5 ml samples were withdrawn with a pipette from the chamber at specified intervals and placed directly in dilution tubes containing 4.5 ml of nutrient broth (Difco).

Two ml. samples were removed from the chamber when testing unprocessed lysates and placed in 7 ml glass centrifuge tubes. The tubes were placed directly in the centrifuge. The refrigeration unit of the centrifuge had been previously started so that the centrifuge was cooled to 4.0°C. After all the samples had been collected they were centrifuged at 9,000 r.p.m. for twenty minutes to remove cellular debris. Five-tenths ml samples of the top supernatant fluid were withdrawn for virus titration from the centrifuge tubes using a sterile 1 ml pipette or a 1 ml syringe.

Assay Method

Virus infectivity titrations were carried out by making serial dilutions using test tubes containing 4.5 ml of nutrient broth (Difco). The 0.5 ml virus sample to be tested was placed in the first tube of the dilution series. A sterile 1 ml pipette was used for each successive transfer. The liquid was drawn into and expelled from the pipette three times, before 0.5 ml was transferred to the next dilution tube, to mix the virus with the diluent. The virus was assayed by the soft agar technique of Gratia (1936).

Test tubes containing 2.5 ml of soft agar (0.7 per cent agar, Difco) (Adams 1950) were melted by steaming in an autoclave. These tubes were transferred to a water bath at 45°C until ready for use.

The E. coli used in the virus infectivity assays was grown in 8 oz medicine bottles containing 40 ml of nutrient agar (Difco) that had been allowed to solidify on the flat side of the bottle. One ml of a nutrient broth suspension of E. coli was placed on the surface of the agar and incubated at 37° C for eighteen to twenty hours. The growth was removed by placing 20 to 25 ml of nutrient broth in the bottle and shaking until the cells were removed from the agar surface. Five-tenths ml of this suspension was pipetted into each of the soft agar tubes.

From each of the virus dilution tubes to be titrated, 0.1 ml was transferred to each of three of the previously inoculated soft agar tubes. The bacteria and virus were mixed by shaking the tubes. The content of each tube was poured into a Petri dish containing

20 ml of solidified nutrient agar (Difco). After the soft agar layer had solidified the plates were inverted and incubated for 18 hours at 37°C. The plaques appearing were then counted. Normally three adjacent dilutions were plated, e.g. 10^{-6}, 10^{-7}, 10^{-8}. If the dilution range was properly chosen one of the three dilutions tested would produce plates with between 10 and 100 plaques per plate. The larger the average number of plaques per plate, the more statistically accurate the titration results will be. The main limitation, however, is that if the number of plaques reaches a certain point they become too numerous to count (Luria 1953). Under the conditions of these experiments plates containing more than 100 plaques could not be counted.

The infectivity titer was computed in the following manner: since 0.1 ml of a given virus dilution was used, then $m = y/vx$, where m = infectious units per ml, y = number of plaques, and v = volume of dilution x.

EXPERIMENT I

SONIC TREATMENT OF PROCESSED LYSATES

The bacteriophage used in these experiments was produced on the surface of "F" agar in Petri dishes. The first two virus lots produced were filtered through HA type Millipore filters, after they had been centrifuged for 20 minutes at 9,000 r.p.m. All subsequent virus lots were centrifuged and not filtered.

The processed lysate to be treated was placed in the previously cooled treatment chamber. The power supply of the oscillator was then energized and when the "ready light" appeared, the plate switch was closed and the treatment begun. After a predetermined number of seconds had elapsed the plate switch was opened and a 0.5 ml sample of the lysate was withdrawn from the chamber. The plate switch was again closed and the treatment continued. This process was repeated until the desired samples had been obtained. The treatment time was recorded by means of a stop watch. Samples were taken as shown in Chart I, IA, and IB.

Fifty ml of processed lysate was treated in test I, Chart I. In tests II through V, 10 to 15 ml of lysate was treated.

Results

During the first several minutes of treatment, in all the tests, the virus titer decreased initially and then rose to an infectivity titer higher than the original. The titer then gradually decreased. After thirty minutes the infectivity titer dropped approximately one log. A virus titer of 10^8 was still present after the lysate had been treated for 45 minutes (Trial II, Chart IB).

Graph I shows the average per cent change in titer of the sonic treated processed lysates. The points on the graph were determined by adding the infectivity titers of the test samples, taken at given periods of time, and computing the numerical average. The percentages were plotted on the basis of the infectivity titer of the zero minutes sample being equal to 100 per cent.

Graph II is plotted on semi-logarithmic graph paper. The points on the graph were determined by computing the numerical average of the virus infectivity titer of the samples taken at given periods of time. The data plotted on this graph confirms the fact that the T-3 bacteriophage is inactivated by sonic oscillations in a non-logarithmic manner. If it were inactivated logarithmically a straight line relationship would exist between the points.

CHART I

EFFECT OF SONIC OSCILLATIONS UPON PROCESSED LYSATES

Trial	Minutes Treatment 0	0.5	1	2	3	4	5
I	2.7×10^{10}		2.2×10^{10}	2.4×10^{10}	2.7×10^{10}	2.9×10^{10}	3.3×10^{10}
II	2.6×10^{10}						
III	4.56×10^{10}		4.63×10^{10}		3.06×10^{10}		2.4×10^{10}
IV	4.83×10^{10}	3.83×10^{10}	3.93×10^{10}	3.80×10^{10}	3.46×10^{10}		
V	1.36×10^{10}	1.0×10^{10}	1.4×10^{10}	1.23×10^{10}		8.23×10^{10}	

CHART IA

EFFECT OF SONIC OSCILLATIONS UPON PROCESSED LYSATES

Trial	Minutes Treatment 7	8	9	10	13	15	16
I	3.3×10^{10}						
II				1.8×10^{10}	9.0×10^{9}		7.0×10^{9}
III	3.26×10^{10}		2.73×10^{10}			1.0×10^{10}	
IV							
V		6.3×10^{9}					

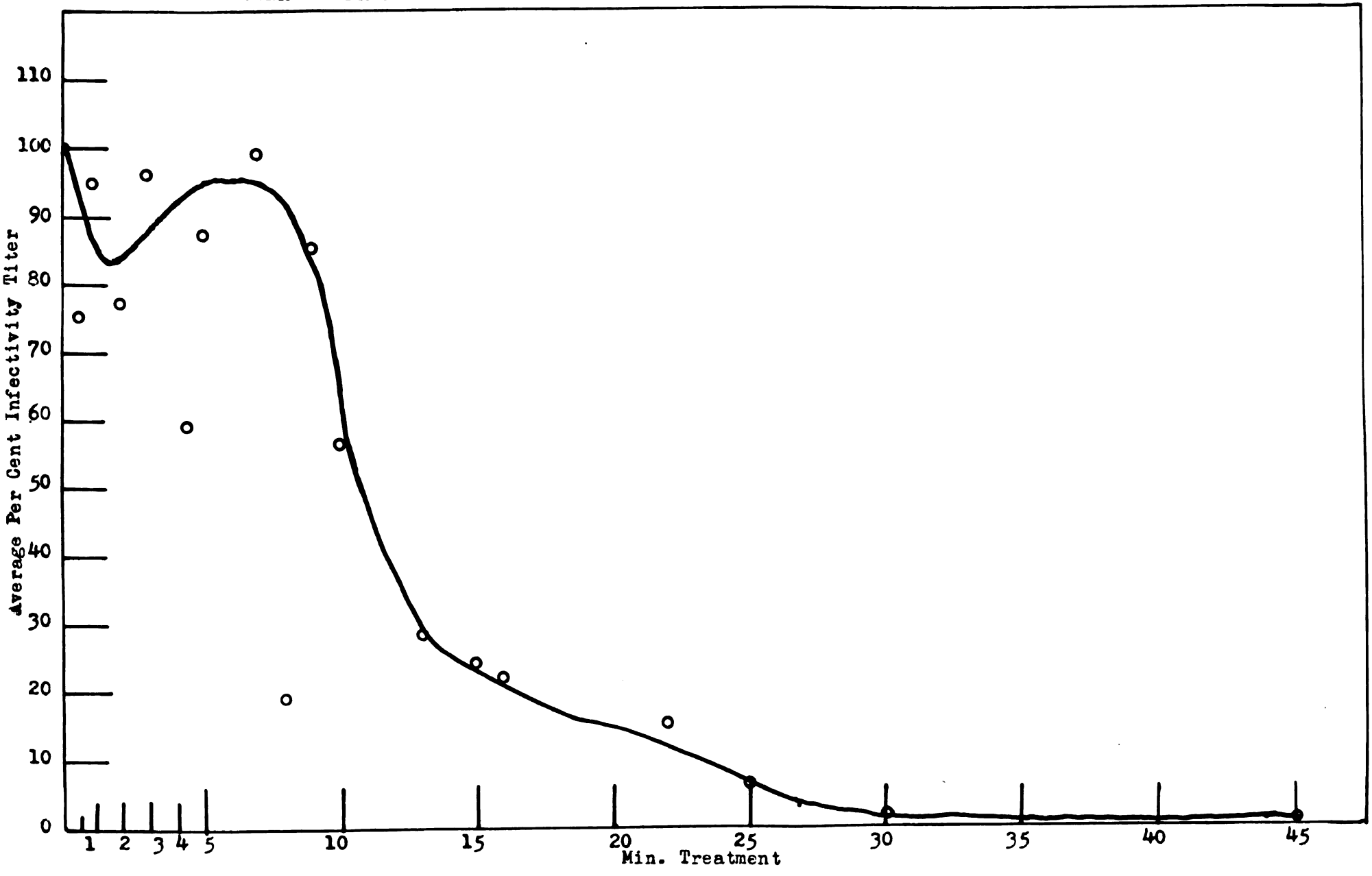
CHART IB

EFFECT OF SONIC OSCILLATIONS UPON PROCESSED LYSATES

Trial	Minutes Treatment 19	22	25	30	45
I					
II	8.0×10^9	4.8×10^9	3.4×10^9	2.7×10^9	3.8×10^8
III					
IV					
V				2.8×10^9	

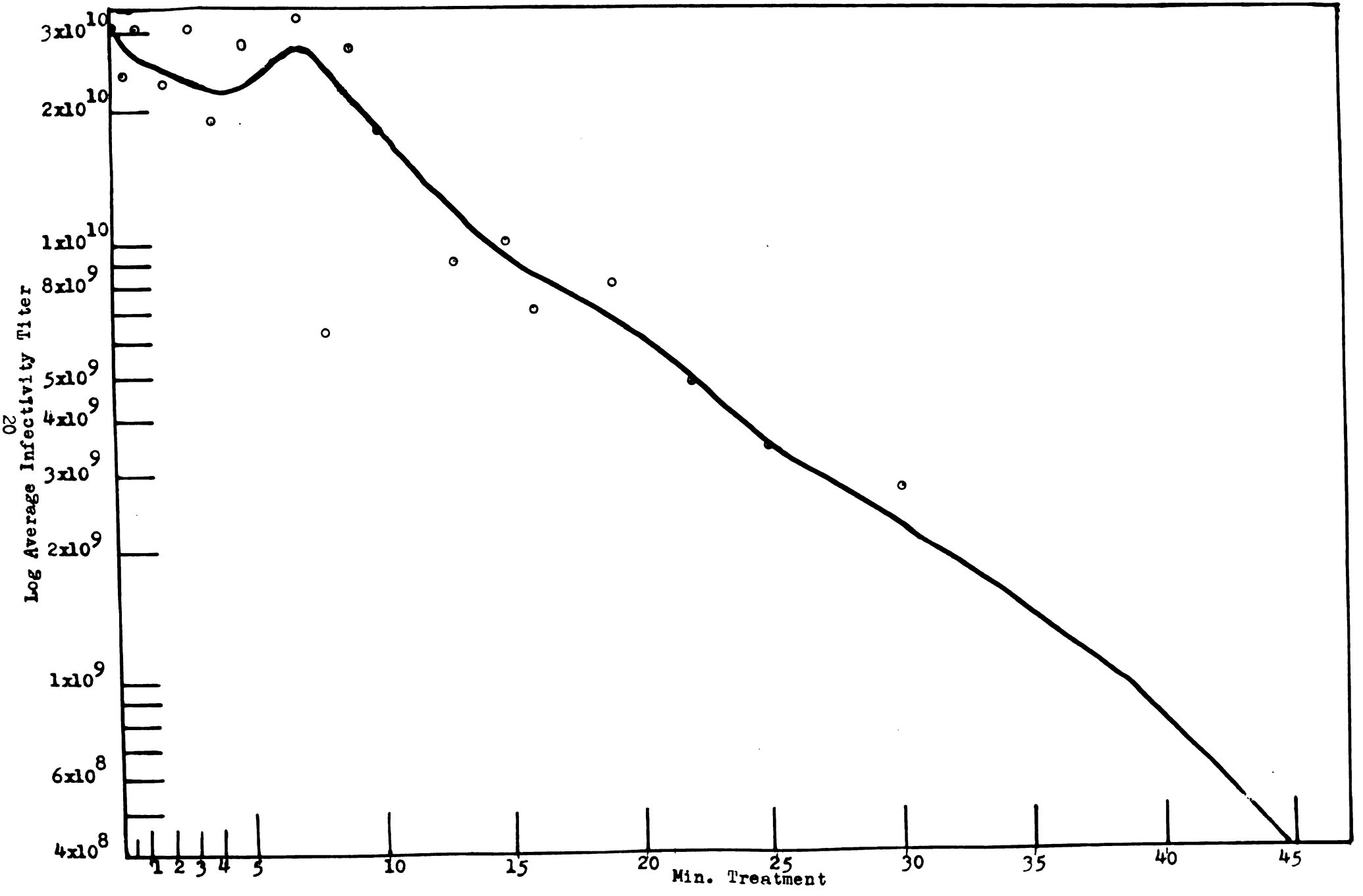
GRAPH I

AVERAGE PER CENT CHANGE IN TITER OF SONIC TREATED PROCESSED LYSATES IN EXPERIMENT I



GRAPH II

AVERAGE INFECTIVITY TITER OF PROCESSED LYSATES TREATED WITH SONIC OSCILLATIONS IN EXPERIMENT I



EXPERIMENT II

SONIC TREATMENT OF PROCESSED LYSATES IN PROTEIN MEDIA

Lysates produced on two different media were used in this experiment. The first was produced on a "high protein" medium consisting of 20 gm peptone (Albimi*), 3.0 gm yeast autolysate (Albimi), 3.0 gm K_2HPO_4, 5.0 gm NaCl, and 1,000 ml of distilled water. One ml of Ringer's solution (291.6 gm NaCl, 12.5 gm KCl, 13.77 gm $CaCl_2$ in 1,000 ml distilled water) was added to each 100 ml of medium.

Fifty ml of the above medium was placed in a flask and 2.0 ml of a stock suspension of E. coli was added. The aerated culture was incubated at 37° C in a water bath. After 18 hours, 2.0 ml of bacteriophage was added to the bacterial culture and the culture was incubated for 24 hours. This lysate was centrifuged for 20 minutes at 6,800 r.p.m. at 4° C to separate the bacterial debris from the virus. A clear supernatant fluid was removed which constituted the processed lysate. Upon standing in the refrigerator for 30 minutes a gray pellicle formed on the surface of the untreated lysate. The tube conteining the lysate was shaken to resuspend the pellicle and 15 ml was placed in the treatment chamber. The oscillator was operated at full power. Samples were withdrawn at the times shown in Chart II and titrated for virus infectivity. All sonic treated samples, after the 0.5 minute sample, were cloudy due to the dispersion of the pellicle throughout the medium.

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*Albimi Laboratories Inc., Brooklyn 2, New York.

A lysate was produced on "F" agar and harvested in nutrient broth. This lysate was centrifuged for 20 minutes at 9,000 r.p.m. at 4° C. Fifteen ml of the processed lysate was placed in the treatment chamber and samples were removed for infectivity titration at the times shown on Chart II. The oscillator was operated at full power.

Results

The results of this experiment are shown in Chart II and Graph III. Graph III shows the per cent change in virus infectivity titer of the treated lysates. The original (0 min) titer is considered to be 100 per cent.

The original titer (0 min) of the virus produced in the high protein medium was 9.0×10^9 infective particles per ml. Sonic treatment of this virus suspension produced an increase in titer to 1.1×10^{10} after one minute. The titer then decreased to 6.76×10^9 at the end of four minutes treatment. The infectivity titer increased 20 per cent after one minute of treatment. A 25 per cent reduction in infectivity titer occurred after four minutes of treatment.

Sonic treatment of the virus produced on "F" agar and harvested in nutrient broth produced different results. The original (0 min) titer of this lysate was 1.36×10^{10} . After a treatment of 0.5 min the titer decreased to 1.0×10^{10} ; this is a 25 per cent decrease. However, after one minute of treatment, the titer rose to 1.4×10^{10} , an increase of 3 per cent over the original titer. Like the high protein lysate infectivity titer, the titer of the lysate produced in nutrient broth rapidly declined after being treated for more than one minute.

THE

CHART II

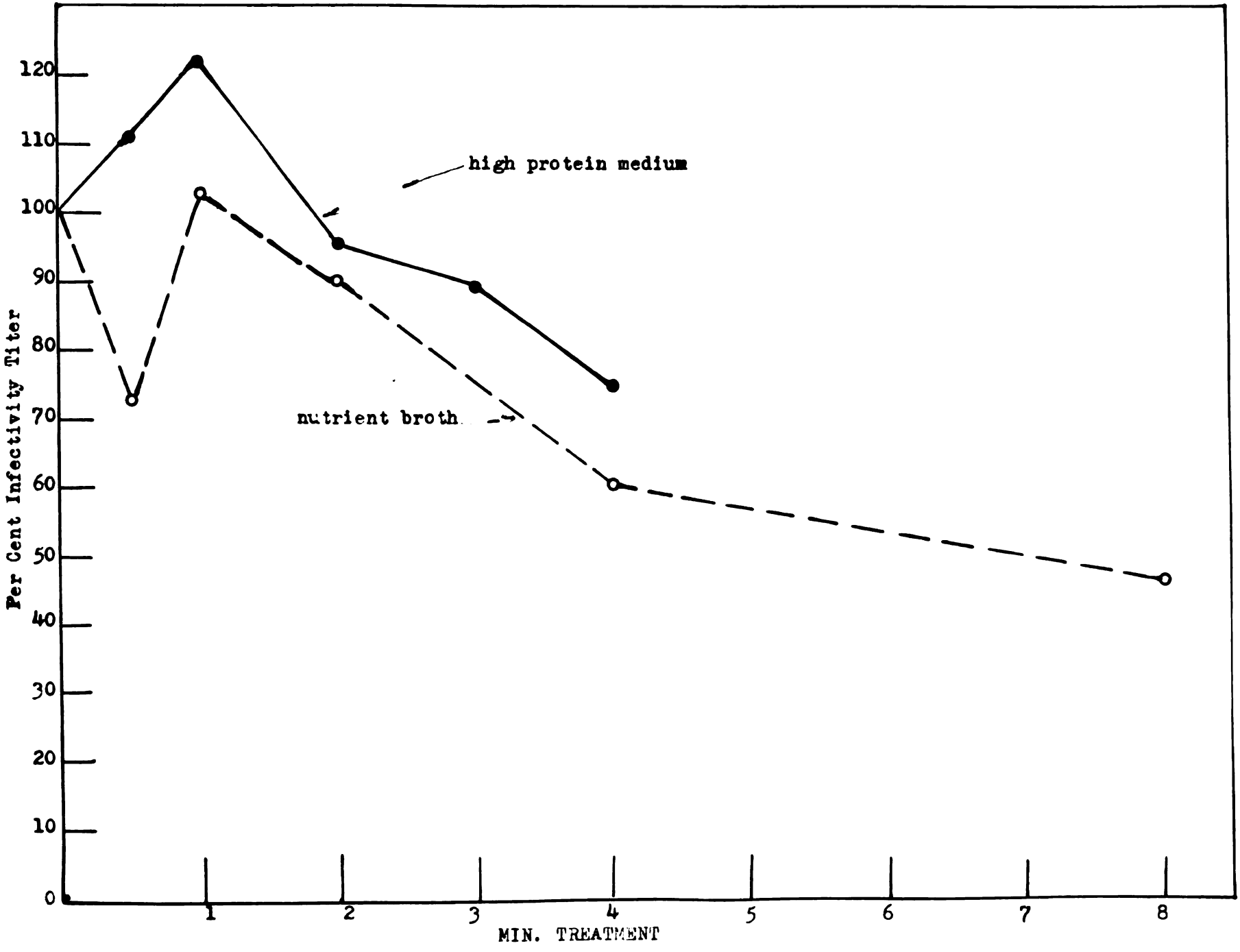
EFFECT OF SONIC OSCILLATIONS UPON PROCESSED LYSATES TREATED IN PROTEIN MEDIA

Medium	Minutes Treatment 0	0.5	1	2	3	4	8
High Protein	9.0×10^9	1.0×10^{10}	1.1×10^{10}	8.6×10^9	8.0×10^9	6.76×10^9	
Nutrient Broth	1.36×10^{10}	1.0×10^{10}	1.4×10^{10}	1.23×10^{10}		8.23×10^9	6.3×10^9

THE

GRAPH III

EFFECT OF SONIC OSCILLATIONS UPON PROCESSED LYSATES TREATED IN PROTEIN MEDIA



EXPERIMENT III

SONIC TREATMENT OF UNPROCESSED LYSATES

After the results of Experiment I were evaluated, it appeared that it would be advisable to investigate the effect of sonic oscillations upon unprocessed lysates. The amount of virus freed from the bacterial cell debris was investigated by harvesting the bacteriophage in buffer systems of varying hydrogen ion concentrations and treating the resulting lysate in the oscillator. If there was a relationship between pH and the release of the virus from the cell debris, the infectivity titer would increase after sonic treatment at a certain pH.

The virus was produced upon the surface of "F" agar in Petri dishes. It was harvested in a buffer system of the desired pH. The buffer solution was placed directly upon the surface of the agar and the growth removed with a triangular glass spreader. The liquid was drawn up in a pipette. Care was exercised to remove all the cellular debris without breaking the agar surface. Approximately 20 ml of unprocessed lysate was transferred to the previously cooled treatment chamber of the oscillator. The oscillator was then started and samples of the virus lysate were withdrawn from the chamber as in Experiment I. Two ml samples were taken from the chamber and placed in glass centrifuge tubes. The tubes were placed in the refrigerated centrifuge at 4.0° C. At the end of the sampling period the treated samples were centrifuged at 9,000 r.p.m. for 20 minutes at 4.0° C. Five-tenths ml of the top supernatant fluid was removed from each tube for titration of virus content.

Results

When the virus and bacterial debris were removed from the plates using buffers at pH 5.0, 6.0, 7.3, 7.5, 8.0 and 9.3, the resulting unprocessed lysate had a homogeneous grayish-white cloudy appearance. In the lysate harvested at pH 4.6, a large granular precipitate was observed to settle out of suspension after the flask containing the lysate was placed in the refrigerator. When buffers of pH 4.3 and 4.1 were used to harvest the lysate, a precipitate was also formed. After standing for a few minutes the suspension was found to consist of two phases. The first phase was a heavy granular precipitate composed of large particles that rapidly settled out of suspension. The second phase was a fine granular suspension that did not settle out, but produced a translucent clouding of the buffer solution.

Treatment of the buffer harvested, unprocessed lysates in the sonic oscillator, produced the results shown in Chart III and Graphs IV and V. Graphs IV and V are plotted on semi-logarithmic paper. The original (0 min) titer of the unprocessed lysate equals 100 per cent. As can be seen in Graphs IV and V the effect of treating the unprocessed lysates with sonic oscillations resulted in infectivity titers varying with the pH of the lysate.

There was little change in appearance of the unprocessed lysates; pH 6.0 to pH 9.3, after sonic treatment. The maximum increase in infectivity titer occurred in the lysate treated at pH 8. The titer increased to 180 per cent after 0.5 minutes of treatment. The next greatest increase in virus titer occurred at pH 6; 170 per cent at one minute. The least increase in infectivity

titer was observed at pH 7.5 and pH 7.3. Both of these lysates exhibited the initial drop in virus titer observed in the treating of processed lysates (Graph I). The maximum increase in infectivity titer occurred in both samples after two minutes of sonic treatment and was 125 and 120 per cent respectively.

The unprocessed lysate, treated at pH 5, although having the same appearance as the lysates in the pH range from 6.0 to 9.3, and showing no change in appearance after sonic treatment, showed a much greater increase in virus infectivity titer. After one minute of sonic treatment the virus titer increased to 650 per cent. The infectivity titer then decreased.

After sonic treatment the virus lysates harvested in buffers pH 4.7, 4.6, 4.3 and 4.1 had a homogeneous grayish-white cloudy appearance. However, after standing in the refrigerated centrifuge for several minutes, prior to centrifugation, a gray flocculent precipitate formed in the centrifuge tubes containing the sonic treated samples. Upon centrifugation the precipitate was completely removed from the solution and a clear supernatant fluid was obtained.

The greatest increase in infectivity titer occurred in the unprocessed lysate treated at pH 4.3. The virus titer increased 37,000 per cent after 4 minutes of treatment. The next greatest increase in virus titer was observed at pH 4.1; 31,000 per cent after 4 minutes of sonic treatment. The smallest increase, 510 per cent, occurred in the virus harvested in buffer pH 4.6 after 3 minutes of treatment.

No attempt was made to treat virus lysates suspended in buffer solutions below pH 4.1 because of the great loss in infectivity titer that occurs below this pH (see Experiment V).

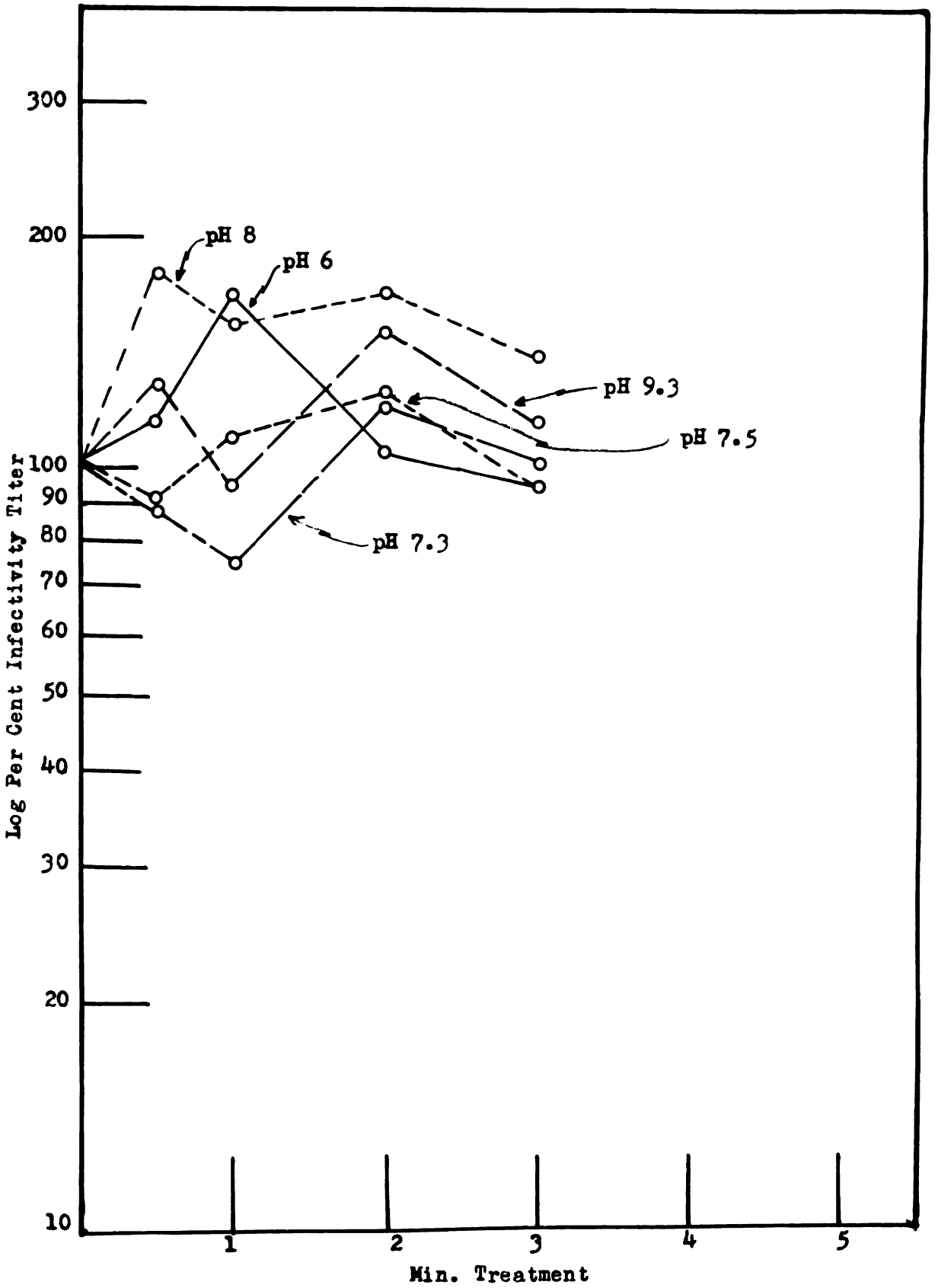
CHART III

CHANGE IN TITER OF UNPROCESSED LYSATES TREATED WITH SONIC OSCILLATIONS

Test	pH Lysate	Duration of Sonic Exposure in Minutes 0	0.5	1	2	3	4	5
I	4.1	1.3×10^{6}		7.6×10^{6}		1.73×10^{8}	4.2×10^{8}	
II	4.3	1.0×10^{7}			1.83×10^{9}	2.83×10^{9}	3.86×10^{9}	3.16×10^{9}
III	4.6	3.73×10^{9}		1.3×10^{10}	1.23×10^{10}	1.93×10^{10}	1.83×10^{10}	
IV	4.7	2.46×10^{9}	1.7×10^{10}	2.53×10^{10}	2.4×10^{10}	3.03×10^{10}		
V	5.0	8.6×10^{9}	4.35×10^{10}	5.6×10^{10}	4.25×10^{10}	3.9×10^{10}		
VI	6.0	3.48×10^{10}	4.0×10^{10}	6.0×10^{10}	3.7×10^{10}	3.3×10^{10}		
VII	7.3	8.3×10^{10}	7.3×10^{10}	6.3×10^{10}	1.0×10^{11}	8.3×10^{10}		
VIII	7.5	1.26×10^{11}	1.13×10^{11}	1.4×10^{11}	3.3×10^{11}	1.2×10^{11}		
IX	8.0	1.26×10^{11}	2.3×10^{11}	1.93×10^{11}	2.23×10^{11}	1.76×10^{11}		
X	9.3	2.75×10^{10}	3.55×10^{10}	2.66×10^{10}	4.13×10^{10}	3.2×10^{10}		

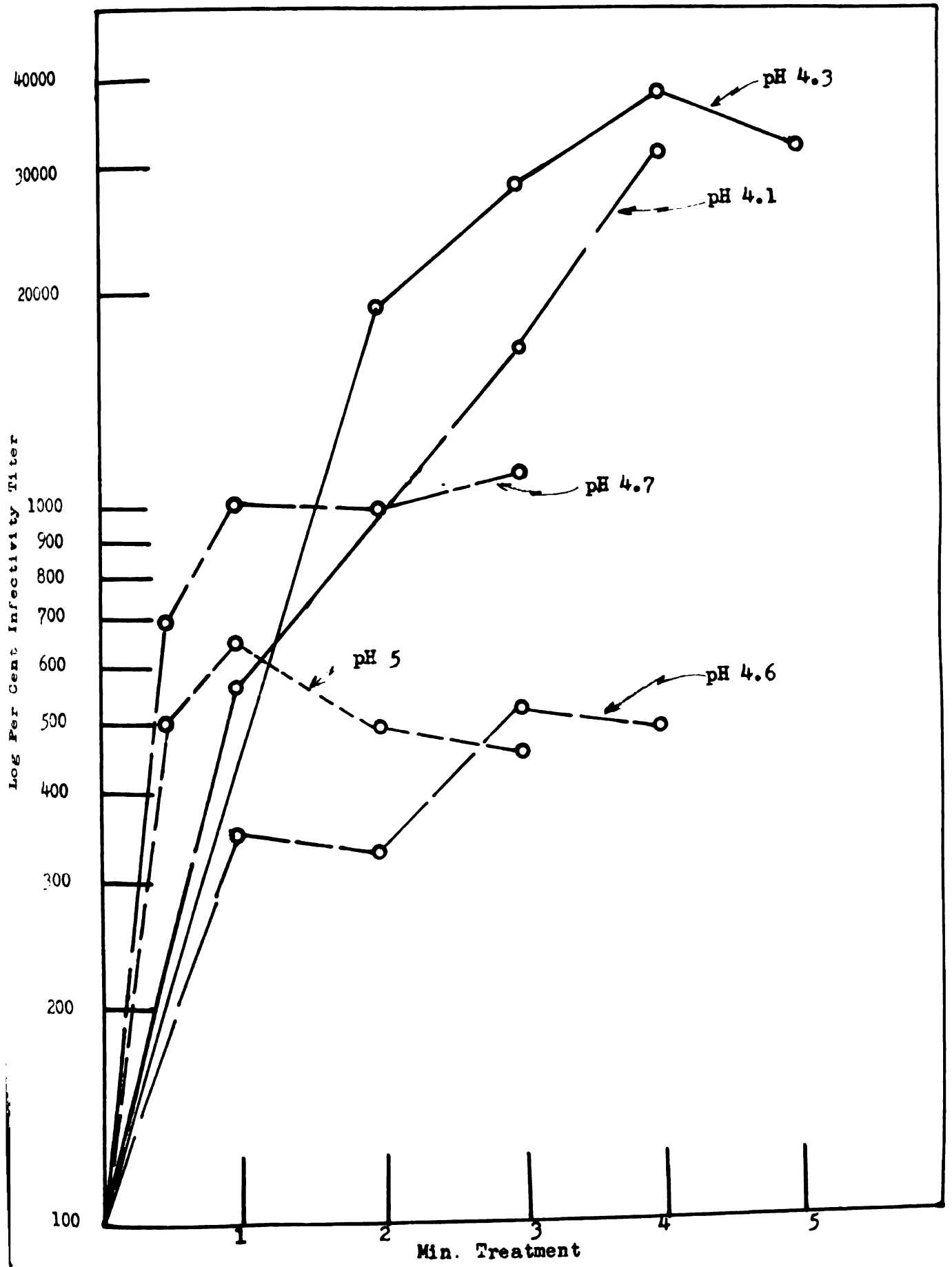
GRAPH IV

EFFECT OF SONIC OSCILLATIONS ON UNPROCESSED LYSATES pH 6 TO pH 9.3



GRAPH V

EFFECT OF SONIC OSCILLATIONS ON UNPROCESSED LYSATES pH 4.1 TO pH 5



EXPERIMENT IV

SONIC TREATMENT OF PROCESSED LYSATES AT pH 4.6 AND pH 4.1

Two bacteriophage lysates were produced on the surface of "F" agar in 150 mm Petri dishes. Both were harvested in 20 ml of sterile distilled water per plate. The lysates were centrifuged at 9,000 r.p.m. for 20 minutes at 4.0° C to remove unlysed cells and bacterial debris. Sterile 0.1 N HCl was added, drop by drop, to the two resulting processed lysates. While the acid was being added, the lysates were vigorously stirred by a magnetic stirrer. The pH of the first lysate was adjusted to 4.6 and the second to pH 4.1.

The lysates were each placed separately in the previously cooled treatment chamber. The oscillator was operated at full power and both lysates were treated under the same conditions. Five-tenths ml samples of treated lysate were withdrawn from the chamber for virus infectivity titration at the times shown on Chart IV.

Results

Before sonic treatment the pH 4.6 virus suspension was found to contain 1.0×10^{10} infectious virus particles per ml. During the first three minutes of treatment there was little change in the virus concentration (Graph VI). Five minutes exposure to sonic oscillations reduced the virus titer to 9.53×10^{9}. When the suspending medium was adjusted to pH 4.1 a marked difference was obtained when the virus was exposed to sonic oscillations. The infectivity titer prior to sonic treatment was 8.5×10^{7}. As can be shown best in Graph VI, the infectivity titer of the virus

rapidly declined and in six minutes decreased to 3.23×10^7. This decline represents an 80 per cent decrease in infectious virus.

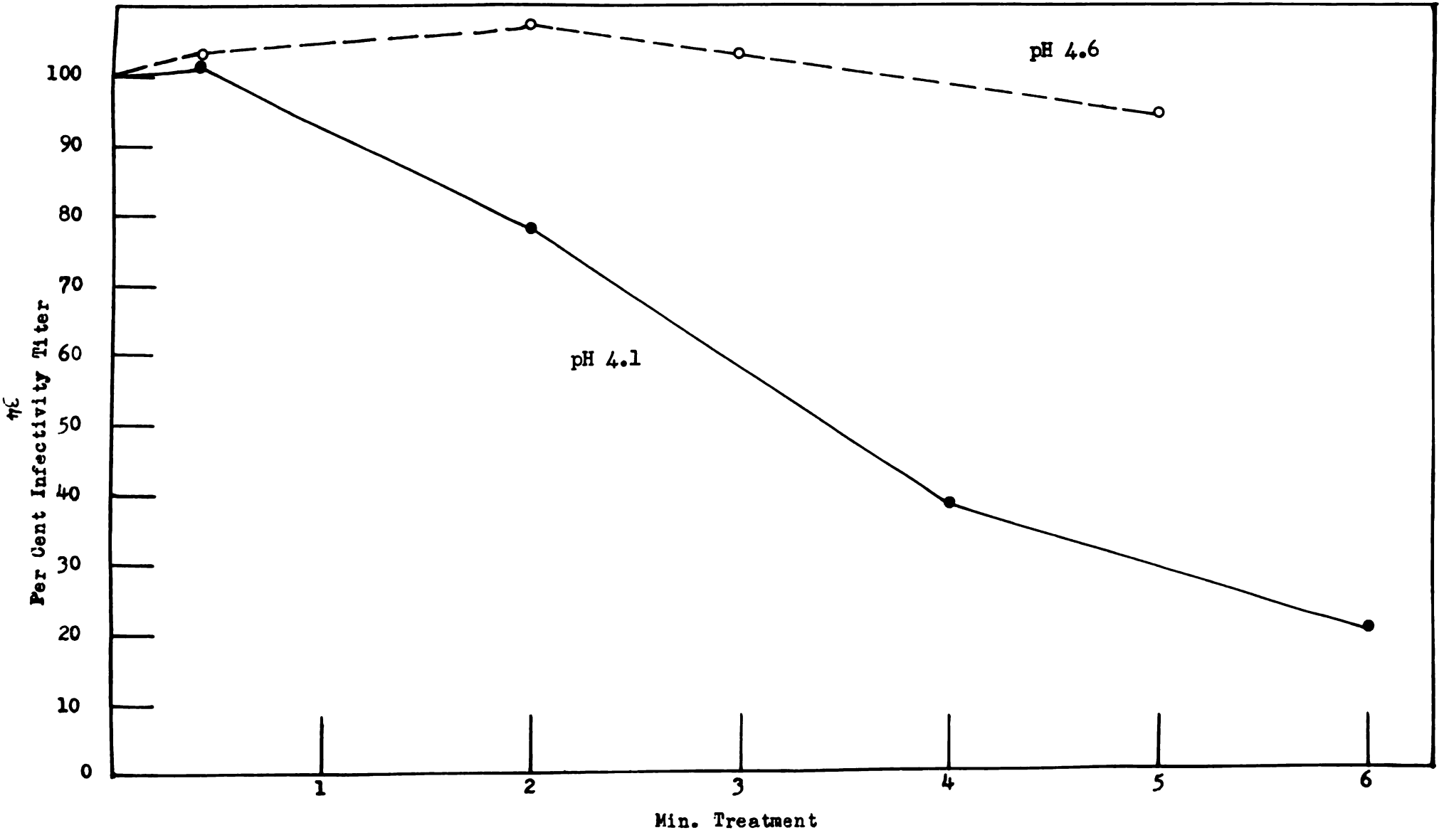
CHART IV

EFFECT OF pH UPON PROCESSED SONIC TREATED LYSATES

Lot No.	pH	Treatment Time in Minutes 0	0.5	2	3	4	5	6
US26	4.1	8.5×10^7	8.6×10^7	6.56×10^7		3.23×10^7		1.73×10^7
US25	4.6	1.01×10^{10}	1.043×10^{10}	1.083×10^{10}	1.046×10^{10}		9.53×10^9	

GRAPH VI

EFFECT OF SONIC OSCILLATIONS UPON PROCESSED LYSATES TREATED AT pH 4.1 AND pH 4.6



EXPERIMENT V

pH INACTIVATION OF T-3 BACTERIOPHAGE PROCESSED LYSATES

After the completion of Experiments III and IV it was deemed desirable to ascertain the effects of pH upon untreated T-3 bacteriophage lysates.

The virus in this experiment was produced on the surface of "F" agar in 150 mm Petri dishes. The lysate was harvested in distilled water and centrifuged at 9,000 r.p.m. for 20 minutes at 4.0° C to remove the unlysed cells and bacterial debris. The resulting processed lysate was equally divided into two sterile 50 ml flasks.

A sterile Teflon coated magnetic stirring bar was placed in the first flask. Three ml of the lysate was transferred to a sterile 5.0 ml beaker and the hydrogen ion concentration determined. The sample was then returned to the flask. Sterile distilled water was used to wash the electrodes after each pH determination. A 0.5 ml sample was then withdrawn for infectivity titration.

Sterile 0.1 N HCl was added drop by drop to the lysate in the first flask. A magnetic stirrer was used to stir the lysate while the acid was being added. After a small amount of acid was added, the pH was determined and a virus sample was removed for infectivity titration. More acid was then added and the process of pH determination and infectivity titration repeated.

The procedure was repeated to raise the pH of the processed lysate in the second flask using 1.0 N NaOH.

Results

The infectivity of the T-3 bacteriophage appears to be quite stable over a pH range from 4.4 to 9.4 (Graph VII). Below pH 4.4 and somewhere between pH 9.4 and 11.3 infectivity is rapidly lost. At pH 4.4 the infectivity titer of the processed lysate was 1.7×10^{10}. When the pH was lowered to 4.1 the titer decreased to 1.26×10^{9} (Chart V). This is a loss of more than one log. At pH 3.9 the infectivity titer precipitously dropped to 9.0×10^{2}. When the hydrogen ion concentration was increased to pH 3.5 no infective virus could be detected when tested at a 10^{-0} dilution.

When the hydrogen ion concentration of the second lysate was decreased to pH 11.3 the infectivity titer dropped to 2.76×10^{9}.

As the pH of the processed lysate was lowered no visible change was observed until the pH reached 4.1. A fine precipitate was formed. It remained dispersed throughout the medium because of the action of the magnetic stirrer.

As NaOH was added to the second lysate, a clouding of the suspension occurred. Clouding was first observed at pH 9.3 and became more pronounced as more NaOH was added to raise the pH to 11.3. When the virus suspension was placed in the refrigerator for 24 hours a fluffy precipitate was produced that settled to the bottom of the flask.

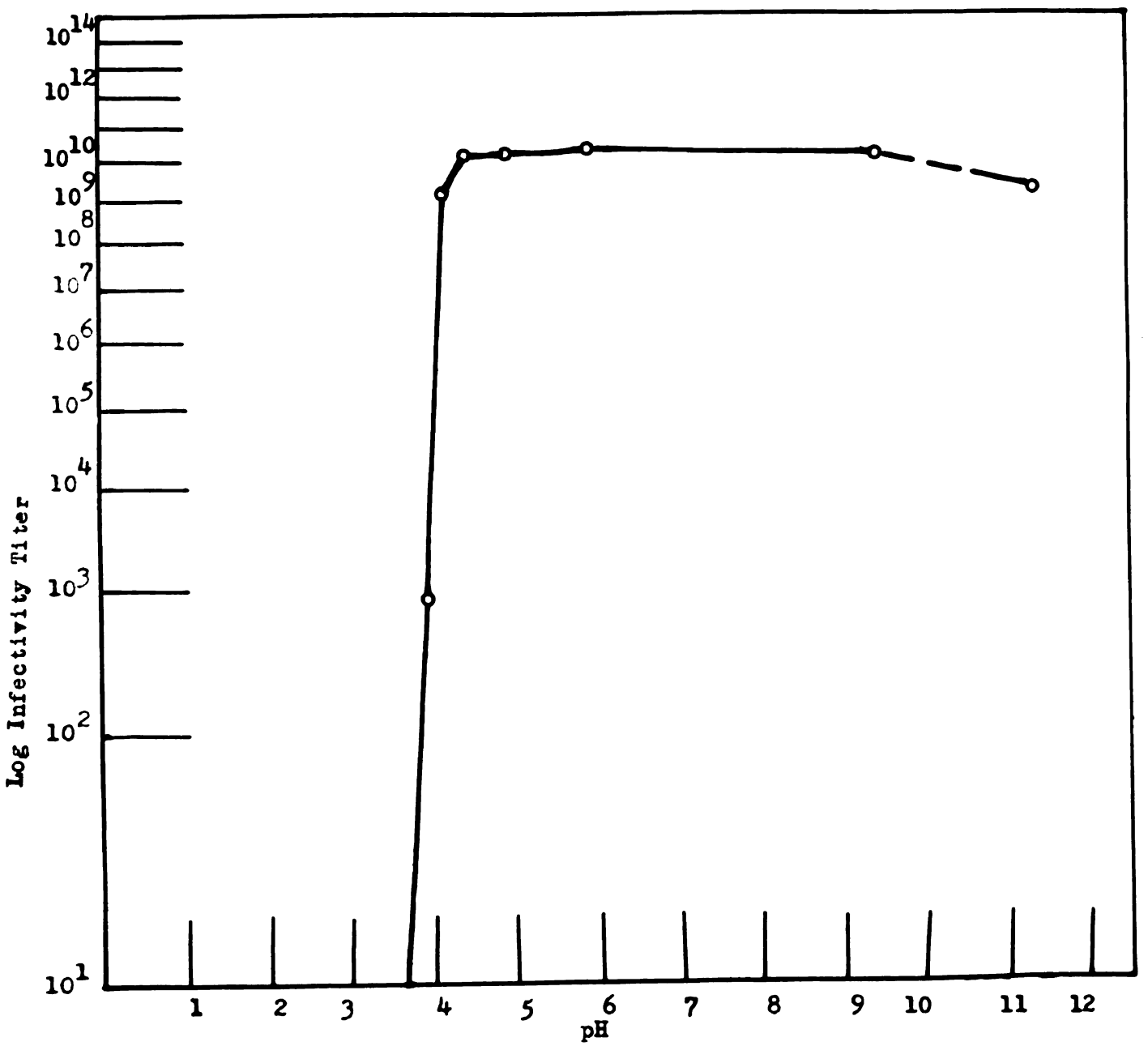
CHART V

pH INFECTIVITY INACTIVATION OF T-3 BACTERIOPHAGE

pH	Virus Dilution Tested	Titer
11.3	10^{-7}	2.76×10^{9}
9.4	10^{-7}	1.81×10^{10}
5.9	10^{-8}	2.56×10^{10}
4.9	10^{-8}	2.06×10^{10}
4.4	10^{-8}	1.70×10^{10}
4.1	10^{-7}	1.26×10^{9}
3.9	10^{-1}	9.00×10^{2}
3.5	10^{-0}	0.00

GRAPH VII

pH INACTIVATION OF T-3 BACTERIOPHAGE



DISCUSSION

DISCUSSION

From the experiments by Anderson, Boggs and Winters (1948) and the information obtained from Experiment I it is apparent that the T-3 bacteriophage is relatively resistant to the effects of sonic oscillations. During the first several minutes of sonic treatment of processed lysates an unexplained reaction occurs. In three of four tests (Trials I, III, IV, and V, Chart I, Graph I) an initial decrease in virus infectivity titer was observed during the first minute of sonic treatment. The virus concentration increased to its original infectivity titer or to a higher titer. Upon further treatment the infectivity titer slowly decreased. After 45 minutes of treatment a decrease in virus concentration of approximately two logs, or 99 per cent occurred (Chart I, Graph I). Although the percentage decrease is great the actual virus loss is not since 10^8 infectious particles per ml remained after treatment; the original lysate contained 10^{10} infectious particles per ml.

It is possible that a rapid inactivation of the more sonic labile particles causes the initial decrease in infectious titer during the first minute of sonic treatment. The subsequent increase in virus infectivity titer is undoubtedly due to the dispersion of naturally occurring aggregates of virus particles.

Since Experiment I indicated that there were aggregates of virus particles in the processed lysates it was deemed desirable to investigate methods of dispersing the aggregated and thus increasing the virus infectivity titer. During the treatment of the aqueous suspensions of bacteriophage, it was possible that the

number of virus particles inactivated during the first several minutes of sonic treatment equaled the number of particles produced by dispersing the aggregates. If the virus could be protected from the deleterious effects of the sonic oscillations then a significant rise in virus infectivity titer might result. Therefore, the virus was suspended for sonic treatment in a "high protein" medium and in nutrient broth (Experiment II). The processed lysate treated in "high protein" medium showed an increase in infectivity titer of 20 per cent and did not exhibit the initial drop in virus infectivity titer (Graph III). The lysate treated in nutrient broth showed the typical initial drop in titer and increase in infectivity of only three per cent (Graph III). The pH of both lysates was approximately the same. The "high protein" medium has a higher viscosity than the nutrient broth. This may explain the greater increase in virus titer in the lysate treated in the "high protein" medium. The "high protein" medium contains 20 gm of peptone per liter compared to 5 gm of peptone per liter in nutrient broth (Difco).

In Experiment III the effect of sonic oscillations on unprocessed lysates at varying hydrogen ion concentrations was investigated. In the initial trials made between pH 6 and pH 8 there was a difference in the infectivity titers obtained after the unprocessed lysates had been treated for varying periods of time at the different hydrogen ion concentrations. There was a correlation between the pH of the lysate and the increase in virus titer. Therefore, treatments were conducted at successively lower, and higher, hydrogen ion concentrations. It was found that as the pH decreased the virus infectivity titer of the treated lysate increased. At

pH 4.3 the maximum increase in virus titer occurred. As buffer systems with successively higher hydrogen ion concentrations were used the virus titer of the original (0 min) lysate sample decreased (Chart III).

The amount of precipitate in the unprocessed lysates increased as the pH was decreased below 4.6. The isoelectric point of E. coli is between pH 4 and pH 5 (Puck 1954). E. coli is positively charged. The T bacteriophage is negatively charged (Putnam et al. 1949, Puck 1954). Puck (1954) stated that the isoelectric point of the T bacteriophage is approximately 4.

As the pH is decreased and the isoelectric point of the bacterium is approached it will become less positively charged. As the isoelectric point of the bacteriophage is approached its negative charge will decrease. The normal electrostatic attractive forces between the bacteriophage and the bacterium will therefore be decreased. Furthermore, the electrostatic repulsive forces between individual bacteria and individual bacteriophage particles will be progressively lessened as their isoelectric points are approached.

Therefore, it is possible that as the isoelectric point of the virus was reached, and all charge was lost, aggregation of the virus particles could occur. The possibility also exists that as the precipitate of the bacterial cell debris is forming, the virus particles will become entrapped in the masses of precipitating debris. The results of Experiment III show that one or both of the aforementioned processes was occurring.

In order to determine the magnitude of the aggregation, Experiment IV was performed. The hydrogen ion concentrations of two processed lysates were adjusted to the points where large increases in virus infectivity titer had occurred during sonic treatment of unprocessed lysates. No significant increase in the virus titer occurred during the sonic treatment of these processed lysates (Chart IV, Graph VI). If aggregation had taken place it was of such a nature that the aggregates formed could not be broken up by sonic oscillations of the magnitude used in these experiments.

The results of Experiment IV show that the great increase in virus infectivity titer occurring in unprocessed lysates treated below pH 4.6 was not due to the disruption of viral aggregates. The increases in titer must have been due to the freeing of bacteriophage particles from the precipitated cell debris.

Ultracentrifugal studies conducted in this laboratory showed that the T-3 bacteriophage sedimented rapidly at pH 5 and below, and that analytical observations were impossible. This could be due to the aggregation of bacteriophage particles. It is interesting to note that this unresolved sedimentation took place at the same pH at which the virus rapidly started to lose infectivity in the pH inactivation experiment (Experiment V).

Bernal and Fankuchen (1941) showed that the distance between adjacent tobacco mosaic virus particles is smallest at their isoelectric point. The intra-particulate distance will increase on either side of the isoelectric point. Pollard (1953) stated that at a distance of six Ångstrom units a strong binding force exists between two southern bean mosaic virus particles. This virus has

approximately the same diameter as the T-3 bacteriophage (Stanley and Lauffer 1952). The above data give evidence that aggregation of virus particles takes place at hydrogen ion concentrations near their isoelectric point.

At hydrogen ion concentrations near neutrality only a small number of bacteriophage could be freed from bacterial debris by sonic treatment (Experiment III, Tests VII, VIII, and Graph IV). Therefore, the lysis process apparently frees a very large number of virus particles and if any remain attached to the cell debris, their attachment is such that it can not be broken by sonic oscillations of the magnitude used in these experiments.

From the results of these experiments, it appears that by decreasing the pH of the suspending medium, irreversible aggregation of T-3 bacteriophage is produced. However, naturally occurring aggregates, in the pH range from 5 to 9 can be dispersed by the use of sonic oscillations.

SUMMARY

SUMMARY

Two types of T-3 bacteriophage lysates were produced on solid "F" medium, (1) processed lysates which were harvested in distilled water and centrifuged to remove bacterial debris, and (2) unprocessed lysates which were harvested with buffer solutions of various hydrogen ion concentrations. The lysates were exposed to sonic oscillations for varying periods of time at the end of which samples were taken and assayed for infectious virus.

Sonic treated processed lysates showed an initial drop in virus titer followed by an increase in infectious titer during the first several minutes of treatment. This increase reached 20 per cent in one case (Experiment II).

Unprocessed lysates were treated at varying hydrogen ion concentrations (Experiment III). These showed large increases in virus infectivity titer when treated between pH 4.6 and pH 4.1. This increase appeared to be due to the freeing of viral particles from precipitated bacterial debris.

Processed lysates treated at pH 4.1 and pH 4.6 did not show significant rise in virus titer (Experiment IV).

The infectivity of the T-3 bacteriophage is stable over a pH range of 4.4 to 9.4.

Below pH 4.9 aggregation of the bacteriophage particles occurs. These aggregates can not be dispersed to any significant extent by sonic oscillations of the magnitude used in these experiments.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams, M.H. 1950 Methods in Medical Research, edited by J.H. Comroe, Jr. 2: 1-73.

Anderson, T. F., Boggs, S., and Winters, B.C. 1948 The relative sensitivity of bacterial viruses to intense sonic vibrations. Science 108: 18.

Bernal, J.D. and Fankuchen, I. 1941 X-ray and crystallographic studies of plant virus preparations. J. of General Physiology 25: 111-146.

Fry, W. J., Wulff, V.J., Tucker, D.T., and Fry, F. J. 1950 Physical factors involved in ultrasonically induced changes in living systems. The J. of the Acoustical Soc. America 22: 867-876.

Gratia, A. 1936 Des relations numerique entre bacteries lysgenes et particules de bacteriophage. Annales De L'institut Pasteur 57: 652-676.

Gregg, E. C. Jr. 1950 Ultrasonics: biologic effects. Medical Physics, edited by Otto Glasser 2: 1132-1138.

Harvey, E. N., Barnes, D. K., McElroy, W. D., Whiteley, A. H., Pease, D. C., and Cooper, K. W. 1944 Bubble formations in animals, I physical factors. J. of Cellular and Comparative Physiology 24: 1-21.

Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W. 1946 Isolation and characteristics of the T-2 bacteriophage of Escherichia coli. J. Biological Chem. 165: 241-258.

Johnson, C. H. 1929 The lethal effect of ultrasonic radiation. J. of Physiology 67: 357-359.

Johnson, F. H., Baylor, M. B., and Fraser, D. 1948 The thermal denaturation of tobacco mosiac virus in relation to hydrostatic pressure. Archives of Biochem. 19: 237-245.

Kabat, E. A. and Mayer, M. M. 1948 Experimental Immunochemistry. C. C. Thomas, Springfield, Ill., p. 538.

Luria, S. E. 1953 General Virology. John Wiley and Sons, Inc., New york, N. Y., pp. 159-160.

Pollard, E. C. 1953 The Physics of Viruses. Academic Press Inc., New York, N. Y., chapts. 7-8.

Puck, T. T. 1954 Cell attachment and penetration by viruses. The Dynamics of Virus and Rickettsial Infections, edited by F. W. Hartman, F. L. Horsfall, and J. G. Kidd. The Blakiston Co. Inc., New York, N. Y., pp. 16-20.

Putnam, F. W., Kozloff, L. M., and Neil, J. C. 1949 Biochemical studies of virus reproduction. J. of Biochem. 179: 303-322.

Schmitt, F. O. and Uhlemeyer, B. 1930 The mechanism of lethal effect of ultrasonic radiation. Proc. Soc. Exp. Biol. Med. 27: 626-628.

Stanley, W. M. 1934 The action of high frequency sound waves on tobacco mosaic virus. Science 80: 339.

Stanley, W. M. and Lauffer, M. A. 1952 Chemical and physical procedures. Viral and Rickettsial Infections of Man, edited by T. M. Rivers. Second Edition. J. B. Lippincott Co., Philadelphia, Pa., p. 20.

Takahashi, W. N. and Christensen, R. J. 1934 The virucidal action of high frequency sound radiation. Science 79: 415.

Williams, R. C. and Fraser, D. 1953 Morphology of the seven T-bacteriophage. J. of Bact. 66: 458-464.