STUDY OF THE TOXIN AND GELATIN-SPLITTING ENZYME OF CLOSTRIDIUM TETANI IN RELATION TO POPULATION

Thesis for the Degree of M. S.

Iva L. Ferguson

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THESIS

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Submitted to the faculty of Michigan State College in partial fulfillment of the requirements for the degree of Master of Science.

By

Iva L. Ferguson

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INTRODUCTION

A study of the many methods of tetanus toxin production leads one to believe that these methods were developed empirically, without a definite aim of discovering the principles of toxin formation. A comparison of the methods employed by producers of tetanus toxin reveals a wide variation in the medium used, in the time of incubation, and in the bacteriological technique. The potencies obtained by the different producers are nearly as varied as the methods employed. Most workers have been content to work toward the regular production of a toxin of uniform potency, with little interest in the mechanism of the production (1, 2, 3, 4, 5).

The literature contains little on the theories of toxin production. It has been customary to accept one of two theories developed in the early days of bacteriology. These theories are:

(1) Toxin is formed in the bacterial cell and liberated through the cell wall during the life of the cell or at its death on autolysis.

(2) Toxin is a substance formed in the medium surrounding the bacterial cell, or it reaches its final form in the surrounding medium.

The most recent publications were by Palitzsch and Walbum (6) and Walbum and Reymann (7) who favor the theory that exotoxins are substances formed, or reach final form, in the medium surrounding the bacterial cell.

This study was initiated as a preliminary effort to gather fundamental data on the mechanism of toxin production. The sound approach appeared to be the accurate measurement of as many as possible of the phenomena occurring during the growth of Clostridium tetanus in ordinary veal infusion broth. To this end an attempt was made to measure, each day, the pH of the medium, the potency of the toxin, the activity of the gelatin-splitting enzyme, and the vegetative and spore cell population by count.

METHODS

1. Anaerobic method:

A modified Fildes jar (8) was used to obtain anaerobic conditions (Fig. 1 and 2). The technique for the operation of the jar was as follows: Hydrogen gas was supplied to the jar from a hydrochloric acid-zinc generator. A fixed volume of gas was passed slowly into the jar with the inlet and outlet valves open. Both valves were then closed. The jar, and a 50 ohm resistance unit were connected in series with a 110 volt light circuit for eight minutes. The circuit was then broken and the inlet valve opened. Additional hydrogen was passed into the jar to re-establish atmospheric pressure. The jar was allowed to cool an additional five minutes and hydrogen was again added to atmospheric pressure. The jar was then placed in the incubator at 37.5°C.

2. Preparation of the seed culture:

A culture of Cl. tetani (serological Type III, Pease) which had been maintained in 0.5% veal infusion semi-solid agar (Appendix 1) for six months was used for the seed culture.

The agar tube was placed in boiling water until the agar was melted. The contents of the tube were pipetted into a tube containing 0.5% dextrose veal infusion broth (Appendix 4) from which the air had been previously removed by boiling. The broth tube was incubated aerobically at 37.5°C. for 24 hours. Five tenths of a cubic centimeter of this broth culture was pipetted to the

Fig. I.

Fig. 2.

bottom of a peptic digest agar slant (Appendix 5) and incubated anaerobically for 48 hours at 37.5°C. At the end of this time growth was visible at the top of the slant.* A loopful of the growth from the top of the slant was placed in a second heated and cooled dextrose broth tube. This broth tube was incubated aerobically at 37.5°C. for 24 hours. The toxin broth used in each experiment was inoculated from a seed tube prepared in this fashion.

The initial seed tube was stored aerobically at 37.5°C. between each batch of toxin broth and was used for the preparation of subsequent seed tubes by the above technique.

3. Preparation and inoculation of tetanus toxin broth:

Five liters of tetanus toxin broth (Appendix 7) were prepared for each experiment. The broth was dispensed in 300 cubic centimeter amounts in sixteen-ounce French square bottles, autoclaved for 25 minutes at 121°C., and cooled rapidly to room temperature in running water. Fifteen cubic centimeters of sterile 20% dextrose solution were added to each bottle of broth following cooling. Sixteen bottles were inoculated with one cubic centimeter of the seed culture and incubated aerobically at 37.5°C.

* All motile strains of Cl. tetanus will grow over the entire surface of a peptic digest agar slant when incubated anaerobically and will rapidly outdistance contaminating organisms which may be present. This characteristic allows easy purification of any motile strain of Cl. tetanus.

Preliminary experiments indicated that more accurate results could be obtained by using single bottles for each day rather than one large bottle for the entire test period. It was found that tests carried out on a large volume of broth were inaccurate because of continuous reduction of volume and because of frequent contamination.

4. Sampling and order of testing:

A 50 cubic centimeter sample of broth was withdrawn from one bottle daily for sixteen days for testing. As each sample was taken the bottle was discarded.

Daily tests were carried out in a constant order and time following removal of the sample. Samples were removed at eight o'clock each morning. Bacterial counts were made immediately after the sample was taken. The balance of the sample was kept at room temperature for four hours during which time enzyme tests from the previous day were completed. The sample was filtered through a sterile Seitz filter and a colorimetric pH determination was made. The enzyme test was next carried out on the filtrate and closely followed by the toxin potency test.

5. Estimation of bacterial population:

Daily counts of the vegetative and spore forms present were made in a Petroff-Hauser counting chamber. Specimens were diluted with physiological saline

(Appendix 8) according to the turbidity of the sample. The maximum dilution factor used was 1:10.

Vegetative and spore forms were counted separately under oil immersion lens (720 diameters) with bright field illumination.

The number of organisms in eighty small squares were enumerated and counts per cubic centimeter were calculated according to the following formula:

$$\frac{\text{Number of organisms} \times \text{dilution} \times 20000}{\text{Number of smallest squares counted}} \times 1000 = \text{Organisms per cubic centimeter}$$

Three daily counts were made and averaged. Bacterial debris present was recorded qualitatively as not present, moderate or marked.

6. pH determination:

Daily measurements of hydrogen ion concentration were made colorimetrically with buffer standards.

7. Gelatin-splitting enzyme determination:

Seven rows of sterile $3\frac{1}{2} \times 5/8$" culture tubes were placed in a rack in a 37°C. water bath. The number of tubes in each row varied from five to eight depending upon the number of dilutions of filtrate measured. The first row was labeled pH 4, the second pH 5 and so on until pH 10 was reached. In the first tube of each row was placed the minimum volume of broth filtrate, in the second row an increased volume, and so on until the last row contained the maximum amount. The volumes of filtrate ranged from 0.01 cubic centimeter to 1.5 cubic centimeters. One cubic centimeter of gelatin-buffer

mixture (Appendix 9) was added to each tube. Each tube was made up to 3 cubic centimeters with sterile distilled water.

Each pH level was controlled by a tube containing one cubic centimeter of gelatin-buffer mixture plus two cubic centimeters of sterile distilled water.

All tubes were thoroughly shaken and incubated for eighteen hours at 37°C. in a constant temperature water bath.

At the end of the incubation period one drop of phenol red indicator (Appendix 12) was added to each tube, including the controls. Tenth normal sodium hydroxide or tenth normal hydrochloric acid was used to adjust each tube to pH 7.0 (faint pink to colorless). Each tube was made up to four cubic centimeters with sterile distilled water and all tubes were placed in a 10-13°C. icebox for thirty minutes. The degree of liquefaction of gelatin was recorded as follows:

± Liquefaction limited to meniscus of gelatin.

± Up to 50% liquefaction.

+ 50-100% liquefaction.

++ Complete liquefaction.

For the purpose of interpreting results the amount of liquefaction was expressed in units. The number of units in each tube was expressed as the reciprocal of the volume of filtrate.

8. Estimation of toxin potency:

The potency of the toxin present in the filtrates was measured in twenty-gram mice. The number of dilutions made on each daily filtrate was governed so as to determine the maximum number of mice minimum lethal doses* present per cubic centimeter of filtrate. All dilutions of the filtrates were made in physiological saline (Appendix 8) according to a standard scheme of dilutions. One cubic centimeter portions of each dilution of filtrate were injected subcutaneously over the abdomen. Dilution levels for each experiment ranged as follows: undiluted, 1:5, 1:50, 1:100, 1:500, 1:1000, 1:3000, 1:5000, 1:7000, 1:10000, 1:12000, 1:15000, 1:20000, 1:25000, 1:30000, 1:35000, 1:40000, 1:45000, 1:50000, 1:55000, 1:60000, No less than five dilutions of filtrate were tested on any one day. Animals were observed daily for symptoms of tetanus until six days had elapsed, at which time all living mice were destroyed.

* For the purpose of this study a mouse minimum lethal dose was defined as the smallest amount of toxin which would kill a twenty-gram mouse within six days with typical symptoms of tetanus.

RESULTS

Eight experiments were carried out but the results from only four were presented in this report. The first four experiments were performed more to standardize the technique and to eliminate experimental error than to yield data for study.

For the sake of clarity, data are presented in two sections: the first, summarizing the findings in each experiment; the second, comparing similar phenomena observed in each experiment.

Early experiments showed that results obtained from the samples withdrawn each day from a single flask containing 1800 cubic centimeters of broth were comparable in pH and toxin potency to samples withdrawn from individual French square bottles over the test period. In two of the four experiments reported here, daily pH measurements were made on a single volume of 1800 cubic centimeters of medium for the purpose of controlling possible pH variations in individual French square bottles. In all cases pH values were comparable.

COMPARISON OF INDIVIDUAL EXPERIMENTS

EXPERIMENT I

Table I

A Comparison of the Vegetative and Spore Population, the Hydrogen Ion Concentration, the Toxin and the Gelatin-Splitting Enzyme, and the Bacterial Debris of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 14 Days.

By Day

Day	Vegetative Cell Count Millions per c.c.	Spore Cell Count Millions per c.c.	pH	Minimum Lethal Doses of Toxin per c.c.	Enzyme Units* per c.c. Measured at pH 7.0	Debris
1	108	-	7.4	50	-	Not
2	71	15	7.4	1000	3	recorded
3	108	63	7.5	5000	3	"
4	145	60	7.6	6000	6	"
5	92	40	7.6	11000	20	"
6	61	43	7.4	15000	20	"
7	36	35	7.6	15000	20	"
8	23	22	7.7	18000	3	"
9	32	35	7.7	20000	20	"
10	18	72	7.7	25000	3	"
11	18	32	7.7	29000	20	Marked
12	6	23	7.7	29000	20	"
13	5	19	7.8	30000	20	"
14	6	12	7.4	30000	20	"

* This table and the tables in following experiments includes the number of units of gelatin-splitting enzyme measured at pH 7.0 since this pH reaction closely approximated the reaction of the medium during incubation. The data on other pH levels is summarized in Table II.

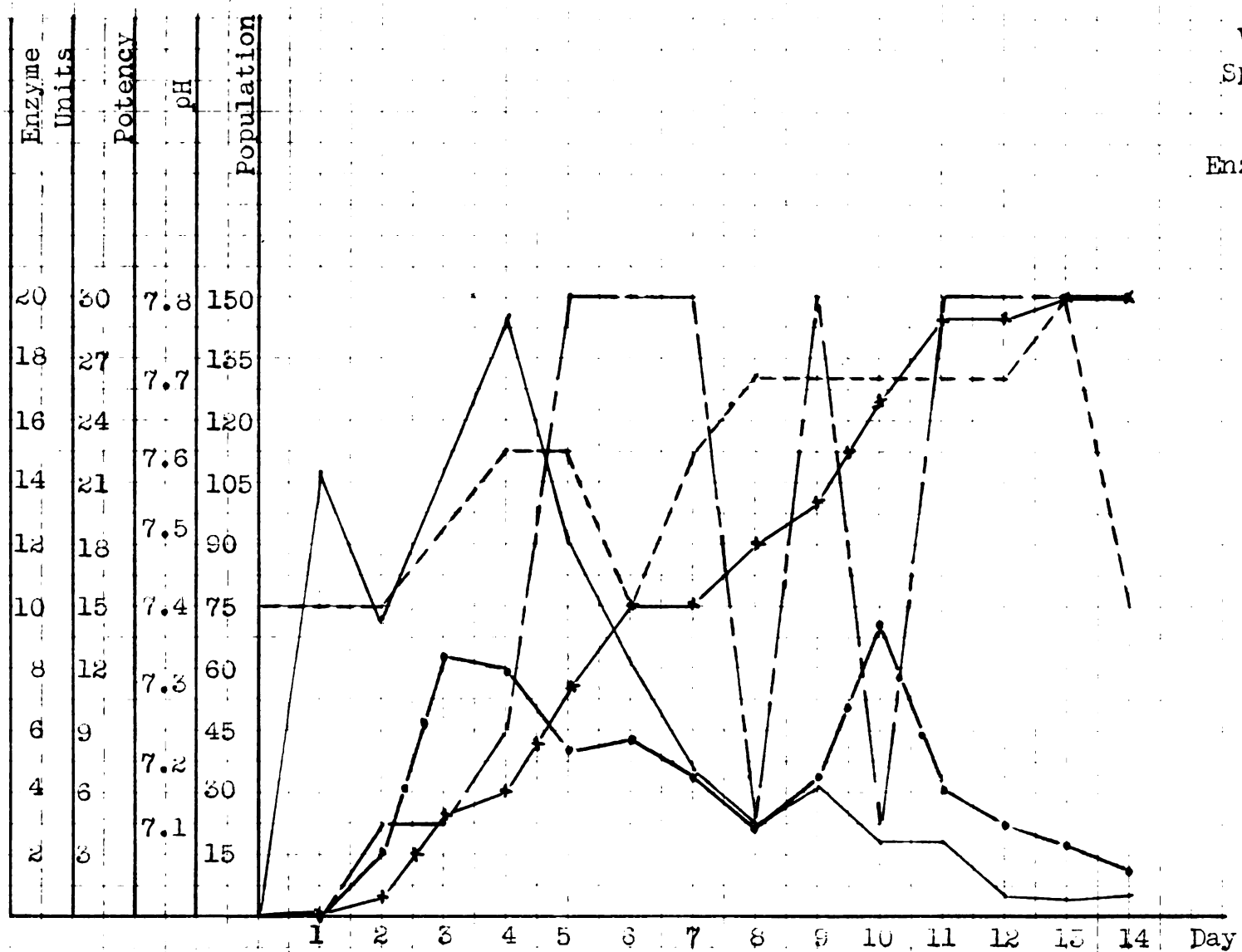


Chart I

A Comparison of the Vegetative and Spore Population, the Hydrogen Ion Concentration, the Toxin and the Gelatin-Splitting Enzyme of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 14 Days. By Day

The vegetative cell population reached a maximum of 145 million cells per cubic centimeter on the fourth day. Thereafter, the count decreased rapidly to reach a constant level at the twelfth day.

The spore cell population rose to 63 million cells per cubic centimeter on the third day, dropped gradually to 22 million cells by the eighth day and rose again to 72 million cells on the tenth day. Thereafter, the spore population diminished gradually to 12 million cells on the fourteenth day. The vegetative cell population reached its maximum one day later than the spore cell population reached its first peak.

The pH rose from 7.4 to 7.6 in the first four days, returned to 7.4 on the sixth day and gradually rose to a maximum of 7.8 on the thirteenth day. The pH reaction on the fourteenth day was again 7.4. There appeared to be some correlation between the peak of the vegetative cell population and the rise in pH in the early stages of incubation. After the sixth day, however, the pH rose as the vegetative cell population diminished.

The potency of the toxin increased gradually to a maximum of 30,000 minimum lethal doses on the thirteenth day. Exact end points were obtained on the fifth, eighth, eleventh and twelfth days only, so that the data can only be interpreted as showing the general trend of toxin production without reference to peak amounts.

It is apparent, however, that the probable peak in potency was reached about nine days after the peak in vegetative cell population. The high toxin potency appeared to correlate well with high pH reaction in the late stages of incubation.

The amount of gelatin-splitting enzyme measured at pH 7.0 rose to 20 units by the fifth day, thus coinciding with the increase in the vegetative and spore cell populations and the pH reaction. Thereafter, the amount of enzyme varied between 20 and 3 units and showed no correlation with any of the other phenomena observed.

Table 2

The Influence of Hydrogen Ion Concentration on the Activity of the Gelatin-Splitting Enzyme of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 14 Days.

By Day

Day	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
1	-	-	-	-	2	20	20
2	-	-	-	3	20	20	20
3	-	-	-	3	20	20	20
4	1	2	2	6	20	20	20
5	2	3	3	20	20	20	20
6	2	6	3	20	20	20	20
7	20	6	20	20	20	20	20
8	1	1	1	3	6	20	20
9	6	20	6	20	20	20	20
10	1	1	3	3	20	20	20
11	20	20	20	20	20	20	20
12	20	20	20	20	20	20	20
13	20	20	20	20	20	20	20
14	3	20	20	20	20	20	20
15							

The influence of the hydrogen ion concentration upon enzyme activity was marked. The enzyme was completely inactivated at pH 4, 5, and 6 for the first three days. Thereafter, the number of units observed varied from 1 to 20 sporadically, reading the highest level of activity in the late stages of incubation. At pH 7.0 the enzyme was inactivated only on the first day. Thereafter, a level of 20 units was reached by the fifth day and, with two exceptions (eighth and tenth days), was maintained.Enzyme activity was greatest at

pH levels 8, 9, and 10 with 20 units measurable by the second day of incubation. In general, a high pH reaction appeared to be much more favorable for enzyme activity than a low pH reaction.

EXPERIMENT 2

Table 3

Chart 2

A comparison of the Vegetative and Spore Population, the Hydrogen Ion Concentration, the Toxin and the Gelatin-Splitting Enzyme, and the Bacterial Debris of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 16 Days.

By Day

Day	Vegetative Cell Count Millions per c.c.	Spore Cell Count Millions per c.c.	pH	Minimum Lethal Doses of Toxin per c.c.	Enzyme Units per c.c. Measured at pH 7.0	Debris
1	52	1	7.2	1000	50	-
2	151	45	7.7	5000	-	-
3	145	40	7.7	10000	2	-
4	102	45	7.3	14000	20	-
5	77	32	7.3	16000	20	Moderate
6	50	35	7.3	18000	3	"
7	50	23	7.2	18000	3	"
8	51	27	7.1	40000	6	"
9	13	11	7.3	40000	2	"
10	32	20	7.2	40000	2	Marked
11	17	14	7.7	40000	3	"
12	3	10	7.7	15000	3	"
13	3	27	7.7	30000	6	"
14	9	19	7.3	30000	2	"
15	3	16	7.7	45000	6	"
16	3	29	7.6	30000	2	"

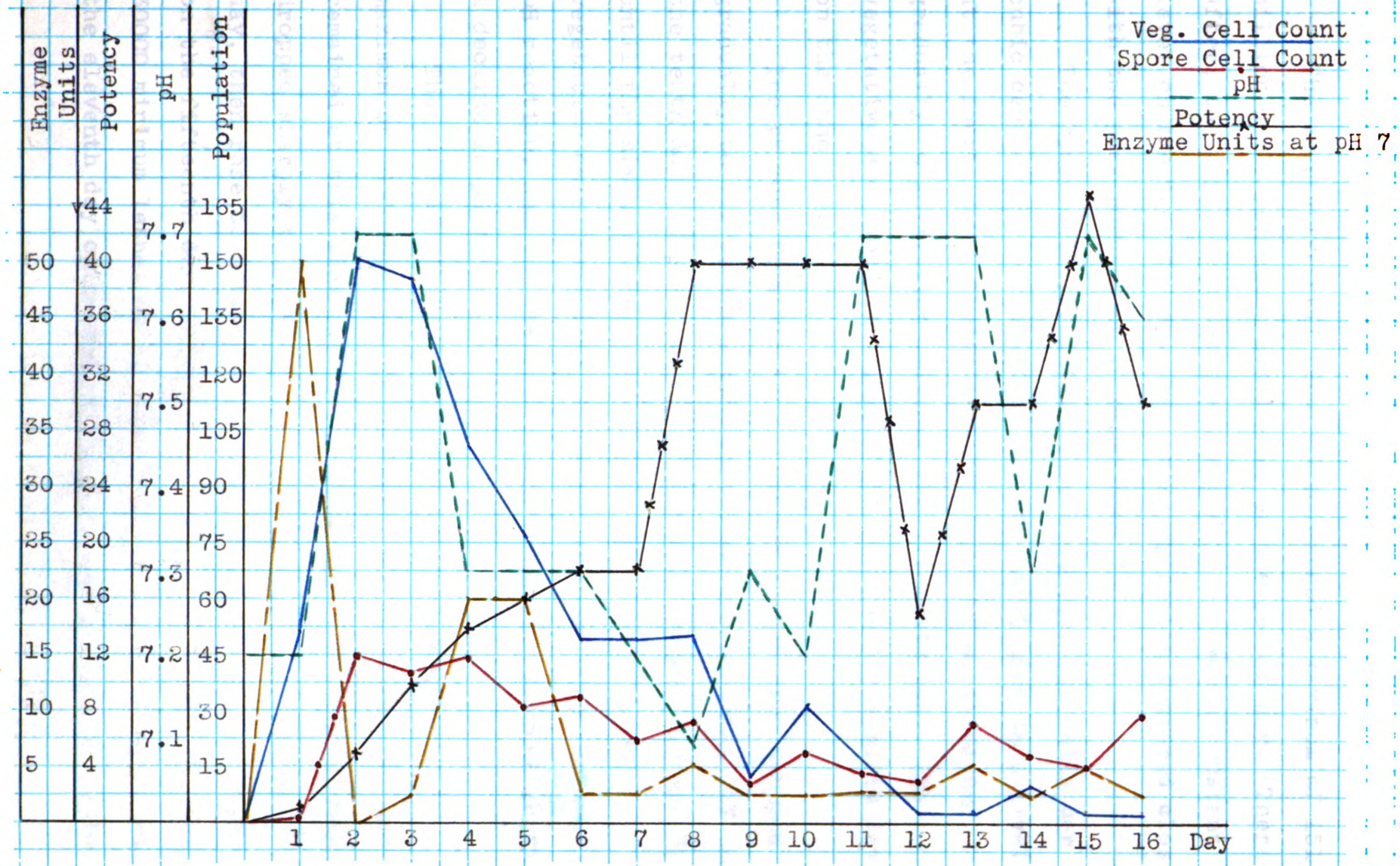


Chart II

A Comparison of the Vegetative and Spore Population, The Hydrogen Ion Concentration, the Toxin and the Gelatin-Splitting Enzyme of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 16 Days. By Day.

The vegetative cell population reached a maximum of 151 million cells per cubic centimeter on the second day. Thereafter, the population decreased rapidly to reach a constant level on the twelfth day. The peak population compared closely with the peak population in Experiment I.

The spore cell population rose to 45 million cells per cubic centimeter on the second day and maintained this level at the fourth day. Thereafter, the population diminished gradually to 16 million cells on the fifteenth day. The vegetative and spore cell population reached the maximum level on the same day.

The pH rose from 7.2 to 7.7 on the second day, dropped gradually to 7.1 by the eighth day, and rose again to 7.7 on the tenth day. Thereafter, it fluctuated between 7.7 and 7.3 until the sixteenth day when the pH was 7.6. The peak vegetative cell population coincided with the first peak in pH reaction, and the decline in population was paralleled by a decline in pH reaction.

The potency of the toxin increased gradually to a maximum of 40000 minimum lethal doses on the eighth day and remained at this level until the eleventh day. The potency dropped sharply to 15000 minimum lethal doses on the twelfth day, then rose to a new level of 45000 minimum lethal doses on the fifteenth day. The potency on the sixteenth day was 30000 minimum lethal doses. The sudden drop in potency after the eleventh day coincided with the second peak in pH reaction

and the subsequent rise in potency coincided with a drop in pH reaction.

The amount of gelatin-splitting enzyme measured at pH 7.0 was 50 units per cubic centimeter at the end of the first 24 hours. There was no measurable enzyme on the second day. Twenty enzyme units per cubic centimeter were present on the fourth day. Thereafter, the number of units fluctuated between 2 and 6 until the end of the test period. There appeared to be a close correlation between the rapid rise in the vegetative cell population and the amount of gelatin-splitting enzyme present in the initial stages of incubation. The sudden drop in enzyme content on the second day was accompanied by a sharp rise in pH reaction.

Table 4

The Influence of Hydrogen Ion Concentration on the Activity of the Gelatin-Splitting Enzyme of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 16 Days.

By Day

Day	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
1	-	-	-	50	50	50	100
2	-	-	-	-	-	25	100
3	-	-	-	2	100	100	100
4	100	33	100	20	100	100	100
5	100	25	100	20	100	100	100
6	100	100	2	3	100	100	100
7	-	-	-	3	100	100	100
8	-	-	-	6	100	100	100
9	-	-	-	2	6	100	100
10	-	-	-	2	6	50	100
11	-	-	2	3	100	100	100
12	-	-	3	3	20	100	100
13	-	-	3	6	20	100	100
14	-	-	2	2	3	6	100
15	-	-	3	6	20	100	100
16	-	-	2	2	3	100	100

The influence of the hydrogen ion concentration upon enzyme activity was marked. The enzyme was completely inactivated at pH 4.0, 5.0, and 6.0 for the first three days. On the fourth, fifth and sixth days there was marked enzyme activity with a maximum of 100 units measurable. No enzyme was measurable at pH 4.0 and 5.0 from the seventh through the sixteenth day, while from 2 to 3 units were observed at pH 6.0 from the eleventh through the sixteenth day.

At pH 7.0 the enzyme activity was of a low order ranging from 2 to 50 units over the test period.

Enzyme activity was greatest at pH levels 8.0, 9.0, and 10.0 with 100 units the most frequent amount present at any measurement.

In general, a high pH reaction appeared to be much more favorable for enzyme activity than a low pH reaction.

EXPERIMENT 3

Table 5

Chart 3

A Comparison of the Vegetative and Spore Population, the Hydrogen Ion Concentration, the Toxin and the Gelatin-Splitting Enzyme, and the Bacterial Debris of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 16 Days.

By Day

Day	Vegetative Cell Count Millions per c.c.	Spore Cell Count Millions per c.c.	pH	Minimum Lethal Doses of Toxin per c.c.	Enzyme Units per c.c. Measured at pH 7.0	Debris
1	38	1	7.3	1000	-	-
2	56	21	7.3	5000	-	-
3	83	28	7.2	7000	0.6	-
4	75	54	7.3	10000	1	-
5	51	16	7.2	20000	1	-
6	30	41	7.3	25000	3	Moderate
7	19	29	7.3	25000	3	"
8	21	23	7.2	25000	3	"
9	13	28	7.3	30000	2	"
10	10	29	7.6	30000	2	"
11	5	9	7.6	30000	1	Marked
12	11	17	7.3	25000	1	"
13	5	25	7.4	40000	1	"
14	6	15	7.3	20000	1	"
15	5	20	7.7	25000	1	"
16	3	15	8.0	25000	2	"

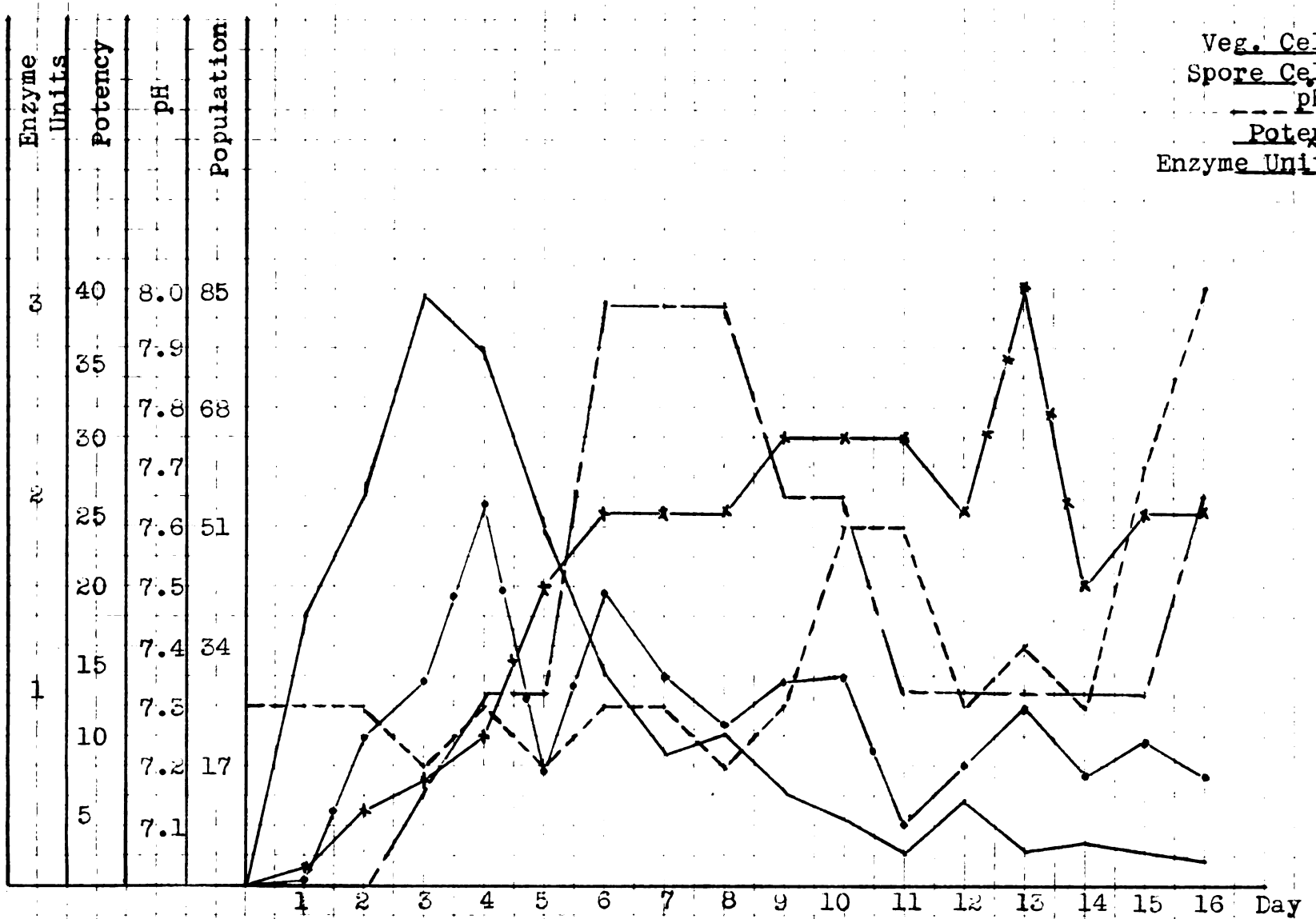


Chart III

A Comparison of the Vegetative and Spore Population, the Hydrogen Ion Concentration, the Toxin and the Gelatin-Splitting Enzyme of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 16 Days. By Day.

The vegetative cell population reached a maximum of 83 million cells per cubic centimeter on the third day. Thereafter, the population decreased rapidly to 3 million cells on the sixteenth day. The cell population in this experiment was much lower than in the two previous experiments, but the peak was reached at approximately the same time.

The spore cell population rose to 54 million cells per cubic centimeter on the fourth day. Thereafter, it decreased gradually to 15 million cells on the sixteenth day.

The pH reaction rose to 7.6 on the tenth day, dropped to 7.3 on the twelfth day, and reached a maximum of pH 8.0 on the sixteenth day. The pH remained more or less constant for the first nine days. Thereafter, there appears to be some correlation between the rise in pH reaction and decrease in the vegetative and spore cell populations. The pH reaction in this experiment and in Experiment 4 was determined immediately after filtration each day. It was discovered that, if the filtrate was allowed to stand in a flask exposing a large surface area of the filtrate, the pH reaction would rise to about 7.7 within two hours. This fact probably accounts for the pH variations in Experiments I and 2 in the early stages of incubation since the pH reactions were determined after the filtrates had stood in 250 cubic centimeter flasks for varying periods of time.

The potency of the toxin increased gradually to a maximum of 40000 minimum lethal doses on the thirteenth day. Exact end points were obtained each day in this experiment. An

increase in potency of the toxin tended to occur from one to two days after a drop in pH reaction.

The amount of gelatin-splitting enzyme measured at pH 7.0 rose to a maximum of 3 units by the sixth day, dropped to one unit on the eleventh day, and increased to 2 units on the sixteenth day. After the sixth day, there appeared to be a correlation between decreased enzyme activity, low pH reaction, vegetative and spore cell populations, and increased toxin potency.

Table 6

The Influence of Hydrogen Ion Concentration on the Activity of the Gelatin-Splitting enzyme of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 16 Days.

By Day

Day	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
1	-	-	-	-	3	3	100
2	-	-	-	-	2	1	100
3	-	-	1	0.6	2	2	100
4	-	-	0.6	1	2	2	100
5	-	0.6	0.6	1	2	3	100
6	-	0.6	1	3	3	20	100
7	-	1	1	3	3	100	100
8	-	0.6	1	3	3	20	100
9	-	0.6	1	2	3	3	100
10	-	0.6	1	2	3	3	100
11	-	0.6	1	1	2	2	100
12	-	0.6	1	1	1	2	100
13	-	0.6	1	1	1	1	100
14	-	0.6	1	1	2	3	100
15	-	1	1	1	2	3	100
16	0.6	1	2	2	2	3	100

No gelatin-splitting enzyme could be measured at pH 4.0 until the sixteenth day. The enzyme was inactivated at pH 5.0 for the first four days, and at pH 6.0 for the first two days. One to three units of enzyme per cubic centimeter were measured at pH 8.0. At pH 9.0, 100 enzyme units were measurable on the seventh day. Thereafter, the number of units present dropped to three on the sixteenth day. One hundred enzyme units were measured every day for sixteen days at pH 10.0.

In general, a high pH reaction appeared to be more favorable for enzyme activity than a low pH reaction.

EXPERIMENT 4

Table 7

Chart 4

A Comparison of the Vegetative and Spore Population, the Hydrogen Ion Concentration, the Toxin and the Gelatin-Splitting Enzyme, and the Bacterial Debris of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 16 Days.

By Day

Day	Vegetative Cell Count Millions per c.c.	Spore Cell Count Millions per c.c.	pH	Minimum Lethal Doses of Toxin per c.c.	Enzyme Units per c.c. Measured at pH 7.0	Debris
1	68	5	7.3	1000	0.6	-
2	105	21	7.1	10000	2	-
3	83	32	7.1	10000	20	-
4	101	33	7.1	15000	20	-
5	40	43	7.3	25000	100	Moderate
6	30	35	7.1	25000	20	"
7	17	20	7.3	50000	20	"
8	20	19	7.1	40000	100	"
9	23	30	7.3	30000	100	"
10	18	21	7.3	30000	20	Marked
11	16	17	7.4	45000	20	"
12	10	16	7.4	30000	20	"
13	10	21	7.6	30000	3	"
14	11	15	7.6	30000	100	"
15	7	18	7.7	30000	20	"
16	8	18	7.7	30000	20	"

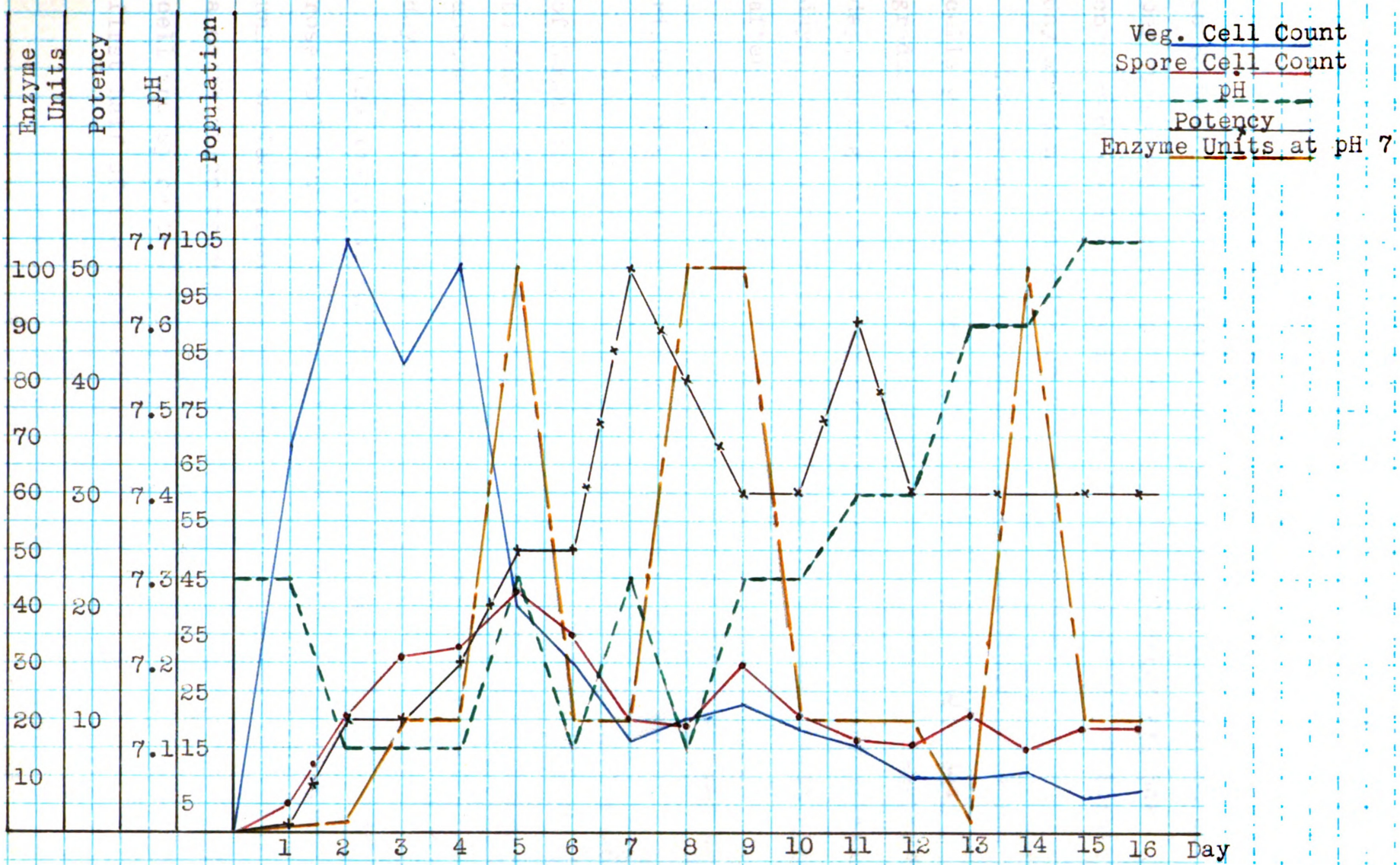


Chart IV

A Comparison of the Vegetative and Spore Population, the Hydrogen Ion Concentration, the Toxin and the Gelatin-Splitting Enzyme of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5°C. for 16 Days. By Day

The vegetative cell population reached a maximum of 105 million cells per cubic centimeter on the second day, dropped to 83 million on the third day, and rose to 101 million cells per cubic centimeter on the fourth day. Thereafter, the count decreased rapidly to 7 million cells on the fifteenth day and 8 million cells on the sixteenth day.

The spore cell population reached a maximum of 43 million cells per cubic centimeter on the fifth day. The count gradually diminished to 15 million cells on the fourteenth day, rising to 18 million cells on the fifteenth and sixteenth days. The spore cell population reached its peak three days after the vegetative cell population.

The pH varied between 7.1 and 7.3 for the first ten days, rising to a maximum of pH 7.7 on the fifteenth day.

The potency of the toxin increased gradually to a maximum of 50000 minimum lethal doses on the seventh day, decreased to 30000 minimum lethal doses on the ninth day and reached another peak of 45000 minimum lethal doses on the eleventh day. End points were obtained for each day.

The amount of gelatin-splitting enzyme measured at pH 7.0 rose to 100 units on the fifth day. Thereafter, the number of units varied between 3 and 100. As in Experiment 3, there was a correlation between low enzyme activity, low pH, low vegetative cell and spore cell counts, and increased potency after the first half of the test period.

Table 8

The Influence of Hydrogen Ion Concentration on the Activity of the Gelatin-Splitting Enzyme of a Strain of Cl. Tetani Growing in Dextrose Veal Infusion Broth at 37.5 C. for 16 Days.

By Day

Day	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
1	-	-	-	0.6	0.6	1	100
2	-	-	1	2	3	2	100
3	-	0.6	2	20	3	100	100
4	-	2	3	20	20	100	100
5	1	2	3	100	100	100	100
6	1	2	3	20	20	100	100
7	1	3	20	20	100	100	100
8	1	3	3	100	20	100	100
9	100	20	3	100	20	100	100
10	1	1	3	20	20	100	100
11	100	3	3	20	100	100	100
12	1	3	20	20	3	100	100
13	0.6	1	2	3	3	20	100
14	20	100	20	100	100	100	100
15	100	3	3	20	100	100	100
16	100	3	20	20	100	100	100

The gelatin-splitting enzyme was completely inactivated until the fifth day at pH 4.0, the third day at pH 5.0, and the second day at pH 6.0. Thereafter, the number of units varied between 0.6 and 100 units per cubic centimeter. The increased enzyme activity in this experiment possibly was due to the fact that the tubes were neutralized in the water bath. When mixtures of this kind are kept at a temperature slightly above room temperature for a short time, the gelatin-splitting process may be initiated or furthered (7). Temperature of the room caused the mixtures to solidify

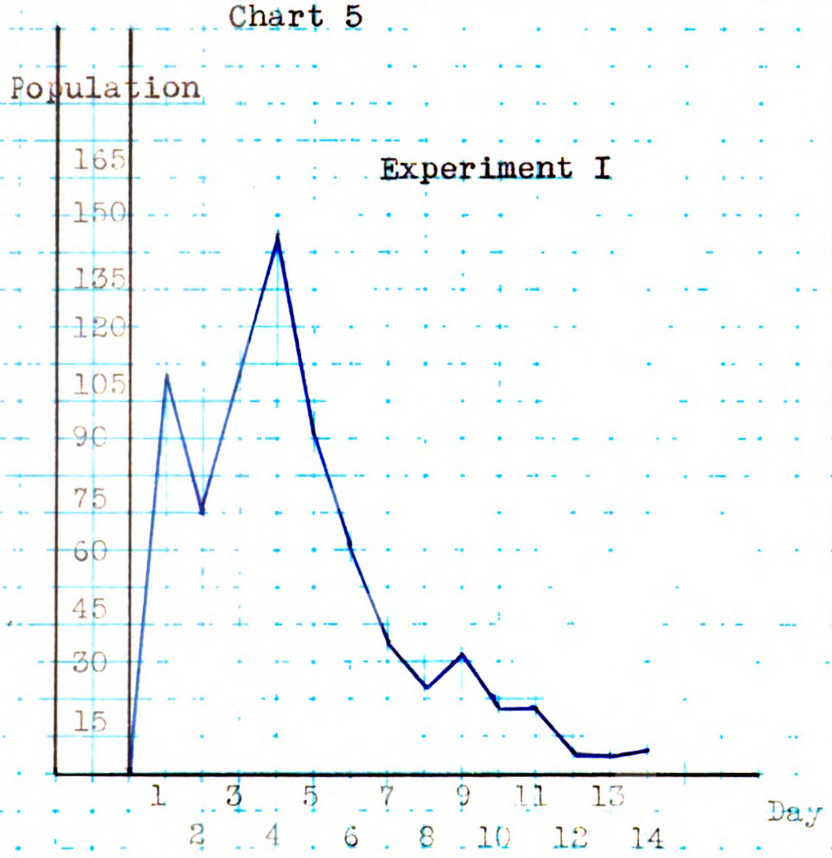
partially before neutralization of all the tubes could be completed. At pH 7.0, 100 units were measured on the fifth day. Thereafter, the number of units present varied from 3 to 100. Similar enzyme behavior was observed at pH 8.0. One hundred enzyme units were consistently present each day at pH 9.0 and 10.0.

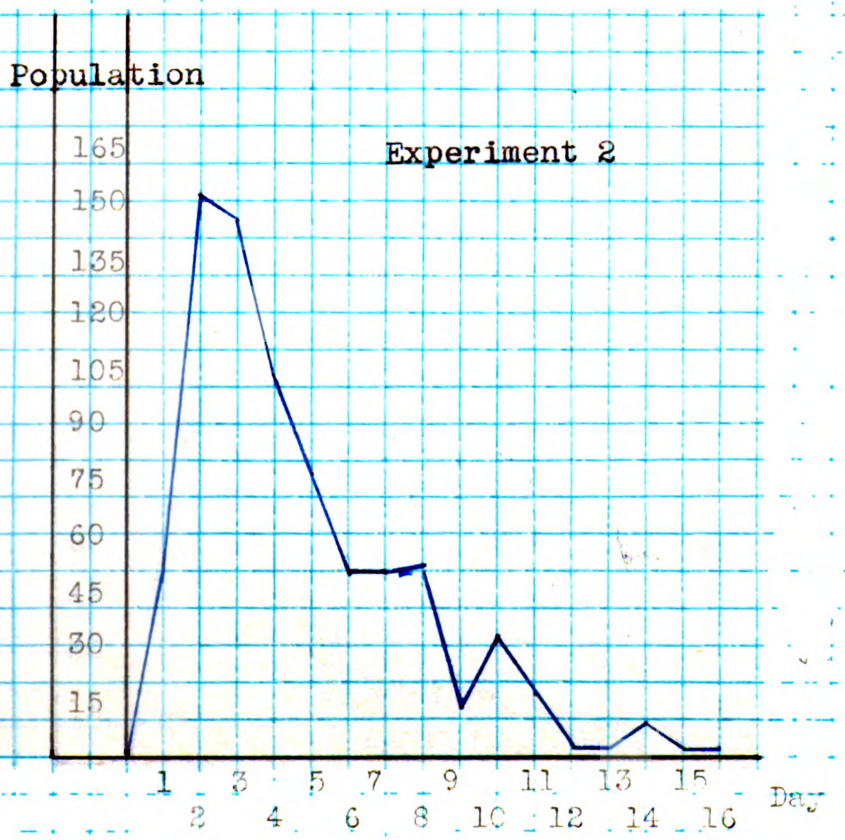
In general, a high pH reaction appeared to be more favorable for enzyme activity than a low pH reaction.

COMPARISON OF SIMILAR PHENOMENA

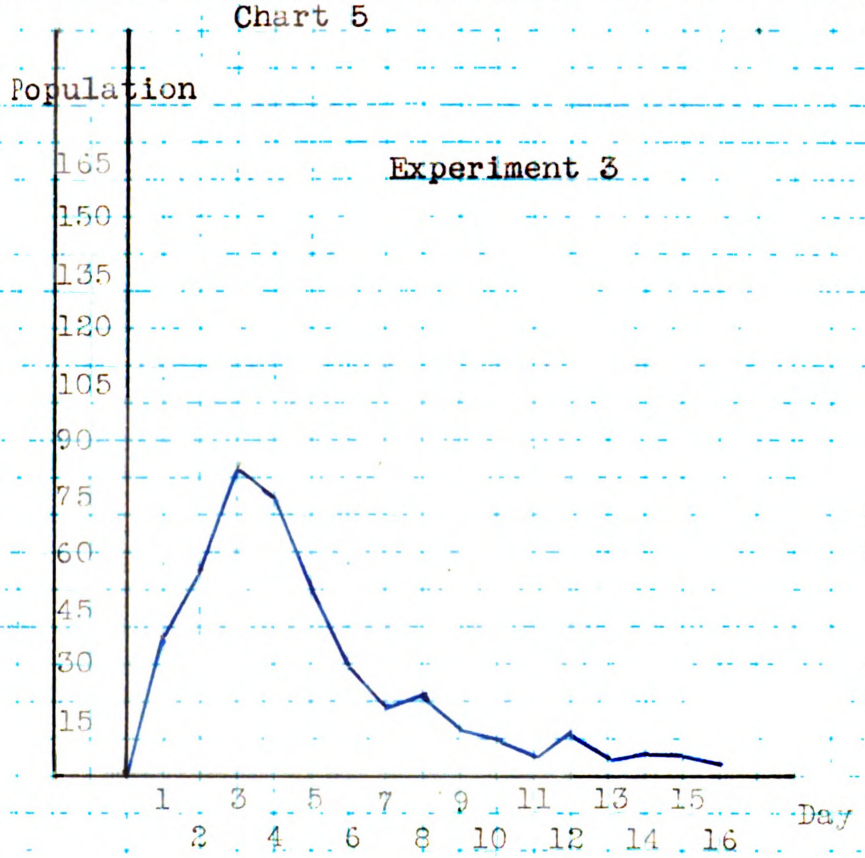
Comparison of Vegetative Cell Populations

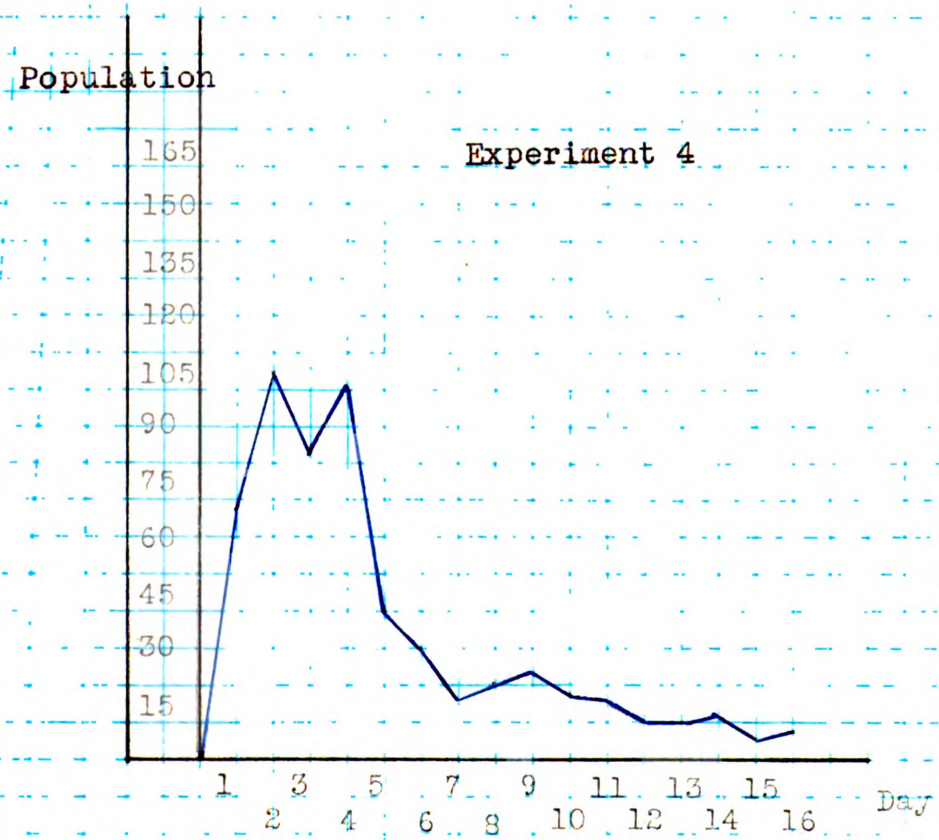
Chart 5





Comparison of Vegetative Cell Populations
Chart 5



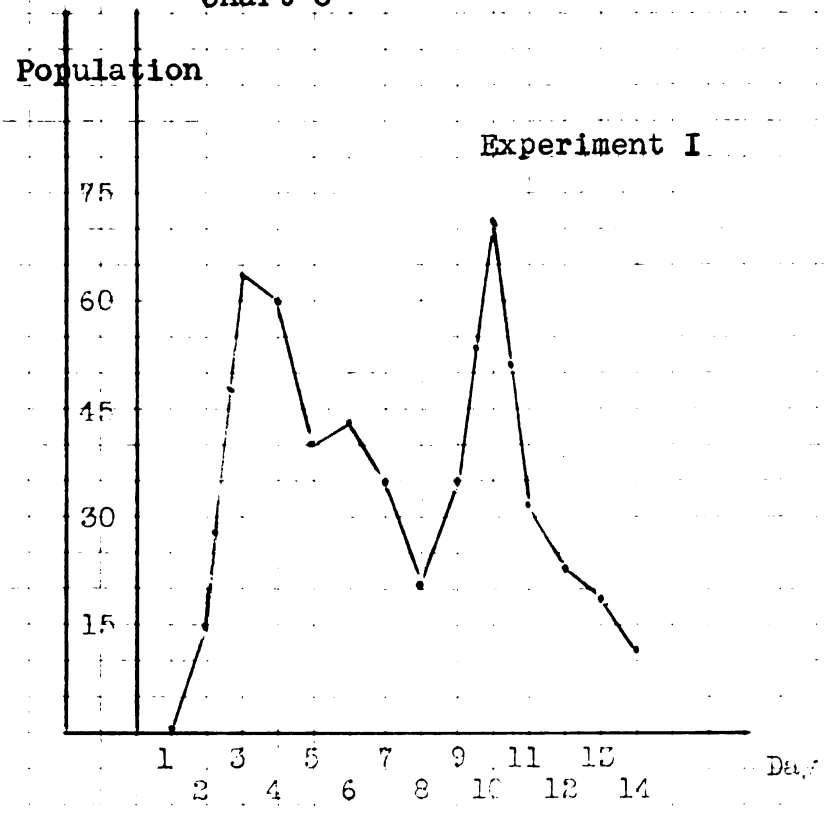


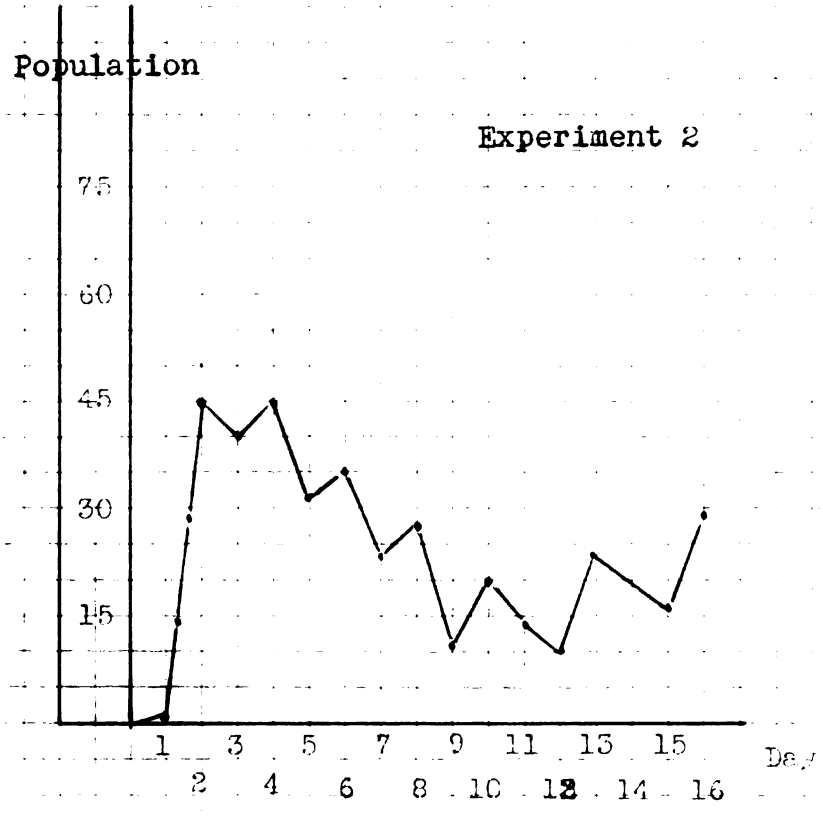
The curves showing the trends of vegetative cell population were nearly identical. Each curve showed a sharp increase in cell population in the early stages of incubation. The population peaks were respectively at four, two, three and two days. At these peaks there were respectively 145 million, 151 million, 83 million and 105 million vegetative cells per cubic centimeter.

Each curve showed a rapid decline in cell population for four to five days after the respective peaks were reached, and each curve indicated a constant level of population after the seventh to ninth days. At fourteen days, the curves had flattened out and the populations were respectively 6 million, 9 million, 6 million and 11 million vegetative cells per cubic centimeter.

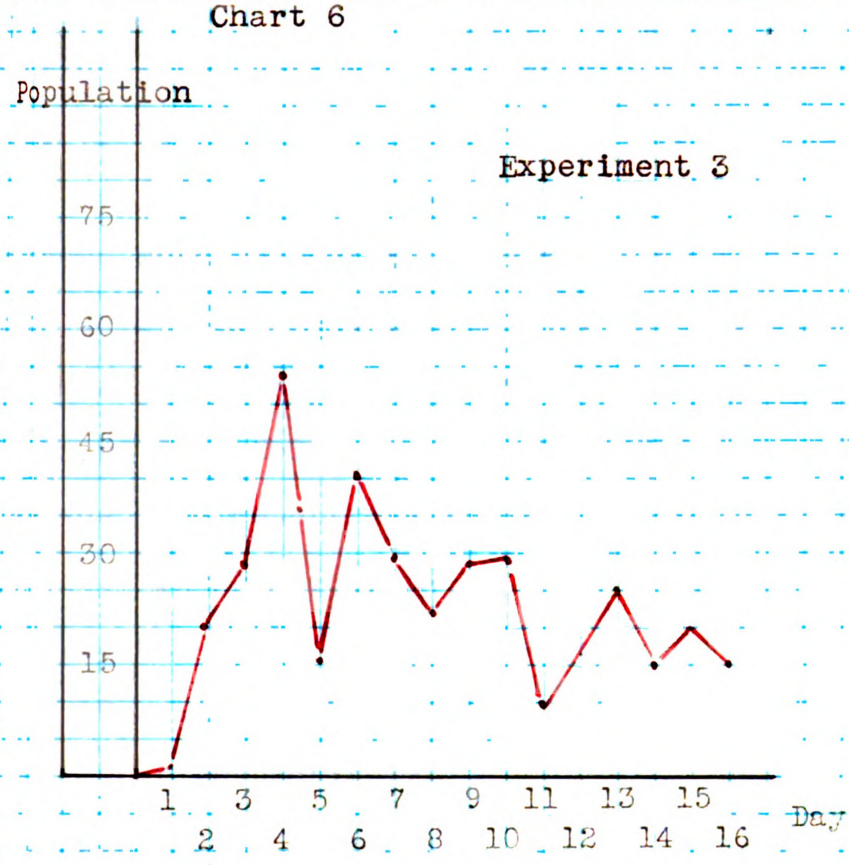
From these curves it is evident that the cell population rises and diminishes rapidly followed by a gradual diminution in number until a relatively constant population is reached.

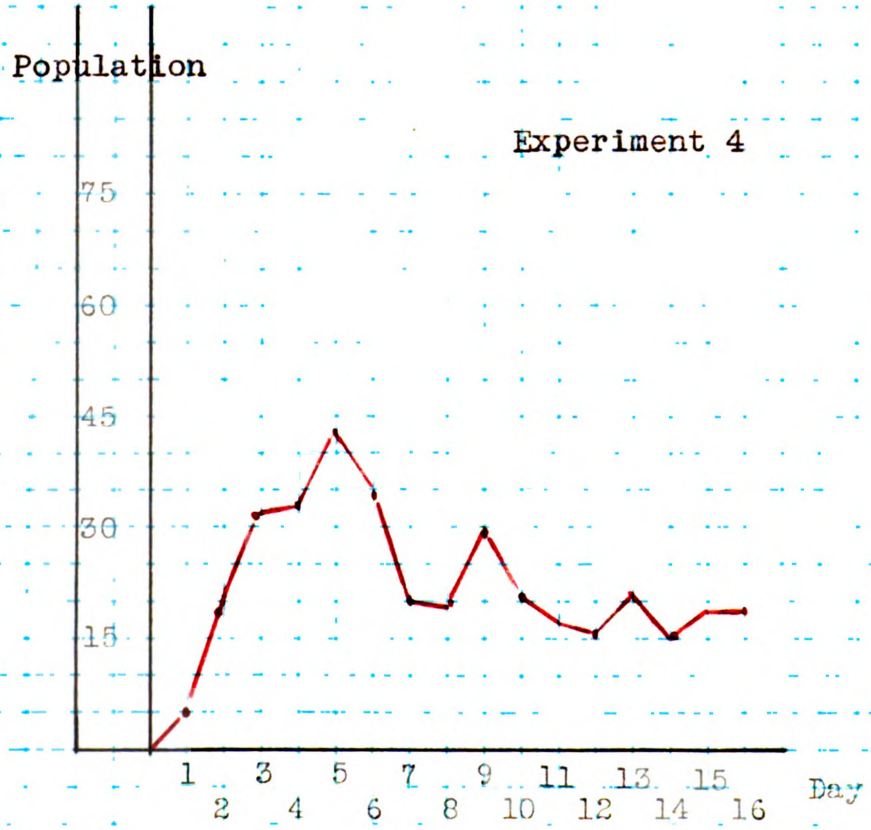
Comparison of Spore Cell Population
Chart 6





Comparison of Spore Cell Population
Chart 6

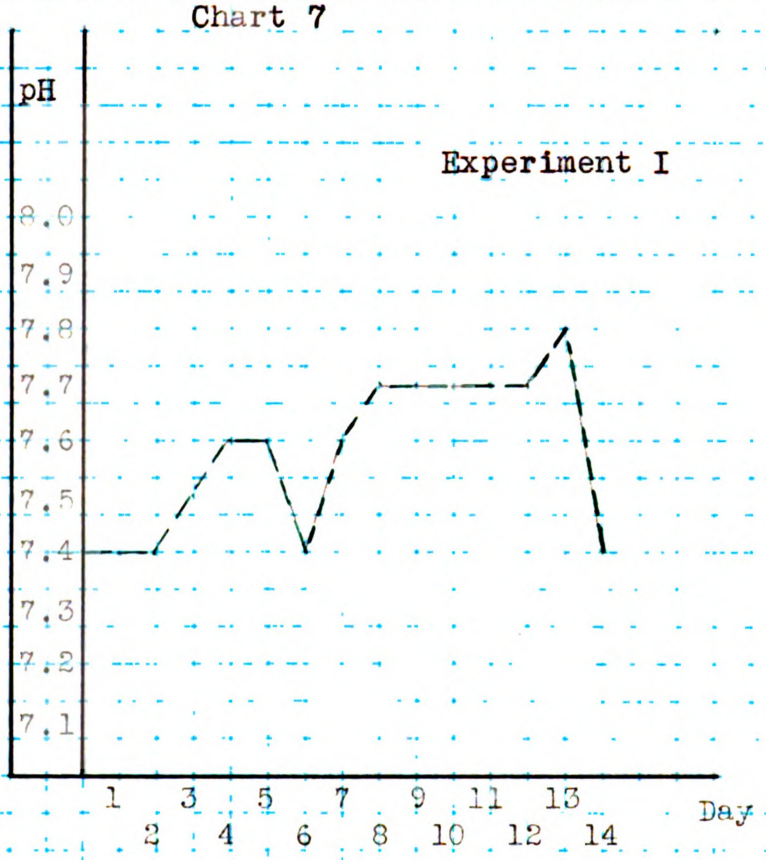


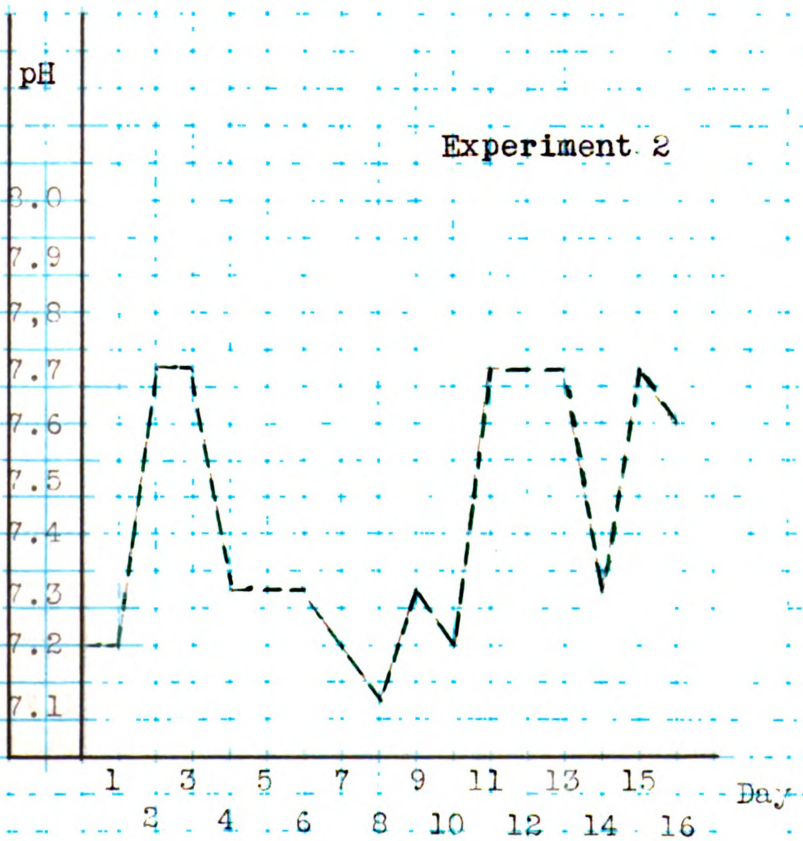


The spore cell population curves were similar in Experiments 2, 3, and 4. These curves were typified by early peaks at two, four, and five days respectively. At these peaks there were respectively 45 million, 54 million, and 43 million cells per cubic centimeter. The curve in Experiment 1 showed a sharp increase to a peak of 63 million cells on the third day, followed by a gradual decrease until the tenth day when the count rose to 72 million cells per cubic centimeter. Thereafter, the count diminished rapidly. At fourteen days, the spore cell populations had diminished to 12 million, 19 million, 15 million, and 15 million cells per cubic centimeter respectively.

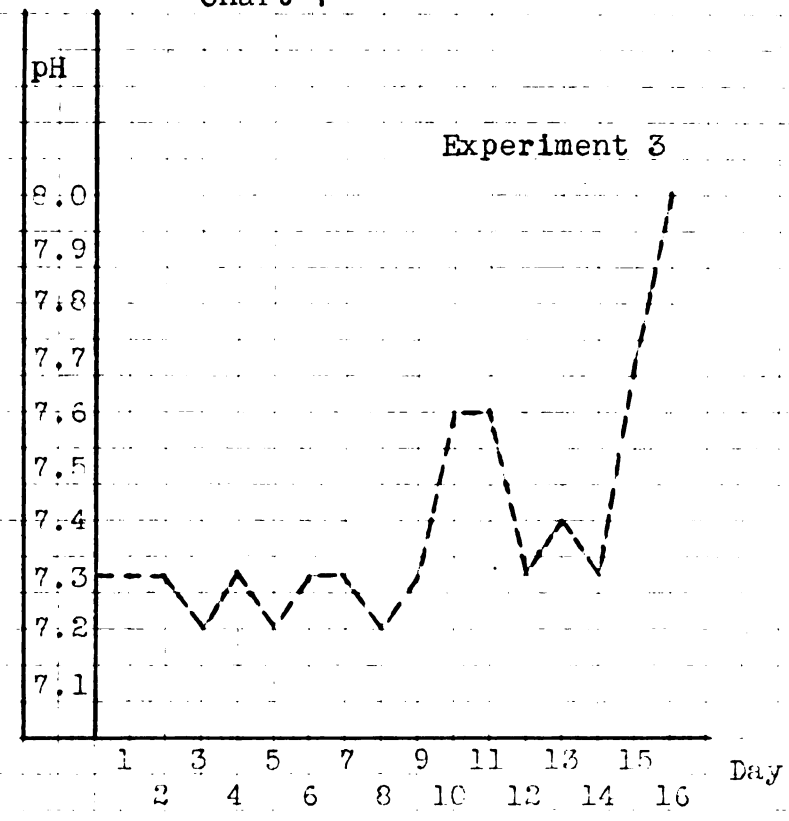
It was evident from these curves that the spore cell population rose rapidly to an early peak and then gradually declined.

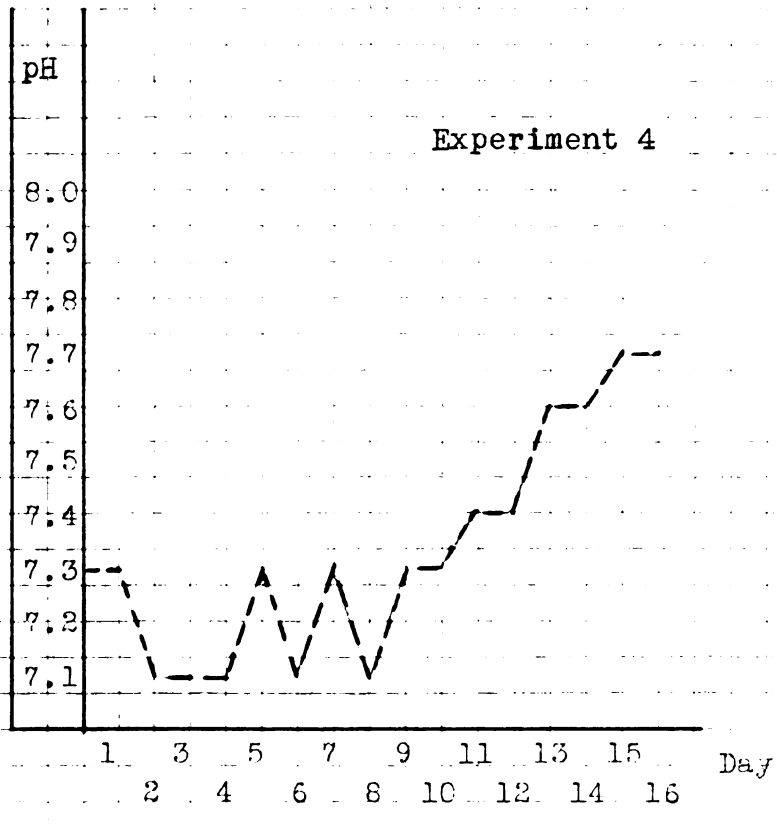
Comparison of pH
Chart 7





Comparison of pH
Chart 7

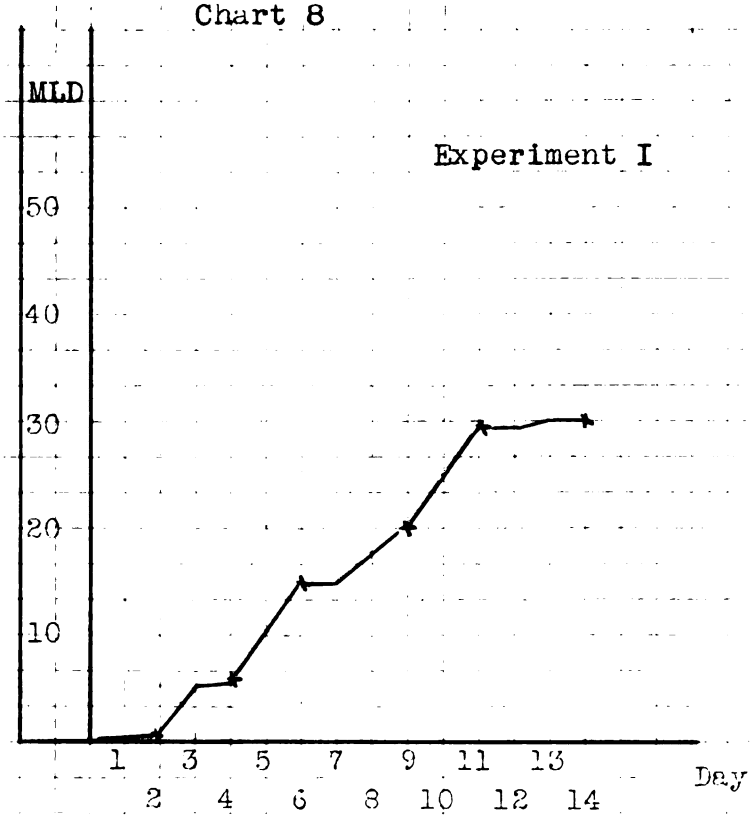


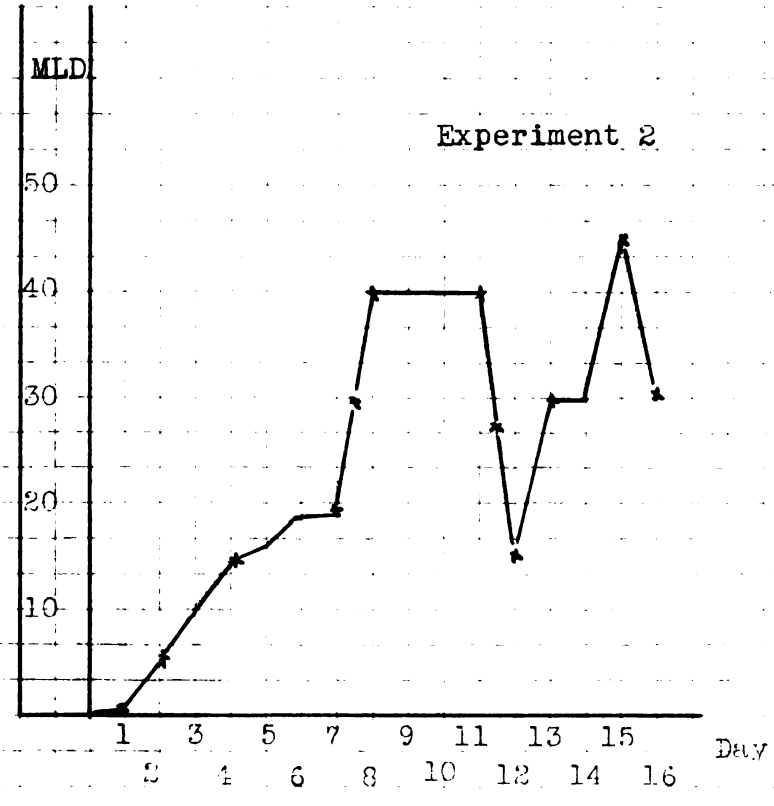


The curves showing the trends in pH varied considerably. The curves in Experiments 3 and 4 showed the same trend for the first eight days. Experiment 3 showed sporadic rise and fall in pH after the eighth day and Experiment 4 a gradual increase in pH to a maximum of 7.7. The peaks in Experiments I and 2 for the first eight days were probably due to the fact that the filtrates stood in 250 cubic centimeter flasks for varying periods of time before measurement.

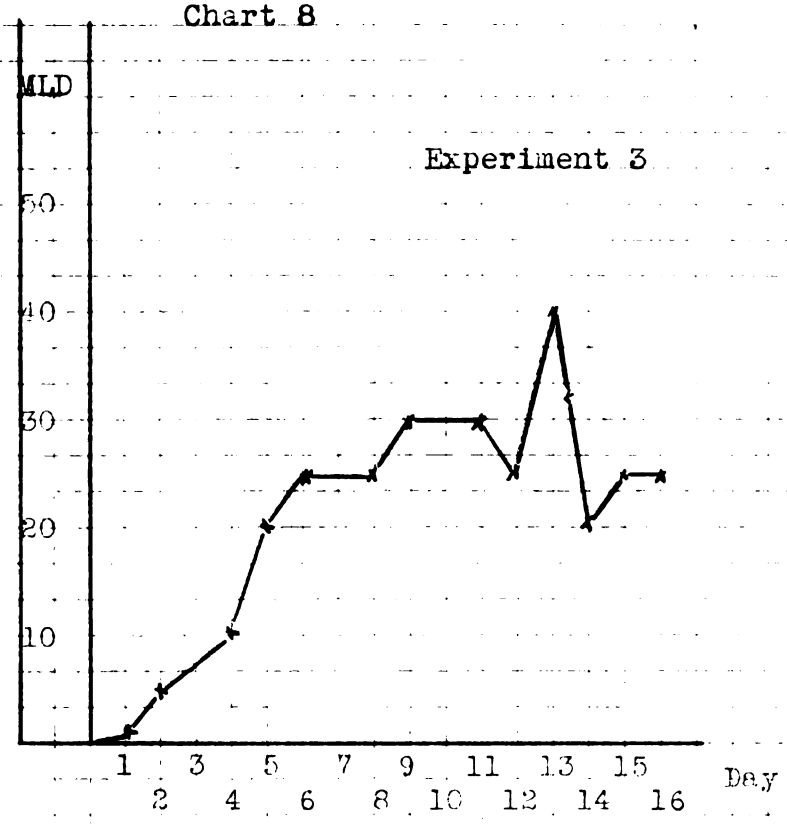
From these curves it appeared that the pH remained at a level of 7.1 to 7.3 for the first eight days followed by sporadic rise and fall in pH during the last half of the test period. In every experiment but Experiment I, pH values at the end of the test period were high. In Experiment I, the pH reaction on the thirteenth day was 7.8, and on the fourteenth day it was 7.4.

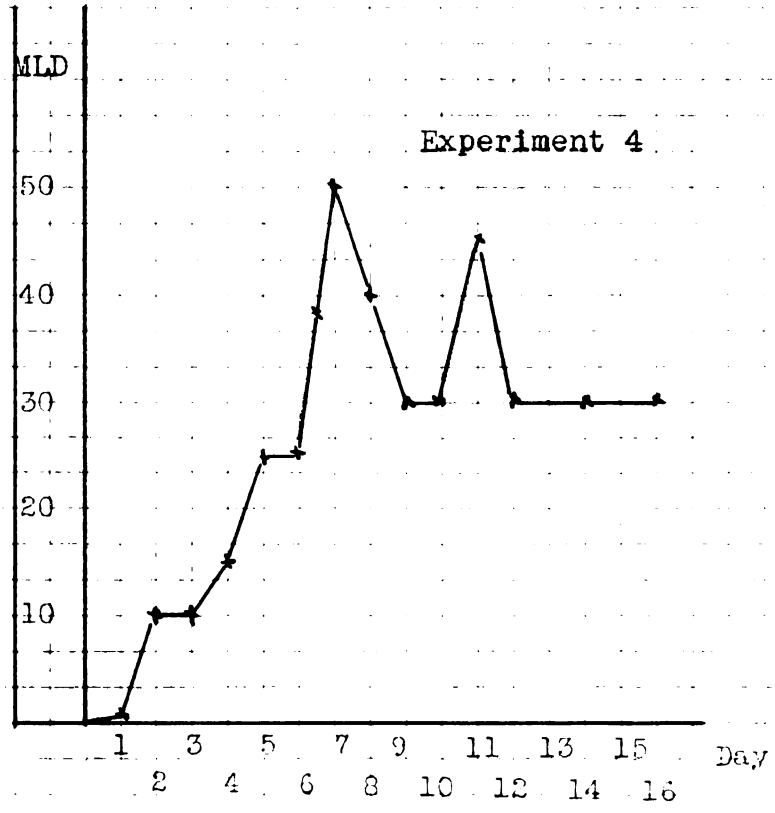
Comparison of Minimum Lethal Doses
of Toxin per Cubic Centimeter
Chart 8





Comparison of Minimum Lethal Doses
of Toxin per Cubic Centimeter
Chart 8

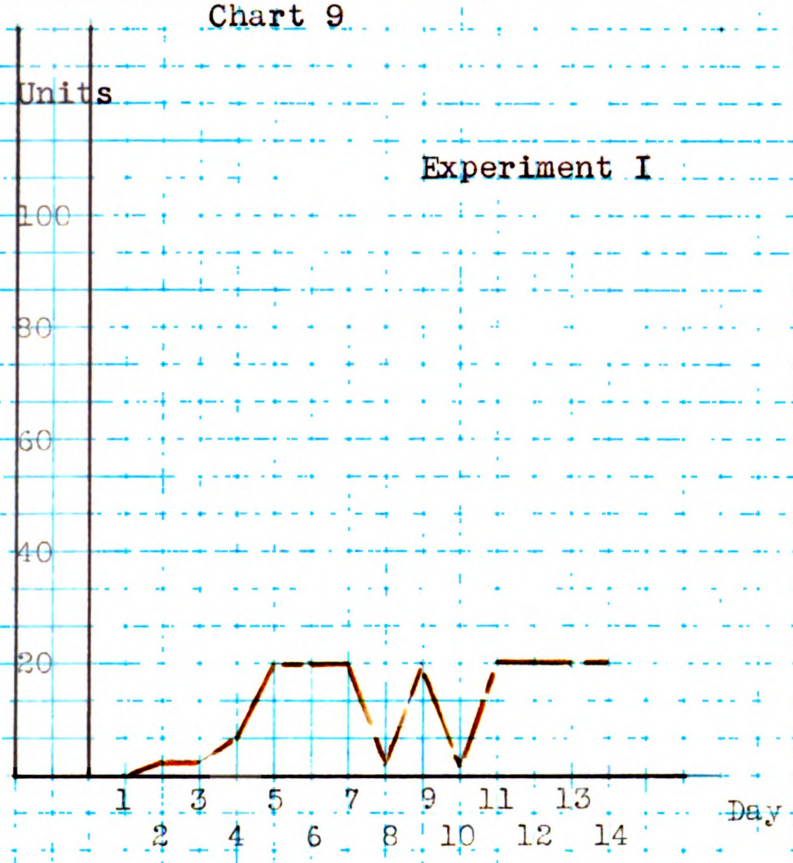


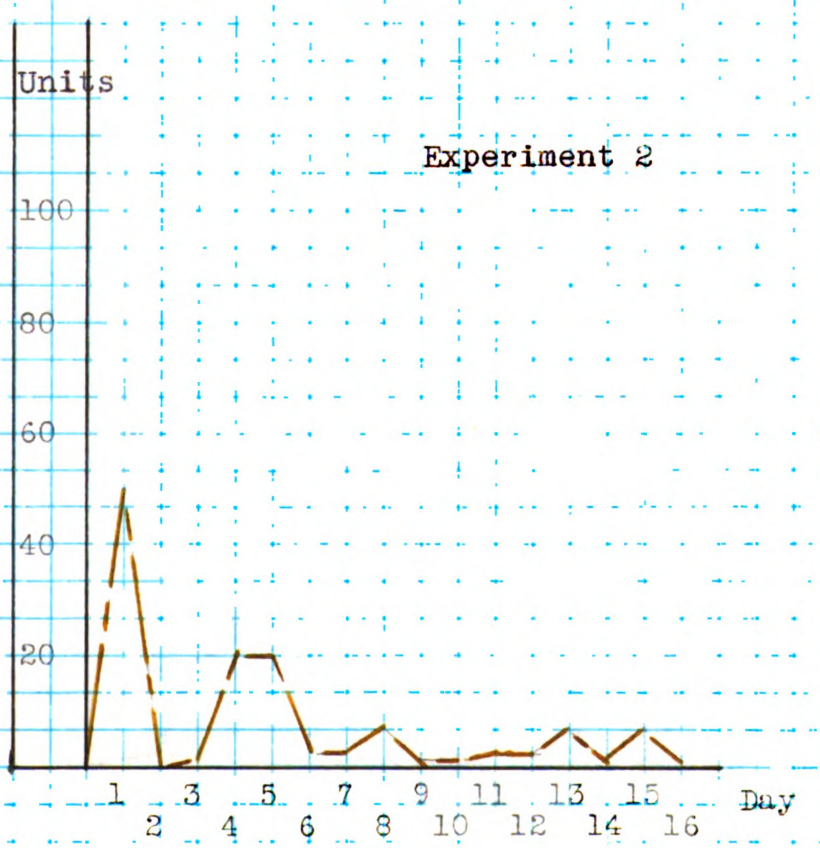


The curves showing the trends of toxin potency for the first six to eight days were nearly identical. Each curve showed a gradual increase in potency. Experiment I continued to show a gradual increase in potency up to 30000 minimum lethal doses on the eleventh day. This potency persisted for the remainder of the test period. Experiments 2, 3, and 4 showed a marked rise and fall in potency in the second half of the test period, reaching a maximum of 45000 minimum lethal doses on the fifteenth day in Experiment 2, 40000 minimum lethal doses on the thirteenth day in Experiment 3, and 50000 minimum lethal doses on the seventh day in Experiment 4. The potencies at fourteen days in the respective experiments were 30000, 30000, 20000, and 30000 minimum lethal doses.

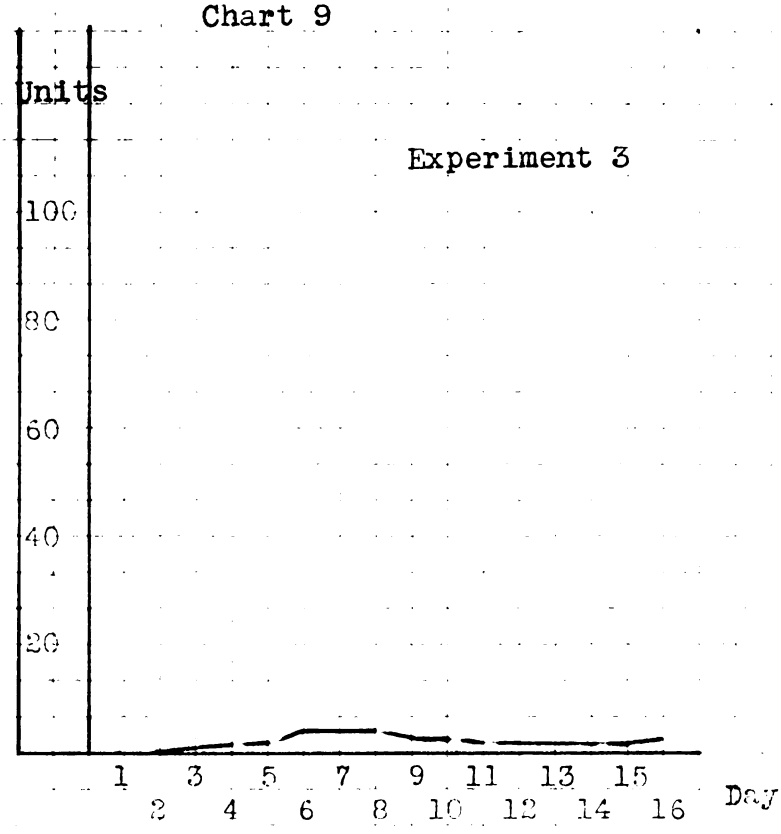
It was evident from these curves that potencies increased gradually for the first six to eight days, then rose and fell sharply one or two times during the last half of the test period.

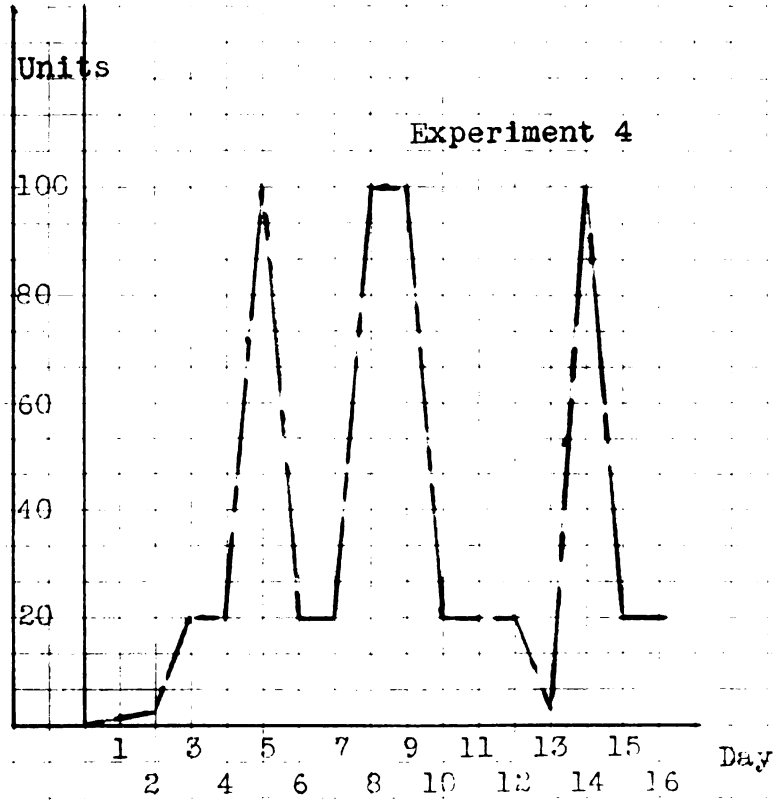
Comparison of Gelatin-Splitting
Enzyme Units Measured at pH 7
Chart 9





Comparison of Gelatin-Splitting
Enzyme Units Measured at pH 7
Chart 9





The curves showing the trends in gelatin-splitting enzyme activity showed no correlation with one another. Enzyme activity varied sporadically throughout the test periods.

There appeared to be no correlation between the enzyme results of one experiment as compared with another.

DISCUSSION

The purpose of this study was to observe daily certain phenomena which occur in tetanus toxin production. It was hoped that such data might pave the way to an understanding of the factors which govern toxin production. Certain assumptions as to the outcome of the various experiments were unavoidable.

It was thought, for example, that the spore population would increase in almost direct proportion to the decrease in vegetative cell population, allowing for a certain amount of autolysis. This was not the case and the influence of autolytic phenomena therefore assumed an important position in theoretical considerations.

It was thought, also, that the pH reaction would show some constant type of behavior. Instead, each experiment showed a wide variation in pH values over the test periods. In no instance did the pH value on the final day of each experiment give any indication of the amount of variation which had taken place. In the routine production of toxin it is customary to measure the pH of the toxin broth at the beginning of the incubation period and at the time of harvest ten to fourteen days later. If this had been the case in these experiments, the initial and final pH values would have been respectively 7.4 and 7.4, 7.2 and 7.6, 7.3 and 8.0, and 7.3 and 7.7. In every case but one these pH values appeared to indicate an increase over the period of incubation.

An inspection of the daily record, however, showed marked fluctuation in pH values between the initial and final days of the test period.

The assumption was that toxin production was a progressive phenomenon indicated by steady increase in the number of lethal doses until some constant level was reached. This assumption was borne out in Experiment I. In the other experiments the progression was orderly for the first six or seven days. Thereafter, the number of lethal doses to be expected on any day was unpredictable. An excellent illustration of this behavior occurred in Experiment 2. The potency on the eleventh day was 40000 minimum lethal doses, on the twelfth day it was 15000 minimum lethal doses. By the fifteenth day, the potency had risen to 45000 minimum lethal doses. The same phenomenon was encountered in Experiment 4. This type of variation emphasizes the difficulty in the selection of the proper day for harvesting toxin. It also illustrates the fallacy of the assumption that a fixed incubation period can be established for routine toxin production. The results have indicated that most of these assumptions were wrong.

Certain phenomena stand out clearly when one considers the actual data collected. For example, the trend in bacterial population was definitely constant. Vegetative cell and spore populations increased and diminished rapidly until a period of nearly constant population was reached. The accumulation of debris occurred progressively. Moderate debris was observed

by the fifth or sixth day. Marked debris appeared on the ninth to thirteenth day.

It was discovered at the beginning of Experiment 3 that exposure of large surfaces of broth filtrate markedly increased pH values. This was not true if the filtrate was stored in test tubes. This factor was controlled in Experiments 3 and 4. pH values varied within narrow limits for the first seven or eight days. Thereafter, pH values varied erratically with a tendancy toward the high levels. The first trend toward high pH levels was accompanied by the appearance of marked debris.

The production of toxin was progressive and rapid for the first six or seven days. Thereafter, the toxin values fluctuated between wide limits. The peak of toxin production was reached five or six days after the peak of the vegetative cell population and was preceded by the appearance of moderate bacterial debris. The peak of toxin production in turn preceded the rise in pH in three of the four experiments.

The amount of gelatin-splitting enzyme measured at pH 7.0 in the various experiments was not constant and did not correlate with any of the other phenomena observed. The enzyme exhibited a much increased activity when measured at pH 8.0 to 10.0.

The data presented in this report lends support to the theory that tetanus toxin is directly evolved by Cl. tetani. The rapidity of evolution of toxin appears to weaken an enzyme hypothesis. It is admitted, however, that the approach

to the enzyme phase of the problem had many shortcomings. Only one enzyme was selected for measurement with no evidence that this particular enzyme was at all associated with toxin production. As Walbum has pointed out, a more accurate approach might be made through the albumose-splitting enzymes. The most that can be said is that the gelatin-splitting enzyme appeared to play no part as a precursor to the formation of toxin.

A broad consideration of the data presented does indicate, however, that simple evolution of toxin by the vegetative cells cannot be considered as the whole story, because it was obvious that some toxin was being produced long after the cell population had been markedly diminished.

The chain of events over an incubation period of fourteen to sixteen days appears to divide into three fairly well defined phases.

Phase I included the first five to six days of incubation. Important indications of this phase were the peak in vegetative and spore cell populations, the relatively constant pH, and the orderly production of toxin. Phase 2 was marked by the appearance of moderate debris, the production of more toxin, and a rise in the general pH level. Phase 3 was marked by wide fluctuations in the toxin potency, high pH levels, and marked debris. The gelatin-splitting enzyme did not appear to be correlated with any of the phases.

Phase I approximates most closely the current theory of exotoxin production. Phase 2 seems to be the result of an autolytic phenomenon because of the continued production of

toxin in the face of low cell counts and considerable cell debris. This toxin might be due either to direct liberation of toxin by remaining bacterial cells or to the action of some autolytic enzyme on naturally formed bacterial debris.

Phase 3 appears to represent a phase following toxin production. No appreciable toxin is produced but the toxin already present fluctuates in potency according to prevailing conditions. Not the least interesting phenomenon is that apparent inactivation and reactivation of the toxin. The factors governing this behavior must be carefully studied if the maximum amount of toxin is to be harvested in routine production.

SUMMARY AND CONCLUSIONS

The vegetative and spore cell populations, pH reaction, potency, activity of the gelatin-splitting enzyme, and the presence of cell debris were observed over a period of fourteen to sixteen days aerobic incubation at 37.5°C. The results led to the following conclusions:

1. Toxin production appeared to be a three-phase phenomenon:
   - Phase I. A period of simple exotoxin production.
   - Phase 2. A period of toxin production apparently due to autolytic phenomenon.
   - Phase 3. A period following toxin production during which the toxin present varied in potency according to prevailing conditions.
2. There was no correlation between vegetative and spore cell populations, and toxin potency.
3. pH values varied within a narrow limit for the first seven to eight days. Thereafter, considerable variation took place with a tendency toward increased pH values as the cell debris became marked.
4. The production of toxin was progressive and rapid for the first six or seven days. Thereafter, the toxin values fluctuated between wide limits.
5. The gelatin-splitting enzyme measured at pH 7.0 showed no constant activity and did not correlate with any of the other phenomena observed.

6. The factors governing the data gathered in these experiments must be carefully studied in future experiments if maximum toxin production is to be obtained.

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APPENDIX

1. Veal Infusion Agar 0.5%. Semi-solid. 0.5% dextrose added.

   (a) Formula:

Veal infusion broth (Appendix 2)	1000 mils.
Agar, Bacto	5 gms.

   (b) Procedure:

   Dissolve agar in broth in steam kettle with constant stirring. When the agar is completely dissolved, adjust reaction to pH 7.5 and boil for 10 minutes. Hold for 2 hours at 60-80°C. Filter through cotton-gauze filter. Add 5 grams of dextrose. Make up to volume and dispense. Autoclave for 25 minutes at 15 lbs. (121°C) pressure. Check reaction. Do not allow agar to solidify before sterilization.

2. Veal Infusion Broth

   (a) Formula:

Ground veal, fat free	(453.6 gms)	1 lb.
Peptone, Bacto		10 gms.
Sodium Chloride C.p.		5 gms.
Distilled water		1000 mils

   (b) Procedure:

   Grind veal and infuse in coldroom over night. Skim off fat and boil in steam kettle until meat is thoroughly cooked. Filter infusion through gauze filter, drain broth out of meat and cool filtrate in kettle until

remaining fat has solidified. Skim off fat and make up to volume. Dissolve peptone and sodium chloride in infusion, adjust to pH 7.4 and boil for 10 minutes. Cool to allow sediment to settle, filter through hard paper and make up to volume. Dispense and autoclave 25 minutes at 15 lbs. (121°C) pressure. Check reaction.

3. Veal Infusion Broth. Sugar-free.

(a) Formula:

Veal infusion broth (Appendix 2)	1000 mils.
B. coli communior, 24 hour culture	2.5 mils

(b) Procedure:

Inoculate the sterile broth with the 24 hour culture of B. coli communior (2.5 c.c. broth culture per liter of broth). Incubate for 24 hours at 37.5°C. Autoclave at 15 lbs. (121°C) pressure for 10 minutes and adjust the reaction to pH 7.5. Boil 10 minutes in steam kettle and then cool to allow sediment to settle. Filter through hard paper filter, then candle, make up to volume, dispense and autoclave for 20 minutes at 15 lbs. (121°C) pressure.

4. Veal Infusion Broth plus 0.5% Dextrose.

(a) Formula:

Ground veal, fat free	(453.6 gms.)	1 lb.
Peptone, Proteose		10 gms.
Sodium Chloride C.p.		5 gms.
Sterile 20% dextrose solution		25 mils.
Distilled water		1000 mils.

(b) Procedure:

Mix ground veal and water and infuse in coldroom over night. Infuse for 1 hour at 45°C. Raise to 100°C. and hold for 1 hour. Strain through gauze, add peptone and sodium chloride and dissolve by stirring. Make up to volume, adjust reaction to pH 7.4 and filter through hard paper. Dispense and autoclave for 25 minutes at 15 lbs. (121°C) pressure. Check reaction. Add sterile dextrose solution aseptically to cooled medium.

5. Peptic Digest Agar (10)

(a) Formula:

Veal infusion agar 2%, pH 7.4 (Appendix 13)	300 mils.
Sterile peptic digest of sheep cells (Appendix 6)	1-3 mils.

(b) Procedure:

Melt sterile agar and cool to 40°C. Add digest aseptically and whirl to mix thoroughly. Dispense

with sterile funnel or pour directly into sterile petri dishes. Incubate 24 hours at 37.5°C. to check sterility.

6. Peptic Digest Solution (10)

(a) Formula:

Physiological saline 0.85 (Appendix 8)	150 mils.
Hydrochloric acid 10 normal	6 mils.
Defibrinated sheep cells	50 mils.
Pepsin, granular, Bacto	1 gm.

(b) Procedure:

Introduce ingredients in the order listed into a 250 c.c. Erlenmeyer flask and shake to dissolve. Place in water bath at 55°C. for 24 hours, shaking occasionally at the beginning of the time interval. Add 12 c.c. of 5N sodium hydroxide accurately prepared and adjust reaction to pH 7.6 using more 5N sodium hydroxide if necessary. To test, the samples are removed from the bottle with a sterile pipette and diluted with distilled water until color is light enough to distinguish the change on the addition of a few drops of indicator. Adjust mixture to pH 7-7.2 with N/1 hydrochloric acid and add 0.25% chloroform. Paper cap the bottle and use in 2-5% amounts in media.

7. Tetanus Toxin Broth (1) (Modified)

(a) Formula:

Ground veal, fat free	(453.6 gms)	1 lb.

Peptone, Witte	10 gms.
Sodium chloride C.p.	5 gms.
Sterile 20% dextrose solution	50 mils
Distilled water	1000 mils

(b) Procedure:

Mix ground veal and water and infuse in coldroom 2 hours. Infuse for 1 hour at 45°C. Raise to 100°C. and hold for 1 hour. Strain through gauze, and cool until fat has solidified. Skim off fat. Heat, add peptone and sodium chloride and dissolve by stirring. Make up to volume, adjust reaction to pH 7.3 and filter through hard paper. Dispense and autoclave 25 minutes at 15 lbs. (121°C) pressure. Check reaction. Add sterile dextrose solution aseptically to cooled medium. Inoculate as soon as cool.

8. Physiological Saline

(a) Formula:

Sodium chloride C.p.	0.85 gm.
Distilled water - up to	100 gms.

(b) Procedure:

Dissolve sodium chloride in distilled water and stir to mix thoroughly. Adjust reaction to pH 7.0 using 0.1% potassium phosphate secondary solution for titration and 1.0% potassium phosphate secondary solution for adjustment. Dispense and autoclave for 20 minutes at 15 lbs. (121°C) pressure. Check reaction.

9. Gelatin-Buffer Mixtures (7)

(a) Formula:

20% gelatin (Appendix 10)	5 gms.
Buffer solution (Appendix 11)	5 mils plus factor

10. 20% gelatin

(a) Formula:

Gelatin, Bacto	400 gms.
Distilled water	2000 gms.
Thymol	1 gm.

(b) Procedure:

Heat distilled water and add slowly 400 grams of gelatin. Make up to near the total volume with distilled water. Adjust reaction to pH 7.0 with N/1 sodium hydroxide. Make up to volume. Add thymol dissolved in a little 95% alcohol.

11. Standard Buffer Mixtures (9)

pH 4 - 50 cc M/5 KH Phthalate
0.4 cc M/5 NaOH
Dilute to 200 cc with distilled water.

pH 5 - 50 cc M/5 KH Phthalate
23.85 cc M/5 NaOH
Dilute to 200 cc with distilled water.

pH 6 - 50 cc M/5 KH_2PO_4
5.70 cc M/5 NaOH
Dilute to 200 cc with distilled water.

pH 7 - 50 cc M/5 KH_2PO_4

29.63 cc M/5 NaOH

Dilute to 200 cc with distilled water.

pH 8 - 50 cc M/5 H_3BO_3, M/5 KCl

3.97 cc M/5 NaOH

Dilute to 200 cc with distilled water.

pH 9 - 50 cc M/5 H_3BO_3, M/5 KCl

21.30 cc M/5 NaOH

Dilute to 200 cc with distilled water

pH 10 - 50 cc M/5 H_3BO_3, M/5 KCl

43.90 cc M/5 NaOH

Dilute to 200 cc with distilled water.

These mixtures were overbalanced with N/1 sodium hydroxide or N/1 hydrochloric acid, so that when mixed with gelatin, broth filtrate, and distilled water, the final pH reaction would be as given above.

12. Phenol Red Indicator

Phenol red indicator	0.02 mg.
95% alcohol	100 mils

13. Veal Infusion Agar 2%

(a) Formula:

Veal infusion broth (Appendix 2)	1000 mils
Agar, Bacto	20 gms.

(b) Procedure:

Dissolve agar in broth in steam kettle stirring

constantly. When agar is completely dissolved, adjust the reaction to pH 7.4 and boil for 10 minutes. Hold for 2 hours at 60-80°C. Filter through cotton-gauze filter. Make up to volume and dispense. Autoclave 25 minutes at 15 lbs. (121°C) pressure. Check reaction. Do not allow agar to solidify before sterilization.