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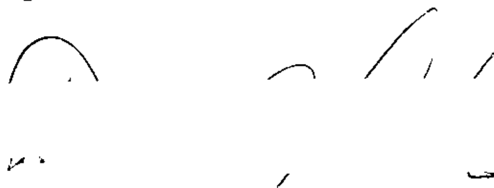

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THE PRODUCTION OF A TRYPSIN-INHIBITING FACTOR BY CLOSTRIDIUM BOTULINUM, TYPE F

A THESIS

Presented to

The Faculty of the Graduate Division

by

David Albert Preston

In Partial Fulfillment

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in The School of Applied Biology

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THE PRODUCTION OF A TRYPSIN-INHIBITING

FACTOR BY CLOSTRIDIUM BOTULINUM, TYPE F

Approved:

Chairman

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SUMMARY

It has been shown in previous works that *Clostridium botulinum*, Types A, B, and E produce a substance which inhibits the enzyme trypsin. The purpose of this research was to demonstrate the production of this substance by *C. botulinum*, Type F.

The trypsin-inhibitory power of cell-free fluids of cultures of *C. botulinum*, Type F was determined by comparing the concentration of products of a standard trypsin-casein digestion with the products of a trypsin-casein digestion containing an aliquot of the culture fluid.

It was concluded that at least one strain of *C. botulinum*, Type F produces a trypsin inhibitor during the exponential phase of bacterial growth. The inhibitor is stable to the cultural conditions of the organism, i.e., it remains indefinitely in the culture fluid.

CHAPTER I

INTRODUCTION

Clostridium botulinum is classified into six types, A, B, C, D, E, and F, according to the immunological reaction of the toxin produced. *C. botulinum*, type F, the most recently found type, was isolated in the laboratories of Møller and Scheibel (1960) after an outbreak of botulism poisoning on the Danish island of Langeland in 1958. Subcultures of this same organism were studied further by Dolman and Murakami (1961). The second and only other reported natural occurrence of this organism to date was in marine sediment off the coast of California (Eklund and Poysky, 1965).

The potency of botulinal toxins varies greatly among types and among strains of the same type. Bonventre and Kempe (1960) demonstrated that at least some strains of *C. botulinum* produce a prototoxin which can be activated by incubation with trypsin, thus greatly increasing the potency of the culture liquid. In those cultures whose toxins show no activation by trypsin, alternate explanations have been proposed: (1) the culture produces its own enzymes which spontaneously activate the toxin to its greatest potency (Bonventre and Kempe, 1960); or (2) the culture produces a trypsin-inhibiting factor (Höyem and Skulberg, 1962).

Höyem and Skulberg (1962) demonstrated the presence of trypsin-inhibiting factors in cultures of *C. botulinum*. Using a standard trypsin-casein digestion as the parameter of measurement, they observed widely

varying amounts of trypsin-inhibiting activity in *C. botulinum*, types A, B, and E. Under the conditions of their experiment they observed the following ranges of maximum trypsin-inhibiting activities:

C. botulinum, type A	13-51 percent
C. botulinum, type B	62-74 percent
C. botulinum, type E	4-35 percent

Using sterile media in the place of a culture sample under the same experimental conditions, they observed no trypsin inhibition.

According to Höyem and Skulberg, the trypsin inhibitor produced by Type E is easily dialysable, leaving the dialysis bag completely within one hour when using a rotating technique. It is also stable to a temperature of 100 C for one to two hours and to autoclaving for 15 minutes at 120 C.

The objective of the research described in this thesis was to demonstrate the presence or absence of similar trypsin-inhibiting factors in cultures of *C. botulinum*, type F, using the procedure of Höyem and Skulberg.

The procedure used by Höyem and Skulberg was modified slightly to conform to the specific apparatus and equipment in this laboratory. A general outline of the experimental procedure, as modified, follows.

A sample of the culture to be tested was observed for growth and then centrifuged to remove the cells and spores from the culture medium. One ml of the supernatant fluid was added to a test tube with 5 ml of 3 percent casein, 1 ml of phosphate buffer (pH 7.6), and 1 ml of the appropriate trypsin solution. The contents of the tube were mixed and allowed to react for one hour. An aliquot of this digestion mixture was mixed with an equal portion of 10 percent trichloroacetic acid and allowed to

stand for 15 minutes. This mixture was centrifuged to separate the precipitated casein from the liquid. An aliquot of the supernatant fluid was then diluted with 5 percent trichloroacetic acid. The absorption of this solution was determined at 275 mμ in the spectrophotometer. The concentration of digestion products thus determined was subtracted from the concentration of products formed in a control digestion mixture without the culture. This difference was attributed to inhibition of the trypsin by the culture and was expressed as a percent of the control.

CHAPTER II

METHODS AND MATERIALS

Organisms Used

Two variants of *Clostridium botulinum*, type F, and one strain of *C. botulinum*, type A, were studied in this work. The designations and sources of these organisms were:

C. botulinum, type F, TOX-IB

C. botulinum, type F, OPY-26

These cultures were obtained from Dr. Lillian Holdeman, Communicable Disease Center, Atlanta, who isolated them as colonial variants from the original Langeland strain.

C. botulinum, Type A, strain Hall.

This culture was obtained from M. A. Cardella, Ft. Detrick, Maryland.

Culture purity was assured by periodically checking smears stained with malachite green spore stain and counterstained with aqueous Safranin as prescribed by the Society of American Bacteriologists (1957).

Serological specificity was confirmed by assay with botulinum antisera types A, B, and F obtained from the Communicable Disease Center, Atlanta, Georgia.

Maintenance of Stock Cultures

Frozen samples of each culture were obtained from Dr. Nancy W. Walls, Georgia Institute of Technology, Atlanta, Georgia. These were

thawed, inoculated into 30 ml each of freshly exhausted cooked meat medium,* and incubated at 30 C for three days. Ten ml of each of the three cooked meat cultures were inoculated into three flasks containing 200 ml each of freshly exhausted cooked meat medium. After five days incubation at 30 C, the culture liquid was dispensed in 2.5 ml quantities into sterile storage vials and stoppered with sterile paraffin coated corks. These were then quick frozen at -80 C in a 2-ethoxyethanol - dry ice bath. The frozen cultures were stored at -15 C for later use.

The strain of C. botulinum to be used in any experiment was chosen from the frozen stock and thawed at room temperature. Two ml of the thawed cultures were aseptically transferred to the appropriate medium.

Growth Measurements

Bacterial growth was observed daily by measuring turbidity at 600 mμ on a Bausch and Lomb "Spectronic 20" colorimeter. The colorimeter was adjusted to indicate 100 percent transmittance with a sterile blank of the same medium being used in any given run. The determination of growth by this method gave a relative value rather than an absolute quantitation of cells per unit volume.

Bacterial Growth Media

Cooked Meat Medium (Difco)

Thirty ml of deionized water were added to 3.75 g of the dry cooked meat medium in a screw-capped culture tube (2.5 cm x 20 cm) and allowed to stand 15 minutes to insure complete wetting of the meat particles before autoclaving 15 minutes at 120 C and 15 psi of pressure. The sterile

* See Bacterial Growth Media, p5.

medium was cooled to room temperature and inoculated immediately with 2 ml of the thawed stock culture.

Trypticase Medium

Ingredients:

Trypticase (BBL) 2.5g

Yeast extract (BBL) 0.5g

Sodium thioglycollate (Difco) 0.05g

Bacto-dextrose (Difco) 0.5g

Water, deionized . . . . .97.5 ml

All the ingredients except the dextrose were dissolved by mixing on a magnetic stirrer. The reaction of the medium was adjusted to pH 7.3 with 10 N NaOH. The medium was then dispensed into screw-capped culture tubes (2 cm x 15 cm) in 9.5 ml quantities and capped. After sterilization for 15 minutes in the autoclave, the tubes were cooled to room temperature. The dextrose was added aseptically as a sterile 20 percent solution in the amount of 0.25 ml per tube. The tubes of medium were inoculated immediately with 0.1 ml of the inoculum culture.

Type A Medium

Ingredients:

N-Z-Amine, type B (Sheffield Chemical Co.) . 2.0g

Autolyzed yeast extract (Vico) 0.5g

Bacto-dextrose (Difco) 0.5g

Deionized water . . . . 94.5 ml

All ingredients except the dextrose were dissolved in the water with gentle heat on a magnetic stirrer. The medium was cooled to room temperature and adjusted to pH 7.0 with 10 N NaOH. The medium was dispensed into

screw-capped culture tubes (2cm x 15 cm) in 9.5 ml quantities and capped. These were sterilized for 15 minutes in the autoclave and cooled to room temperature. The dextrose was added aseptically as a sterile 20 percent solution in the amount of 0.25 ml per tube. Each tube of medium was immediately inoculated with 0.5 ml of the inoculum culture.

The Standard Trypsin-Casein Digestion

The endopeptidase trypsin characteristically catalyses the hydrolysis of interior peptide bonds of casein to a variety of products consisting primarily of an assortment of polypeptides (Conn and Stumpf, 1963). This experiment is based on the quantitative spectrophotometric measurement of these peptides as products of a standard trypsin-casein digestion (Northrup, et al., 1948).

The typical digestion mixture consisted of 5 ml of stock 3 percent casein,* 1 ml of phosphate buffer (pH 7.6*), 1 ml of aqueous trypsin*, and 1 ml of the material whose effect on the reaction was to be observed.

All of the above ingredients except the trypsin were placed into a screw-capped test tube (1.5 x 12.5 cm), mixed thoroughly, and placed into a 37.5 C ± 0.5 C water bath for 5 minutes. The trypsin solution was added at an observed time. The mixture was agitated thoroughly and replaced in the water bath. After 60.0 minutes, the digestion was stopped by adding 3 ml of the digestion mixture to 3 ml of a 10 percent (w/v) aqueous solution of trichloroacetic acid in a centrifuge tube (1.5 x 17.5 cm) and mixing thoroughly. After 15 minutes, the mixture was centrifuged in the cold (1 C) at 4200 x g for 15 minutes. The products of the digestion remained dissolved in the supernatant fluid.

* See Appendix

Spectrophotometric Quantitation of Digestion Products

One ml of the supernatant fluid described above was diluted in 5 ml of 5 percent (w/v) trichloroacetic acid. The absorption of this solution was determined at 275 mμ in a Beckman Ratio-Recording Spectrophotometer, Model DK-2.

Products of the digestion of casein by trypsin absorb ultraviolet light strongly at 275 mμ. This absorption varies directly with the concentration of the products (Northrup, et al., 1948). The digestion products may be quantitated by comparing the absorption at 275 mμ of a solution of the products to the absorption of a control or reference solution. The reference solution was prepared by mixing the same proportions of ingredients as present in the test solution. However, in this case, the casein was precipitated with trichloroacetic acid before the addition of trypsin, thereby precluding any digestion.

Calibration of the Trypsin

A series of trypsin solutions was prepared containing 2.5, 5, 7.5, 10, 12.5, and 15 mg trypsin per 100 ml phosphate buffer (pH 7.6). Each of these was allowed to digest casein in the previously described manner. The absorbances of the products were plotted against the various concentrations of trypsin. The curve fitted by inspection to these points (Fig. 1) was used to calculate the specific activity of the trypsin.

The specific activity, expressed in tryptic units, was defined as the amount of trypsin which gives rise, under the conditions described, to an increase of one unit of absorbance at 275 mμ per minute of digestion. The slope of the first part of the curve divided by the digestion time in

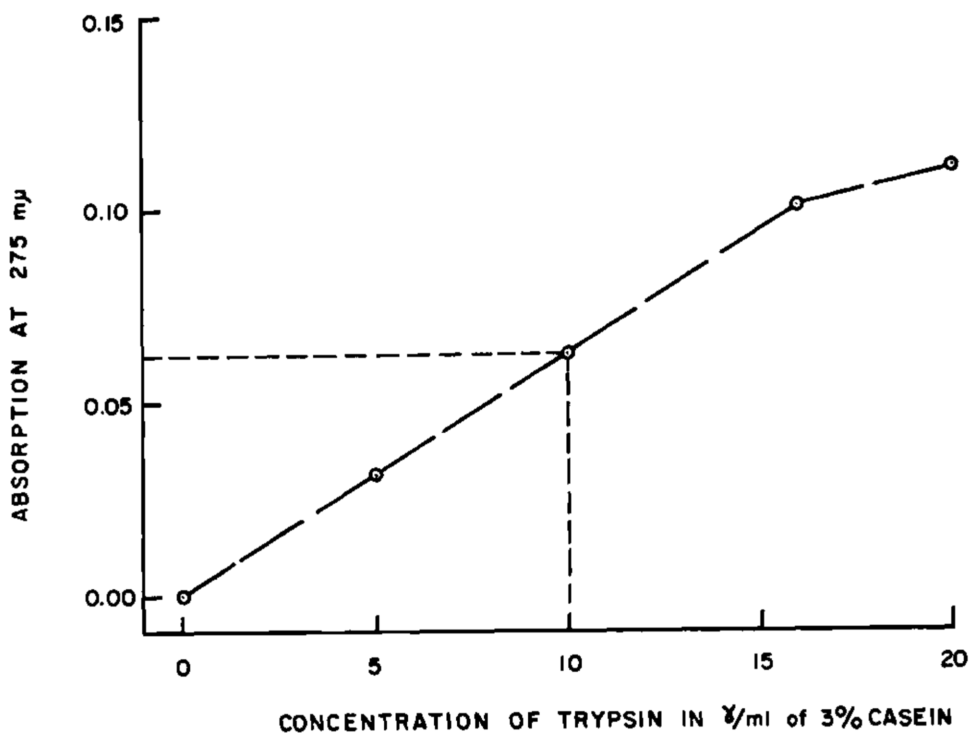

Figure 1. Calculation of Specific Activity of Trypsin The points designate experimental values relating concentration of trypsin to absorption of light at 275 mμ by soluble digestion products. The slope of the first part of the curve divided by the digestion time in seconds gives the specific activity of the trypsin:

$$[TU]^{Cas.}_{\gamma\ Trypsin} = \frac{6.2}{10 \times 60.0} = 1.03 \times 10^{-2}$$

minutes gives the activity per microgram of trypsin, i.e., the specific activity. Using the appropriate information read from the first curve, another curve (Fig. 2) was then plotted by inspection relating absorbance to tryptic units. If a sample of trypsin is allowed to digest casein under the previously described conditions and is subjected to spectrophotometric analysis, the apparent number of tryptic units acting on the casein may be read from this second curve. The concentration of trypsin chosen for use in the experimental runs was 15 mg trypsin per 100 ml buffer. This amount is equivalent to 30 γ trypsin per ml of 3 percent casein in the digestion mixture. Because inhibition was expected, the trypsin activity was anticipated to be less than this, thus yielding observations which quantitatively are consistent with the straight portion of the curve in Figure 1.

Preparation of the Culture Sample

Seven ml of the broth culture of *C. botulinum* were placed into a centrifuge tube and centrifuged in the cold at 4200 x g for 20 minutes. Five ml of the supernatant fluid were drawn off and placed in a test tube and mixed vigorously on the Vortex mixer. This solution was used as the supernatant culture fluid in the determination of trypsin inhibition.

Determination of Trypsin Inhibition

Trypsin inhibition was determined by comparing the amounts of digestion products given by a set of three digestion mixtures containing 1 ml each of a centrifugate of the bacterial culture being tested with those given by a set of three control digestion mixtures; i.e., digestion mixtures without the culture centrifugate. Less products in the experimental

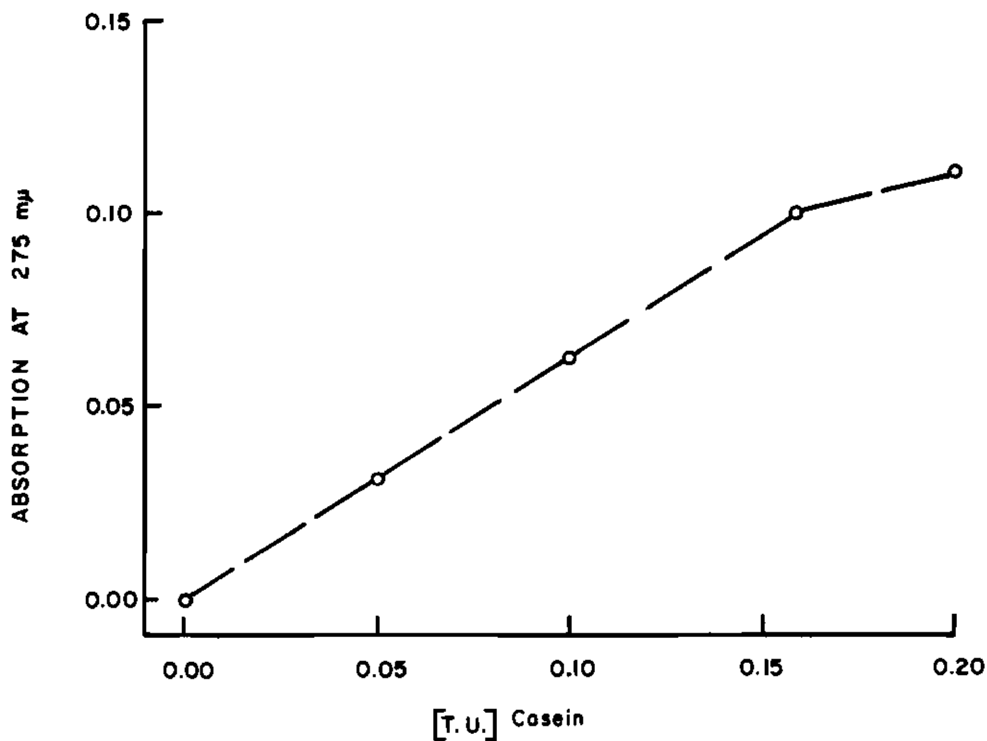

Figure 2. General Curve For Determination of Tryptic Units The points are derived from the points in Figure 1.by changing the abcissal units from γ Trypsin/ml 3% casein to Tryptic Units and replotting. The curve, fitted by inspection, then gives direct reading of Tryptic units for a given absorption at 275 mμ.

mixtures than in the control mixtures implied inhibition. The apparent tryptic units acting in an experimental sample were expressed as a percentage of the tryptic units acting in the control samples and designated percent inhibition.

Compensation for the Effect of Endogenous Proteolytic Enzymes

Included in each run was a pair of digestion mixtures to which no trypsin was added. Digestion products appearing in these tubes could be attributed to proteolytic enzymes produced by the culture. This amount of digestion in each case could be quantitated and subtracted from the total amount of digestion products in the experimental samples. This method was expected to compensate for the effect of the endogenous proteolytic enzymes.

CHAPTER III

RESULTS

Trypsin-inhibition and Growth

Type F, OPY-26

Figures 3 and 4 represent the cultural growth and trypsin-inhibition demonstrated by two identical consecutive runs with OPY-26. In each case, the inhibition was first seen after 24 hours. On the second day of each run, the level of inhibition reached about 20 percent and remained near this value through the seventh day.

Type F, TOX-IB

The first run with TOX-IB demonstrated trypsin-inhibition of about 35 percent (Fig. 5) in a pattern similar to that of OPY-26. But in three subsequent runs, very erratic results were observed. Figure 6 represents the growth and inhibition observed in one such case. From day to day the culture appeared to inhibit or enhance the activity of the trypsin in a manner with no apparent pattern.

A striking difference was noted between the first run with TOX-IB and subsequent experiments with this strain. In the latter runs, the culture fluid itself was capable of digesting casein without any trypsin being added. This, of course, implied the production of proteolytic enzymes by the cultures. These trypsin-like enzymes appeared in quantities up to an equivalence of 0.12 tryptic units per ml of culture supernatant, equivalent to almost half of the activity of the trypsin added in the

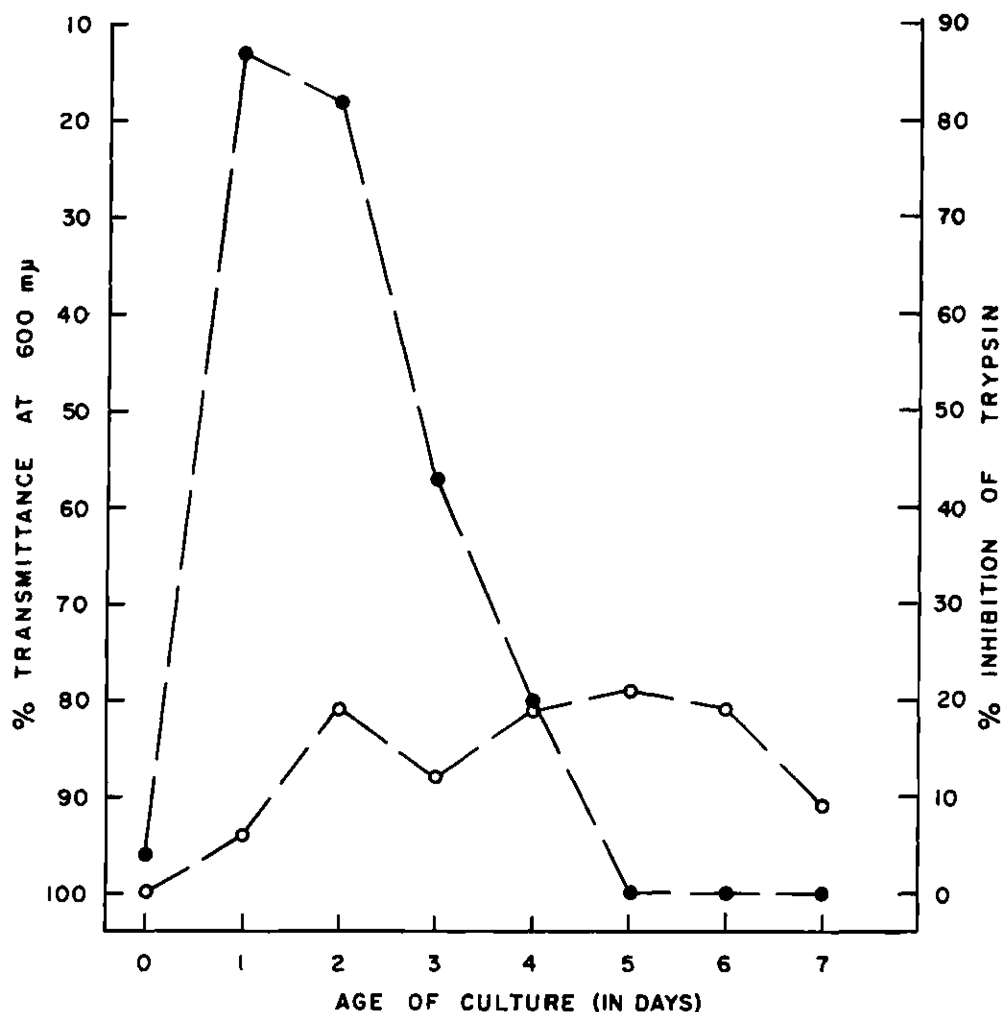

Figure 3. Bacterial Growth and Trypsin Inhibition by Clostridium botulinum Type F, OPY-26 (Run I). The solid circles (●) designate growth of the culture by turbidity in percent transmittance at 600 mμ. The turbidity is recorded relative to the transmittance of a sterile blank of the medium used in this run. The open circles (o) represent the calculated percent inhibition of the trypsin used in the test.

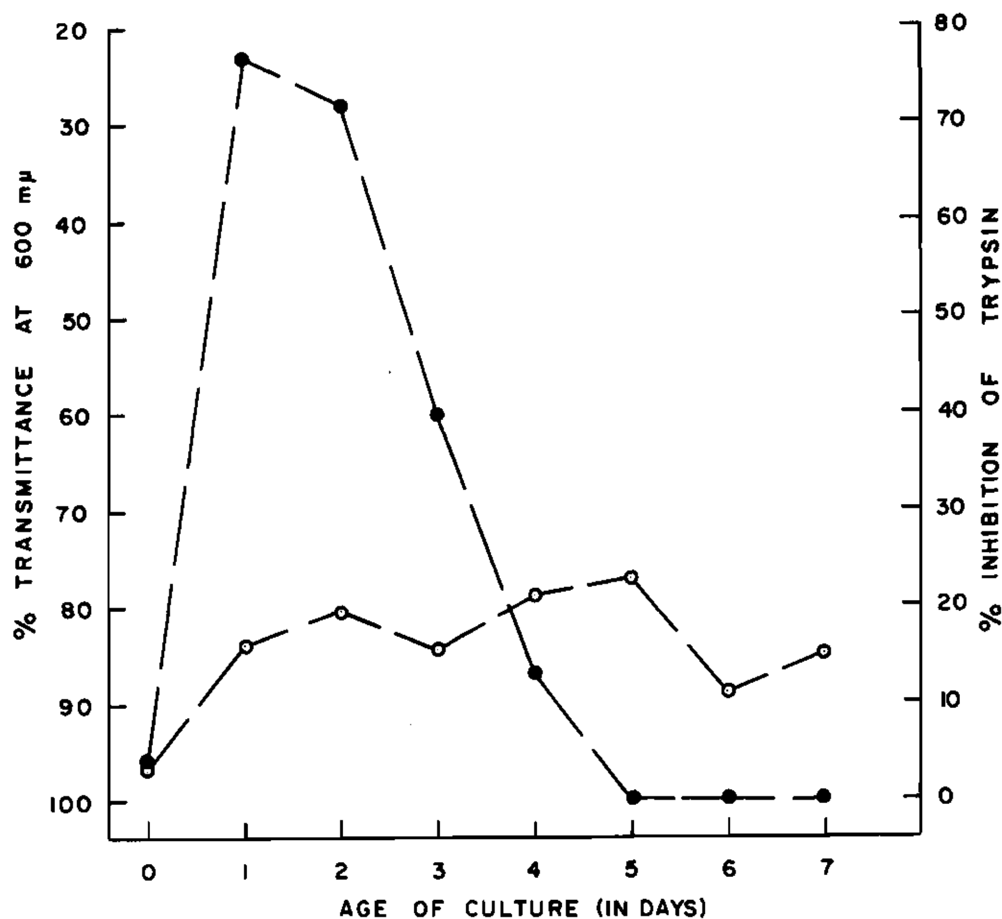

Figure 4. Bacterial Growth and Trypsin Inhibition by Clostridium botulinum Type F, OPY-26 (Run II). The solid circles (•) designate growth of the culture by turbidity in percent transmittance at 600 mμ. The turbidity is recorded relative to the transmittance of a sterile blank of the medium used in this run. The open circles (o) represent the calculated percent inhibition of the trypsin used in the test.

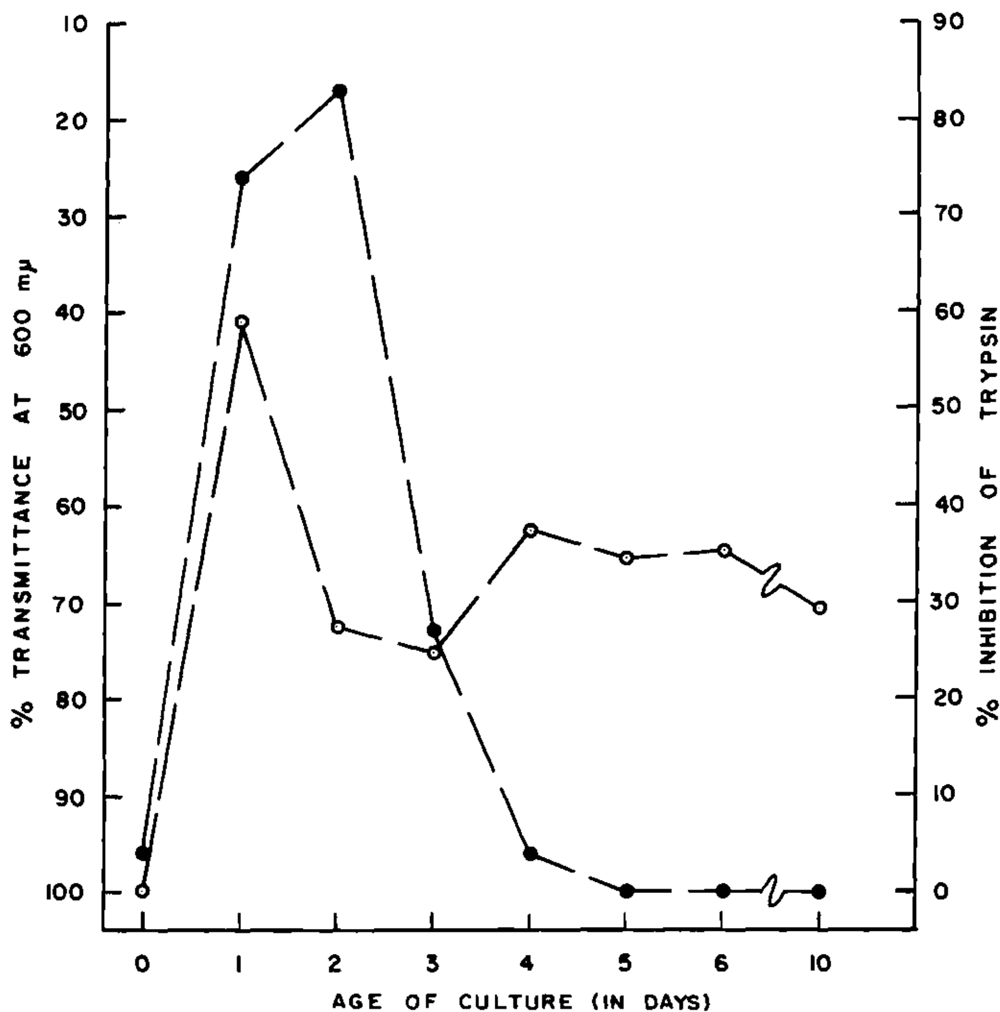

Figure 5. Bacterial Growth and Trypsin Inhibition by <u>Clostridium botulinum</u>, Type F, TOX-IB (Run I). The solid circles (•) designate growth of the culture by turbidity in percent transmittance at 600 mμ. The turbidity is recorded relative to the transmittance of a sterile blank of the medium used in this run. The open circles (o) represent the calculated percent inhibition of the trypsin used in the test.

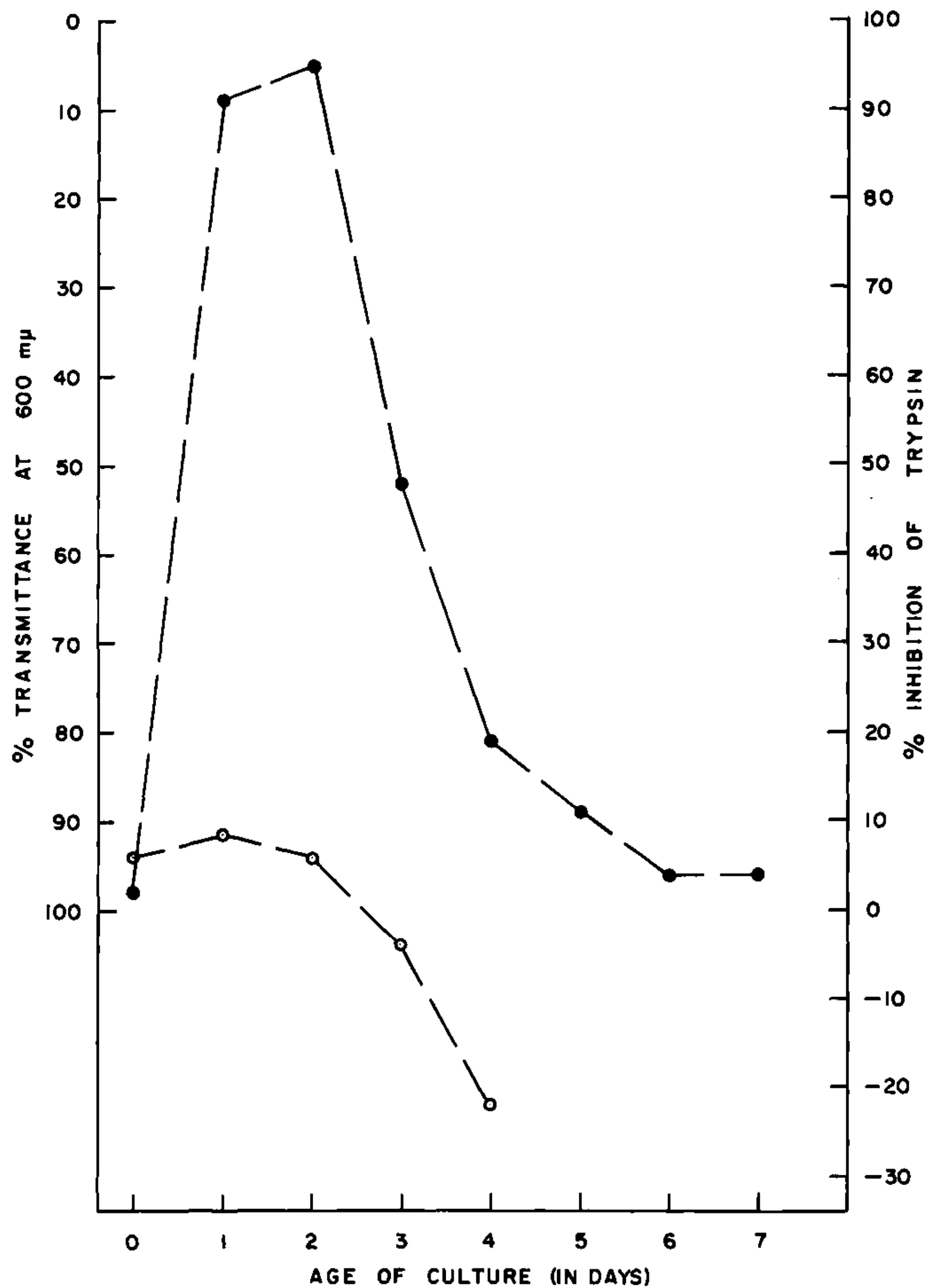

Figure 6. Bacterial Growth and Trypsin Inhibition by *Clostridium botulinum*, Type F, TOX-IB (Run IV). For explanatory notes see Figure 5.

experimental digestion samples. These proteolytic enzymes did not appear in the first run with TOX-IB.

Type A, Hall

In runs with Type A, Hall very erratic results were observed with little or no inhibition of trypsin (Fig. 7 and 8). In Holdeman's trypticase medium and in Type A medium (used by Höyem and Skulberg), Type A, Hall produced large amounts of proteolytic enzymes, sometimes reaching an equivalence of 0.16 tryptic units per ml of culture fluid.

Characteristics of Inhibitor Production

The trypsin inhibitor produced by *C. botulinum*, Type F, OPY-26 apparently is produced during the logarithmic phase of the bacterial growth (Fig. 3, 4, and 5). Because the level of inhibition remained approximately the same from the second day through the seventh day, it was concluded that the inhibitor is a stable compound which, after being produced, remains in the culture fluid. Averaging all the inhibition data for *C. botulinum*, Type F, OPY-26 yields the curve shown in Figure 9. The first derivative of this curve describes the rate of inhibitor production. The plot of this information, shown in Figure 10, demonstrates that the rate of production is maximum during the logarithmic growth phase of the bacterial cells and ceases altogether after the second day, i.e., after initiation of autolysis.

Statistical Analysis of Data

The data in this experiment were subjected to a multiple variance F test (Croxton, 1953) to ascertain the significance of the differences between: experimental samples and controls; daily runs; identical samples

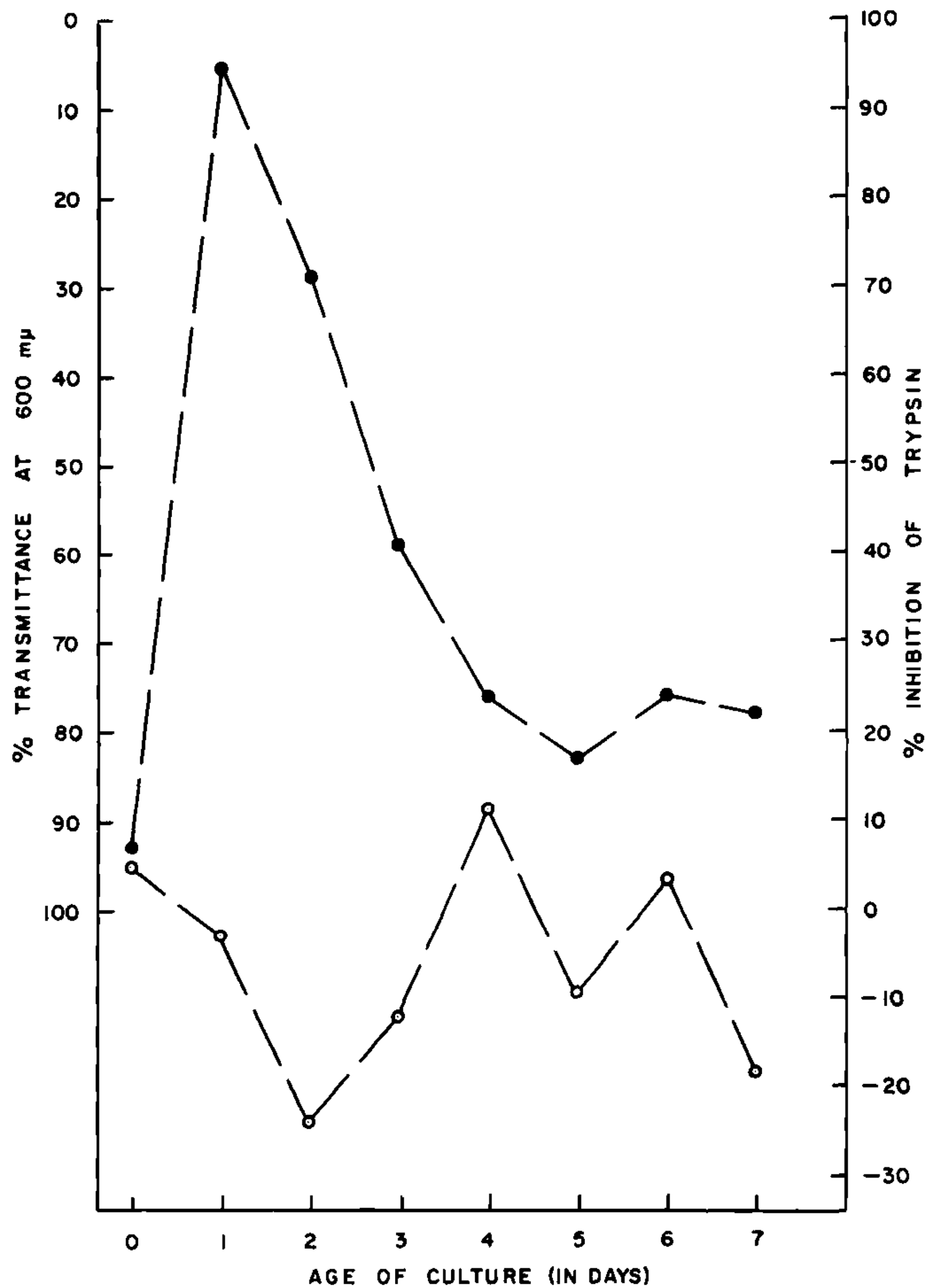

Figure 7. Bacterial Growth and Trypsin Inhibition by *Clostridium botulinum*, Type A, Hall (in Trypticase Medium).
For explanatory notes see Figure 5.

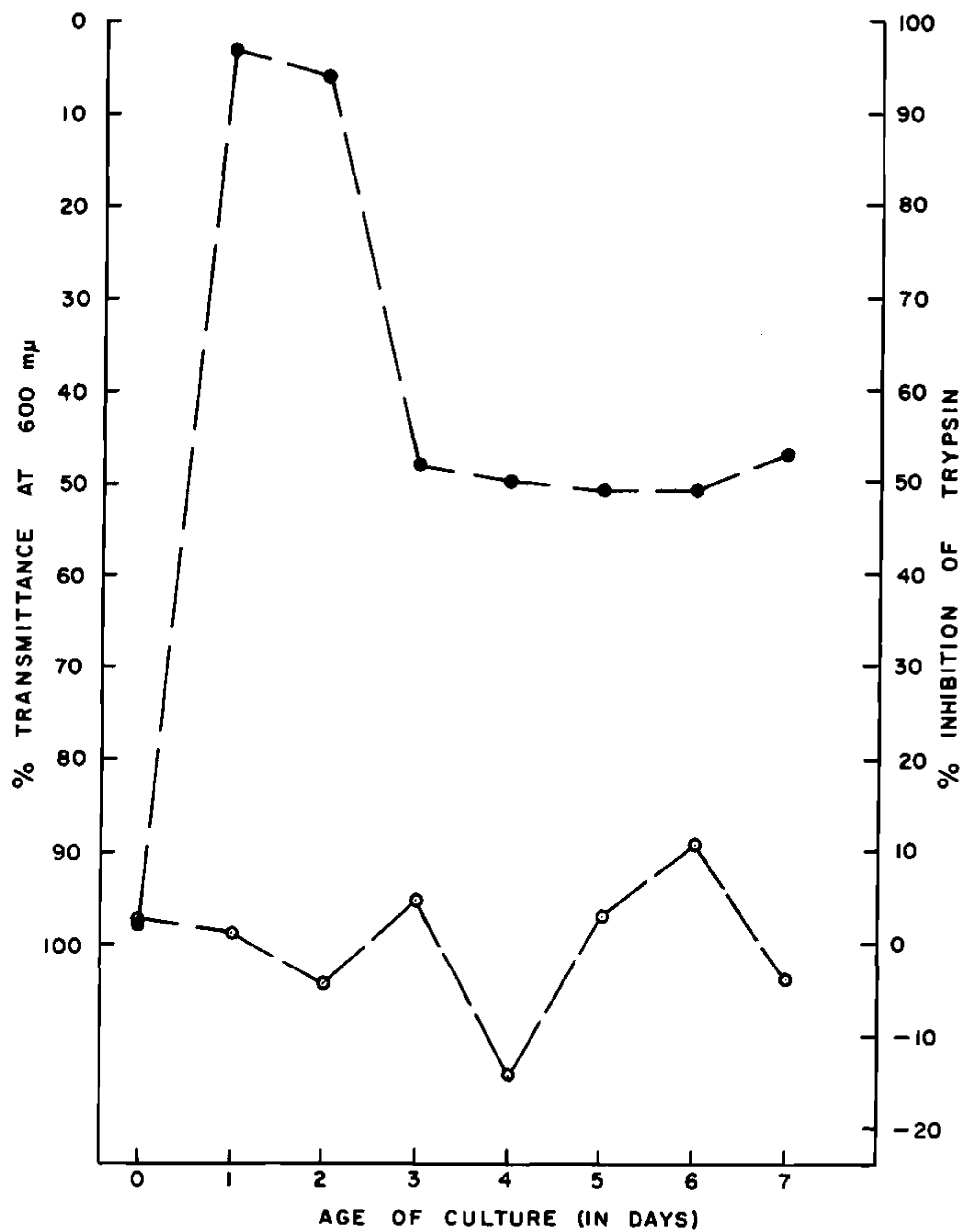

Figure 8. Bacterial Growth and Trypsin Inhibition by *Clostridium botulinum*, Type A, Hall (in Type A Medium).
For explanatory notes see Figure 5.

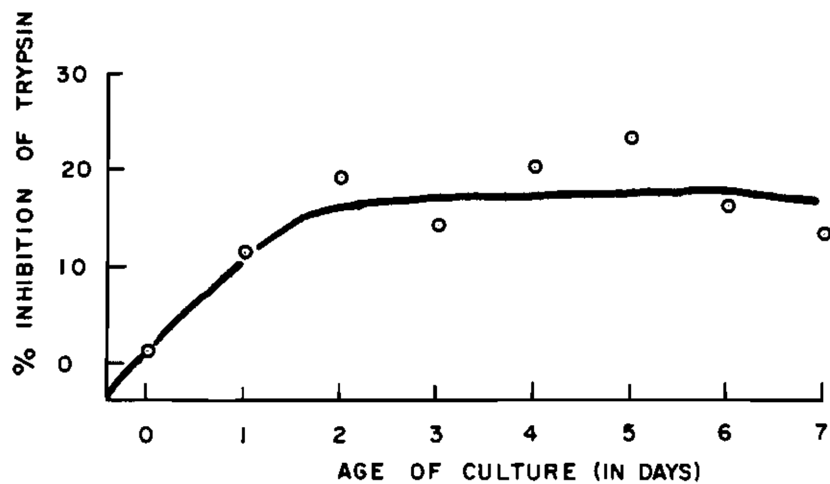

Figure 9. General Curve for Daily Amount of Trypsin Inhibitor Found in the Culture Fluid in Two Runs with *Clostridium botulinum*, Type F, OPY-26. The curve was fitted by inspection.

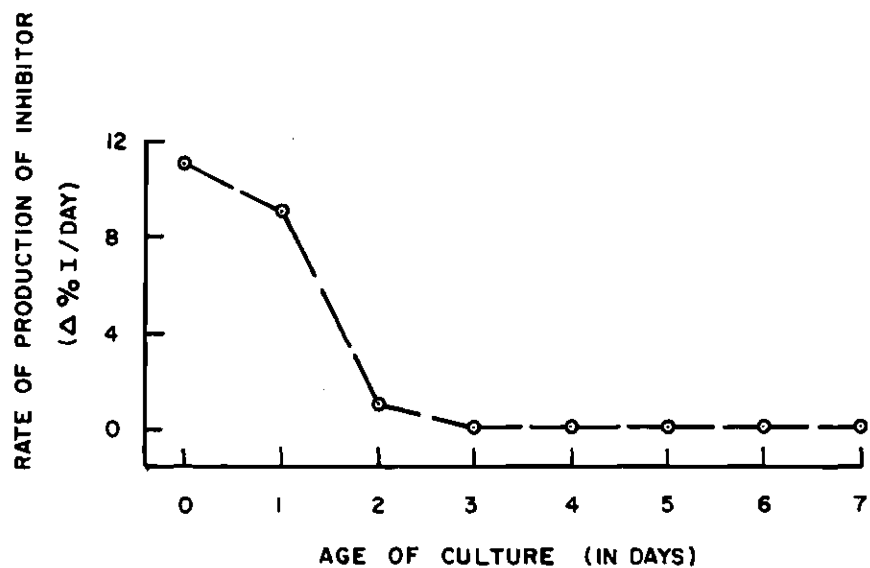

Figure 10. Daily Rate of Production of Trypsin Inhibitor by *Clostridium botulinum*, Type F, OPY-26. The points represent the scope of the curve in Figure 9 at daily intervals.

of each day of a series of daily runs (see Appendix). In all runs which yielded erratic results, statistical treatment of the data verified the unreliability of the information. However, in those runs which yielded consistent daily results, statistical treatment of the data validated the reliability of similarities and differences at the 95 percent confidence level.

CHAPTER IV

DISCUSSION

Inhibition of the activity of a standard trypsin solution by samples of culture fluid of Clostridium botulinum, Type F, OPY-26 and TOX-IB was measured by observing the difference between the amount of digestion products in a digestion mixture containing culture fluid and the amount of digestion products in a control digestion containing no culture fluid. The maximum inhibition values for inhibitors produced by these organisms varied between 20 and 37 percent; values similar to those obtained by Höyem and Skulberg (1962) for types A, B and E.

Cultures of Type F, OPY-26 inhibited trypsin activity 21 percent and 24 percent in two experimental runs. The two runs yielded very similar inhibition curves. In both cases the inhibition reached a maximum of about 20 percent by the second day and remained near this value through the seventh day.

One experimental run with Type F, TOX-IB yielded inhibition of 58 percent on the second day and thereafter fluctuated between 24 and 37 percent through the tenth day. The jump to 58 percent on the second day was regarded as the result of an experimental error. Subsequent experiments with Type F, TOX-IB yielded statistically insignificant differences between means of controls and experimentals. In each of the latter experiments, high levels of endogenous trypsin-like enzymes were indicated.

All experiments with C. botulinum, Type A, Hall yielded statistically

insignificant differences between means of control and experimentals. In each experiment with this organism, high levels of endogenous trypsin-like enzymes were indicated. The strain of Type A used in this experiment was designated the same as Höyem and Skulberg's Type A. However, it may be noted that the strain used here was not obtained directly from Höyem and Skulberg and therefore may have undergone a mutation which could account for the difference between the results obtained here and those obtained by Höyem and Skulberg (1962).

It is interesting to note that in each run which yielded erratic data, the cultures produced relatively large amounts of trypsin-like enzymes, and that in those which yielded consistent data, the culture produced very small amounts of these enzymes. It was not shown conclusively that these enzymes were responsible for the erratic data, but this is the probable answer. Failure of attempts to compensate for the presence of these enzymes implies that their presence in the digestion mixture poses a problem more complex than the simple augmentation of the activity of the digesting enzymes. Perhaps the inhibitor is "used up," i.e., chemically inactivated or denatured, during its inhibition of trypsin or similar enzymes. This could lead to obvious complications in the interpretation of the observed data.

The characteristic way in which the trypsin inhibitor appears in the culture fluid may shed some light on the mechanism of its production. The curve shown in Figure 9 can be interpretated as a two phase function, i.e., (1) production of inhibitor at a somewhat constant rate from initiation of growth to the second day, and (2) cessation of production after the second day. Interpretation of the curve in this light clearly indicates

that the inhibitor is produced and liberated into the medium during the logarithmic phase of the bacterial growth. The second phase of the curve implies that the inhibitor is stable to the existing conditions in the culture fluid at incubation temperature; once produced, it remains unchanged in the culture fluid.

Results of the test for trypsin inhibition used in this experiment have a single definite meaning only in the positive case. If the test indicates trypsin inhibition, then there must be an inhibitor present. If, however, no inhibition is indicated by the test, the assumption that no inhibitor exists is not necessarily valid. In such a case, one of two conditions could exist. There could be an absence of trypsin inhibitor, or the presence of an inhibitor could be masked by some other conditions. For instance, if the culture produced proteolytic enzymes, and if the inhibitor is "used up" during its action on these enzymes, then the test may not indicate inhibition of the trypsin used in the test.

CHAPTER V

CONCLUSIONS

Under the experimental conditions set forth here, *Clostridium botulinum*, Type F, OPY-26 produced an inhibitor of the proteolytic enzyme trypsin. *C. botulinum*, Type F, TOX-IB produced a trypsin inhibitor in one instance. However, further experimentation with this organism proved inconclusive. All experiments with *C. botulinum*, Type A, Hall proved inconclusive.

The production of trypsin inhibitors by *C. botulinum*, Type F, OPY-26 and TOX-IB occur as a two-stage function.

The production of a trypsin inhibitor by *C. botulinum*, Type F, OPY-26 and TOX-IB occurs during the logarithmic growth phase of the cultures.

The trypsin inhibitors produced by *C. botulinum*, Type F, OPY-26 and TOX-IB are stable, remaining in the culture fluid after production.

CHAPTER VI

RECOMMENDATIONS

There are several areas related to the work described here which are worthy of further consideration. The role of the endogenous trypsin-like enzymes in the effectiveness of the trypsin inhibitors produced by cultures of *Clostridium botulinum* is provocative of further study. In a study of this nature one might begin by determining whether or not the inhibitor is inactivated or "used up" during its action on trypsin or trypsin-like enzymes. Information of this nature might facilitate the development of a more sophisticated method of assay of trypsin inhibitor in culture fluids than was used in this experiment, i.e., one which would yield intelligible data from cultures containing appreciable amounts of endogenous proteolytic enzymes.

Another problem of interest would be to further substantiate the proposed mechanism of production of inhibitor by daily comparisons of amounts of extracellular and intracellular trypsin inhibitors in cultures. Extracellular trypsin inhibitors could be isolated for assay in the manner described in this thesis, i.e. by centrifugation. Intracellular trypsin inhibitors could be isolated for assay by washing the cells from the spun down culture with buffer. After the cells were washed one could resuspend the cells in an appropriate medium and disintegrate the cells by sonic means. The liberated intracellular material could then be assayed for trypsin inhibitors.

A last but most obvious recommendation is to isolate, purify, and chemically analyze the trypsin inhibitors produced by *C. botulinum*.

APPENDIX

Preparation of Solutions

Stock 3 percent Casein Solution

Casein, purified (Difco) . . . . 3 g.

Deionized water . . . . 100 ml.

NaOH, 10 N solution . . . . as needed.

The casein was added to the water in a 200 ml beaker on a magnetic stirrer. Base was added dropwise with stirring until the casein began to dissolve. Enough base was then added to bring the final pH to 7.6. The solution, placed into a screw-capped Erlenmeyer flask, was sterilized 10 minutes at 120 C and 15 psi of pressure, and stored at 4 C in the refrigerator.

Phosphate Buffer, pH 7.6

Aqueous Na_2HPO_4, 0.067 M . . . . 870 ml.

Aqueous KH_2PO_4, 0.067 M . . . . 130 ml.

The two phosphate solutions were mixed in a screw-capped Erlenmeyer flask, sterilized 15 minutes at 120 C and 15 psi of pressure, and stored at 4 C in the refrigerator.

Trypsin Solutions

Trypsin 1:250 (Difco) . . . . as indicated

PO_4 buffer, pH 7.6 . . . . q.s. to 100.0 ml.

The desired amount of trypsin was dissolved in buffer in a 100 ml volumetric flask. This solution was stored at 4 C in the refrigerator overnight before use. To insure good replication between identical

digestion mixtures, the trypsin solution was always mixed vigorously before removing an aliquot.

Statistical Analysis

Symbols Used in Statistical Analysis

k_b: the number of boxes.

k_c: the number of columns.

k_r: the number of rows.

n_1: degrees of freedom associated with $\hat{\sigma}_I^2$.

n_2: degrees of freedom associated with $\hat{\sigma}_I^2$.

N_b: number of items in a box.

N_c: number of items in a column.

N_r: number of items in a row.

Σ: upper-case Greek sigma, meaning "take the sum of."

$\sum_{1}^{k_b}$: a summation over the k_b boxes.

$\sum_{1}^{k_c}$: a summation over the k_c columns.

$\sum_{1}^{k_r}$: a summation over the k_r rows.

$\sum_{1}^{N}$: a summation over all items. Same as Σ.

$\sum_{1}^{N_b}$: a summation over the N_b items in a box.

$\sum_{1}^{N_c}$: a summation over the N_c items in a column.

$\sum_{1}^{N_r}$: a summation over the N_r items in a row.

X: an observed value.

Table 1. Computation of Values Required for Analysis of Variance of Data of the First Experiment on *Clostridium botulinum*, Type F, OPY-26

A. Observed Data and Sums for Columns and Rows

Day	Control	Experimental	$\sum_{1}^{N_r} X$
0	20.0 23.0 21.0	21.0 22.0 21.0	128.0
1	22.5 21.5 22.5	20.0 20.0 22.0	128.5
2	25.0 22.5 24.0	19.0 18.0 18.0	126.5
3	20.0 17.0 20.0	17.5 17.0 15.0	107.5
4	17.5 18.0 17.0	14.0 14.5 13.5	94.5
5	20.0 22.5 27.0	17.0 17.0 16.0	119.5
6	20.0 16.5 17.5	15.0 15.5 13.0	97.5
7	22.5 24.5 23.0	21.0 20.0 22.0	133.0
$\sum_{1}^{N_c} X$	505.0	430.0	935.0 = ΣX

Table 1. (Continued)

B. Squares and Sums for Columns and Rows

Day	Control	Experimental	$\sum_{1}^{N_r} X^2$
0	400.00 529.00 441.00	441.00 484.00 441.00	2736.00
1	506.25 462.25 506.25	400.00 400.00 484.00	2758.75
2	625.00 506.25 576.00	361.00 324.00 324.00	2716.25
3	400.00 289.00 400.00	306.25 289.00 256.00	1940.25
4	306.25 324.00 289.00	196.00 210.25 182.25	1507.75
5	400.00 506.25 729.00	289.00 289.00 256.00	2469.25
6	400.00 272.25 306.25	225.00 240.25 169.00	1612.75
7	506.25 600.25 529.00	441.00 400.00 484.00	2960.50
$\sum_{1}^{N_c} X^2$	10809.50	7892.00	18701.50 = ΣX^2

Table 1. (Concluded)

C. Sums and Squares of Sums for Boxes

Box		$\sum_{1}^{N_b} X$	$\left(\sum_{1}^{N_b} X\right)^2$
Row 0,	Col. 1	64.0	4096.00
	Col. 2	64.0	4096.00
Row 1,	Col. 1	66.5	4422.25
	Col. 2	62.0	3844.00
Row 2,	Col. 1	71.5	5112.25
	Col. 2	55.0	3025.00
Row 3,	Col. 1	57.0	3249.00
	Col. 2	50.5	2550.25
Row 4,	Col. 1	52.5	2756.25
	Col. 2	42.0	1764.00
Row 5,	Col. 1	69.5	4830.25
	Col. 2	50.0	2500.00
Row 6,	Col. 1	54.0	2916.00
	Col. 2	43.5	1892.25
Row 7,	Col. 1	70.0	4900.00
	Col. 2	63.0	3969.00
TOTAL		935.0	55922.50 $= \sum_{1}^{k_b} \left(\sum_{1}^{N_b} X\right)^2$

Computation of Variations of Data of the First Experiment on Clostridium botulinum, Type F, OPY-26

$$\text{Total Variation} = \Sigma X^2 - \frac{(\Sigma X)^2}{N}$$

$$18701.50 - \frac{(935.0)^2}{48} = 488.48$$

$$\text{Variation Between Column Means} = \frac{\sum_{1}^{k_c}\left(\sum_{1}^{N_c} X\right)^2}{N_c} - \frac{(\Sigma X)^2}{N}$$

$$\frac{(505.0)^2 + (430.0)^2}{24} - \frac{(935.0)^2}{48} = 117.18$$

$$\text{Variation Between Row Means} = \frac{\sum_{1}^{k_r}\left(\sum_{1}^{N_r} X\right)^2}{N_r} - \frac{(\Sigma X)^2}{N}$$

$$\frac{(128.0)^2 + (128.5)^2 + (126.5)^2 + (107.5)^2 + (94.5)^2 + (119.5)^2 + (97.5)^2}{6} + \frac{(133.0)^2}{6} - \frac{(935.0)^2}{48} = 3.34$$

$$\text{Variation Within the Boxes} = \Sigma X^2 - \frac{\sum_{1}^{k_b}\left(\sum_{1}^{N_b} X\right)^2}{N_b}$$

$$18701.50 - \frac{55,922.50}{3} = 60.67$$

Computation of Interaction

Interaction may be computed by subtracting variation between column means, variation between row means, and variation within boxes from total variation.

Table 2. Summary of Amounts of Variation, Degrees of Freedom, and Estimated Variances for each Source of Variation of Data of The First Experiment on Clostridium botulinum, Type F, OPY-26.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance
Between Column Means	117.18	1	117.18
Between Row Means	3.34	7	0.47
Interaction	307.29	7	12.24
Within Boxes	60.67	32	4.33
TOTAL	488.48	47	...

$$488.48 - (117.18 + 3.34 + 60.67) = 307.29$$

Computation of Estimated Variances

The estimated variance may be computed by dividing the variation by the degrees of freedom for that source of variation. (See Table 2)

Tests of Variances for Significance

Test of Interaction for Significance

$$F = \frac{12.24}{4.33} = 2.82 \quad n_1 = 7, \quad n_2 = 32$$

Probability of $F \geqq 2.82$ is < 0.025

Therefore interaction is significant.

Test of Estimated Variance between Column means for Significance

$$F = \frac{117.18}{12.24} = 9.57 \quad n_1 = 1, \quad n_2 = 7$$

Probability of $F \geqq 9.57$ is < 0.025

Therefore estimated variance is significant.

Test of Estimated Variance between Row means for Significance

$$F = \frac{0.47}{12.24} = 0.038 \quad n_1 = 7, \quad n_2 = 7$$

Probability of $F \geqq 0.038$ is > 0.1

Therefore estimated variance is not significant.

Table 3. Computation of Values Required for Analysis of Variance of Data of the Second Experiment on *Clostridium botulinum*, Type F, OPY-26.

A. Observed Data and Sums for Columns and Rows

Day	Control	Experimental	$\sum_{1}^{N_r} X$
0	20.0 19.5 20.0	19.5 19.5 19.0	117.5
1	21.0 19.5 18.5	16.0 17.0 16.5	108.5
2	18.0 18.0 17.0	15.0 13.5 14.5	96.0
3	19.0 18.0 19.0	15.5 14.0 17.5	103.0
4	17.0 19.0 17.0	14.5 14.5 13.0	95.0
5	20.0 21.0 19.0	15.5 15.5 15.5	106.5
6	18.5 20.0 18.5	18.0 16.0 16.5	107.5
7	18.5 17.5 19.5	15.5 15.5 15.5	100.5
$\sum_{1}^{N_c} X$	452.5	383.5	836.0 = ΣX

Table 3. (Continued)

B. Squares and Sums for Columns and Rows

Day	Control	Experimental	$\sum_{1}^{N_r} X^2$
0	400.00 380.25 400.00	380.25 380.25 361.00	2301.75
1	441.00 380.25 342.25	256.00 289.00 272.25	1980.75
2	324.00 324.00 289.00	225.00 182.25 210.25	1554.50
3	361.00 324.00 361.00	240.25 196.00 306.25	1788.50
4	289.00 361.00 289.00	210.25 210.25 169.00	1528.50
5	400.00 441.00 361.00	240.25 240.25 240.25	1922.75
6	342.25 400.00 342.25	324.00 256.00 272.25	1936.75
7	342.25 306.25 361.00	240.25 240.25 240.25	1730.25
$\sum_{1}^{N_c} X^2$	8561.75	6182.00	14743.75 = ΣX^2

Table 3. (Concluded)

C. Sums and Squares of Sums for Boxes

Box	$\sum_{1}^{N_b} X$	$(\sum_{1}^{N_b} X)^2$
Row 0, Col. 1	59.5	3540.25
Col. 2	58.0	3365.00
Row 1, Col. 1	59.0	3481.00
Col. 2	49.5	2450.25
Row 2, Col. 1	53.0	2809.00
Col. 2	43.0	1849.00
Row 3, Col. 1	56.0	3136.00
Col. 2	47.0	2209.00
Row 4, Col. 1	53.0	2809.00
Col. 2	42.0	1764.00
Row 5, Col. 1	60.0	3600.00
Col. 2	46.5	2162.25
Row 6, Col. 1	57.0	3249.00
Col. 2	50.5	2550.25
Row 7, Col. 1	54.0	2916.00
Col. 2	46.5	2162.25
TOTAL	834.5	44051.25 $= \sum_{1}^{k_b} (\sum_{1}^{N_b} X)^2$

<u>Computation of Variations of Data of the Second Experiment on Clostridium botulinum, Type F, OPY-26</u>.

Total Variation = $\Sigma X^2 - \frac{(\Sigma X)^2}{N}$

$$14743.75 - \frac{(836.0)^2}{48} = 183.42$$

Variation Between Column Means = $\frac{\sum_{1}^{k_c}\left(\sum_{1}^{N_c} X\right)^2}{N_c} - \frac{(\Sigma X)^2}{N}$

$$\frac{(452.5)^2 + (383.5)^2}{24} - \frac{(836.0)^2}{48} = 99.19$$

Variation Between Row Means = $\frac{\sum_{1}^{k_r}\left(\sum_{1}^{N_r} X\right)^2}{N_r} - \frac{(\Sigma X)^2}{N}$

$$\frac{(117.5)^2 + (108.5)^2 + (96.0)^2 + (103.0)^2 + (95.0)^2 + (106.5)^2 + (107.5)^2}{6} + \frac{(100.5)^2}{6} - \frac{(836.0)^2}{48} = 10.87$$

Variation Within the Boxes = $\Sigma X^2 - \frac{\sum_{1}^{k_b}\left(\sum_{1}^{N_b} X\right)^2}{N_b}$

$$14,743,75 - \frac{44,051.25}{3} = 60.00$$

<u>Computation of Interaction</u>

Interaction may be computed by subtracting variation between column means, variation between row means, and variation within boxes from total variation.

$$183.42 - (99.19 + 10.87 + 60.00) = 13.36$$

Computation of Estimated Variances

The estimated variance may be computed by dividing the variation by the degrees of freedom for that source of variation (see Table 4).

Tests of Variances for Significance

Test of Interaction for Significance

$$F = \frac{0.53}{4.28} = 0.124 \quad n_1 = 7, \quad n_2 = 32$$

Probability of $F \geqq 0.124$ is > 0.1

Therefore interaction is not significant.

Combined Variation within boxes and Variation due to interaction:

$$13.36 + 60.00 = 73.36 \quad n = 39$$

Combined estimated variance = 1.88

Test of Estimated Variance between Row means against combined estimated variance of within boxes variation and variation due to interaction for significance.

$$F = \frac{1.55}{1.88} = 0.83 \quad n_1 = 7, \quad n_2 = 39$$

Probability of $F \geqq 0.83$ is < 0.1

Therefore differences are not significant.

Test of Estimated Variance between Column means against combined estimated variance of within boxes variation and variation due to interaction for significance.

$$F = \frac{99.19}{1.88} = 52.7 \quad n_1 = 1 \quad n_2 = 39$$

Probability of $F \geqq 52.7$ is < 0.001

Therefore differences are significant.

Table 4. Summary of Amounts of Variation, Degrees of Freedom, and Estimated Variances for each Source of Variation of Data of the Second Experiment on *Clostridium botulinum*, Type F, OPY-26.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance
Between Column Means	99.19	1	99.19
Between Row Means	10.87	7	1.55
Interaction	13.36	7	0.53
Within Boxes	60.00	32	4.28
TOTAL	183.42	47	...

Table 5. Computation of Values Required for Analysis of Variance of Data of the First Experiment on Clostridium botulinum, Type F, TOX-IB.

A. Observed Data and Sums for Columns and Rows

Day	Control	Experimental	$\sum_{1}^{N_r} X$
0	20.5 20.5 20.5	24.0 21.0 20.5	127.0
1	17.0 14.0 14.0	6.5 6.5 6.0	64.0
2	20.0 20.0 18.0	15.0 13.0 14.0	100.0
3	17.0 20.0 18.0	13.5 12.0 13.5	94.0
4	18.0 18.0 15.0	10.0 11.0 10.5	82.5
5	16.0 16.0 15.5	11.0 10.0 11.0	79.5
6	11.0 11.0 11.0	11.0 7.0 7.0	58.0
10	13.0 14.0 14.0	9.0 10.0 10.0	70.0
$\sum_{1}^{N_c} X$	392.0	283.0	675.0 = ΣX

Table 5. (Continued)

B. Squares and Sums for Columns and Rows

Day	Control	Experimental	$\sum_{1}^{N_r} X^2$
0	420.25 420.25 420.25	576.00 441.00 420.25	2698.00
1	289.00 196.00 196.00	42.25 42.25 36.00	801.50
2	400.00 400.00 324.00	225.00 169.00 196.00	1714.00
3	289.00 400.00 324.00	182.25 144.00 182.25	1521.50
4	324.00 324.00 225.00	100.00 121.00 110.25	1204.25
5	256.00 256.00 240.25	121.00 100.00 121.00	1094.25
6	121.00 121.00 121.00	121.00 49.00 49.00	582.00
10	169.00 196.00 196.00	81.00 100.00 100.00	842.00
$\sum_{1}^{N_c} (X)^2$	6628.00	3829.50	10457.50 = ΣX^2

Table 5. (Concluded)

C. Sums and Squares of Sums for Boxes

Box		$\sum_{1}^{N_b} X$	$\sum_{1}^{N_b} X^2$
Row 0,	Col. 1	61.5	3782.25
	Col. 2	65.5	4290.25
Row 1,	Col. 1	45.0	2025.00
	Col. 2	19.0	361.00
Row 2,	Col. 1	58.0	3364.00
	Col. 2	42.0	1764.00
Row 3,	Col. 1	55.0	3025.00
	Col. 2	39.0	1521.00
Row 4,	Col. 1	51.0	2601.00
	Col. 2	31.5	992.25
Row 5,	Col. 1	47.5	2256.25
	Col. 2	32.0	1024.00
Row 6,	Col. 1	33.0	1089.00
	Col. 2	25.0	625.00
Row 7,	Col. 1	41.0	1681.00
	Col. 2	29.0	841.00
TOTAL		675.0	31,242.00 $= \sum_{1}^{k_b} \left(\sum_{1}^{N_b} X\right)^2$

Computation of Variations of Data of the First Experiment on Clostridium botulinum, Type F, TOX-IB.

$$\text{Total Variation} = \Sigma X^2 - \frac{(\Sigma X)^2}{N}$$

$$10,457.50 - \frac{(675.0)^2}{48} = 965.32$$

$$\text{Variation Between Column Means} = \frac{\sum_{1}^{k_c} \left(\sum_{1}^{N_c} X \right)^2}{N_c} - \frac{(\Sigma X)^2}{N}$$

$$\frac{(392.0)^2 + (283.0)^2}{24} - \frac{(675.0)^2}{48} = 247.52$$

$$\text{Variation Between Row Means} = \frac{\sum_{1}^{k_r} \left(\sum_{1}^{N_r} X \right)^2}{N_r} - \frac{(\Sigma X)^2}{N}$$

$$\frac{(127.0)^2 + (64.0)^2 + (100.0)^2 + (94.0)^2 + (82.5)^2 + (79.5)^2 + (58.0)^2}{6}$$

$$+ \frac{(70.0)^2}{6} - \frac{(675.0)^2}{48} = 582.77$$

$$\text{Variation Within the Boxes} = \Sigma X^2 - \frac{\sum_{1}^{k_b} \left(\sum_{1}^{N_b} X \right)^2}{N_b}$$

$$10457.50 - \frac{31242.00}{3} = 43.5$$

Computation of Interaction

Interaction may be computed by subtracting variation between column means, variation between row means, and variation within boxes from the total variation.

$$965.32 - (247.52 + 582.77 + 43.5) = 91.53$$

Computation of Estimated Variances

The estimated variance may be computed by dividing the variation by the degrees of freedom for that source of variation (see Table 6).

Tests of Variances for Significance

Test of Interaction for Significance

$$F = \frac{13.07}{1.35} = 9.68 \quad n_1 = 7, \quad n_2 = 32$$

Probability of $F \geqq 9.68$ is < 0.001

Therefore interaction is significant.

Test of Estimated Variance between Column means for significance

$$F = \frac{247.52}{13.07} = 18.93 \quad n_1 = 1, \quad n_2 = 7$$

Probability of $F \geqq 18.93$ is < 0.01

Therefore the Estimated Variance is Significant.

Test of Estimated Variance between Row means for Significance.

$$F = \frac{83.25}{13.07} = 6.36 \quad n_1 = 7, \quad n_2 = 7$$

Probability of $F \geqq 6.36$ is < 0.025

Therefore the Estimated Variance significant.

Table 6. Summary of Amounts of Variation, Degrees of Freedom, and Estimated Variances for each Source of Variation of Data of the First Experiment on *Clostridium botulinum*, Type F, TOX-IB.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance
Between Column Means	247.52	1	247.52
Between Row Means	582.77	7	83.25
Interaction	91.53	7	13.07
Within Boxes	43.50	32	1.35
TOTAL	965.32	47	...

Table 7. Computation of Values Required for Analysis of Variance of Data of the Fourth Experiment on Clostridium botulinum, Type F, TOX-IB.

A. Observed Data and Sums for Columns and Rows

Day	Control	Experimental	$\sum_{1}^{N_r} X$
0	23.5 23.5 25.5	23.5 22.5 22.5	141.0
1	20.0 21.0 24.0	22.0 22.0 23.0	132.0
2	15.0 15.0 15.0	18.0 18.5 19.5	101.0
3	20.0 21.5 20.5	24.0 24.0 24.0	134.0
4	19.0 21.0 17.5	16.0 17.0 18.0	108.5
5	17.5 17.0 18.0	19.5 19.0 18.5	109.5
6	17.0 16.5 19.5	17.0 16.5 16.5	103.0
7	13.5 14.0 13.0	16.0 16.0 16.0	88.5
$\sum_{1}^{N_c} X$	448.0	469.5	917.5 = ΣX

Table 7. (Continued)

B. Squares and Sums for Columns and Rows

Day	Controls	Experimentals	$\sum_{1}^{N_r} X^2$
0	552.25 552.25 650.25	552.25 506.25 506.25	3319.50
1	400.00 441.00 576.00	484.00 484.00 529.00	2914.00
2	225.00 225.00 225.00	324.00 342.25 380.25	1721.50
3	400.00 462.25 420.25	576.00 576.00 576.00	3010.50
4	361.00 441.00 306.25	256.00 289.00 324.00	1977.25
5	306.25 289.00 324.00	380.25 361.00 342.25	2002.75
6	289.00 272.25 380.25	289.00 272.25 272.25	1775.00
7	182.25 196.00 169.00	256.00 256.00 256.00	1315.25
$\sum_{1}^{N_c} X^2$	8645.50	9390.25	18035.75 = ΣX^2

Table 7. (Concluded)

C. Sums and Squares of Sums for Boxes

Box		$\sum_{1}^{N_b} X$	$\left(\sum_{1}^{N_b} X\right)^2$
Row 0,	Col. 1	72.5	5256.25
	Col. 2	68.5	4692.25
Row 1,	Col. 1	65.0	4225.00
	Col. 2	67.0	4489.00
Row 2,	Col. 1	45.0	2025.00
	Col. 2	56.0	3136.00
Row 3,	Col. 1	62.0	3844.00
	Col. 2	72.0	5184.00
Row 4,	Col. 1	67.5	3306.25
	Col. 2	51.0	2601.00
Row 5,	Col. 1	52.5	2756.25
	Col. 2	57.0	3249.00
Row 6,	Col. 1	53.0	2809.00
	Col. 2	50.0	2500.00
Row 7,	Col. 1	40.5	1640.25
	Col. 2	48.0	2304.00
TOTAL		917.5	54017.25 $= \sum_{1}^{k_b} \left(\sum_{1}^{N_b} X\right)^2$

Computation of Variations of Data of the Fourth Experiment on Clostridium botulinum, Type F, TOX-IB.

Total Variation = $\Sigma X^2 - \frac{(\Sigma X)^2}{N}$

$$18,035.75 - \frac{(917.5)^2}{48} = 498.12$$

Variation Between Column Means = $\frac{\sum_{1}^{k_c} \left(\sum_{1}^{N_c} X \right)^2}{N_c} - \frac{(\Sigma X)^2}{N}$

$$\frac{(448.0)^2 + (469.5)^2}{24} - \frac{(917.5)^2}{48} = 9.63$$

Variation Between Row Means = $\frac{\sum_{1}^{k_r} \left(\sum_{1}^{N_r} X \right)^2}{N_r} - \frac{(\Sigma X)^2}{N}$

$$\frac{(141.0)^2 + (132.0)^2 = (101.0)^2 + (134.0)^2 + (108.5)^2 + (109.5)^2 + (103.0)^2}{6}$$

$$+ \frac{(88.5)^2}{6} - \frac{(971.5)^2}{48} = 406.66$$

Variation withinthe Boxes = $\Sigma X^2 - \frac{\sum_{1}^{k_b} \left(\sum_{1}^{N_b} X \right)^2}{N_b}$

$$18035.75 - \frac{54017.25}{3} = 30.00$$

Computation of Interaction

Interaction may be computed by subtracting variation between column means, variation between row means, and variation within boxes from total variation.

Table 8. Summary of Amount of Variation, Degrees of Freedom, and Estimated Variance for Each Source of Variation of the Data of the Fourth Experiment on *Clostridium botulinum*, Type F, TOX-IB.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance
Between Column Means	9.63	1	9.63
Between Row Means	406.66	7	58.09
Interaction	51.83	7	7.44
Within Boxes	30.00	32	0.94
TOTAL	498.12	47	...

$$498.12 - (9.63 + 406.66 + 30.00) = 51.83$$

Computation of Estimated Variances

The estimated variance may be computed by dividing the variation by the degrees of freedom for that source of variation (see Table 8).

Test of Variances for Significance

Test of Interaction for Significance

$$F = \frac{7.44}{0.94} = 7.91 \quad n_1 = 7, \; n_2 = 32$$

Probability of $F \geqq 7.91$ is < 0.001

Therefore interaction is significant

Test of Estimated Variance between Column means for significance.

$$F = \frac{9.63}{7.44} = 1.29 \quad n_1 = 1, \quad n_2 = 7$$

Probability of $F \geqq 1.29$ is > 0.10

Therefore the Estimated Variance is not significant.

Test of Estimated Variance Between Row means for Significance.

$$F = \frac{58.09}{7.44} = 7.8 \quad n_1 = 7, \quad n_2 = 7$$

Probability of $F \geqq 7.8$ is < 0.01

Therefore the estimated variance is significant.

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* Abbreviations used here follow the form employed by World List of Scientific Periodicals 1900-1960, 4th ed. Butterworths, Washington, 1965.