An approach to a computer-analyzed bioassay using axenic cultures of the protozoa Tetrahymena pyriformis

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AN APPROACH TO A COMPUTER-ANALYZED BIOASSAY USING AXENIC CULTURES OF THE PROTOZOA *Tetrahymena pyriformis*

by

Gerald Kane O'Bryan

B.S. Bradley University, 1966

Presented in partial fulfillment of the requirements for the degree of Master of Science in Zoology

UNIVERSITY OF MONTANA

1968

Approved by:

[Signature]

Chairman, Board of Examiners

[Signature]

Dean, Graduate School

August 6, 1968

Date
I wish to thank the following persons for their help during the course of this study:

Dr. W. B. Rowan for suggesting the original problem and guiding the initial stages;

Dr. A. G. Canaris for his able guidance of the terminal stages of this study and his many constructive criticisms;

Dr. M. J. Nakamura for suggesting the sterility tests;

Dr. H. E. Reinhardt for extensive advice on the use of various statistics and the derivation of the calculation formulae in the analysis of covariance for use with a weighted regression;

Dr. R. S. Hoffmann for his advice and encouragement.

Special thanks are due Dr. R. P. Banaugh and the entire staff of the University of Montana Computer Center. Without the advice and many hours of machine time afforded me, much of this study would have been impossible.

Any errors in the statistics and the computer programs used are my responsibility.

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**Please Note: This dissertation is not a publication, and no material herein may be used without the written consent of the author and the Department of Zoology.
INTRODUCTION

Most protozoan bioassays are based directly or indirectly on the growth curve of a population. While investigating the utility of cultures of protozoa for an insecticide assay, a question was raised concerning the possible use of the slope of the population growth curve as an indicator. The idea was to compare the mean growth curve slopes from an assay with the mean slopes of populations of cultures grown with known concentrations of a chemical standard. This might allow more precise interpretations than the qualitative assays described in the literature. The reasoning was that any change in the growth of a population of protozoa would be reflected in the slope of the curve describing the growth of that population. If the natural growth curve under ideal conditions was known, the use of an appropriate statistical analysis would allow the detection of natural variations in growth. That the use of the slope should allow a more quantitative description of the cause and effect relationship concerning the assayed material was considered important because of the nature of the materials to be assayed. Also, this method would allow the comparison of interlaboratory results without extensive interpretation because the results of an assay would be described in terms of a known chemical standard.

Axenic cultures of Tetrahymena pyriformis were selected for use in this study. The choice was prompted by the extensive utilization of this organism in current research.

An investigation of the available literature for assays utilizing T. pyriformis disclosed several that appeared to be reasonably good.
 Those described by Baker et al. (1966), Epstein et al. (1965), Epstein, Saporoschetz, and Hutner (1967), Nardone and Blaszczynski (1954), and Seaman (1953) are typical. None of the above assays is based on the slope of the observed growth curve per se. This would not be possible because the methods that were used did not compensate for counting errors and natural variation in the growth of the cultures. Unless the above errors are considered and accounted for, no bioassay can be any better than qualitative.

The literature also indicated that most of the studies which described the growth curve of T. pyriformis were for culture volumes much larger than those which would be convenient to use in an assay. There were variations in the described curves which appeared to be associated with physiological differences between the strains of T. pyriformis that were studied. The above information indicated that the mean growth curve of T. pyriformis would have to be determined under the conditions of this study.

Since pilot studies indicated that the data from the cultures appeared to be in a linear relationship, a linear regression analysis was used to obtain an approximation of the growth curves. The slopes of the regression lines were used as the slope of their respective growth curves. This decision also simplified the statistical analysis that was required to detect natural variation in the growth of the cultures.

The problems associated with the definition of an estimate of the mean natural growth curve required a change in the original objectives.
of this study. The main question which had to be answered prior to any actual assay work was: What was the regression line estimate of the mean growth curve of \textit{T. pyriformis} under the conditions of this study? This paper is primarily concerned with the answer to this question.

Also discussed is a subsequent pilot assay used to determine the effect of daily sample removal and the utility of an analysis of covariance for detecting natural variation in the population of the cultures.
MATERIALS AND METHODS

I. Culture Source.

An axenic culture of *Tetrahymena pyriformis*, variety Hall, was obtained in June, 1966, from Dr. W. H. Furgason, Department of Zoology, University of California at Los Angeles. This culture has been maintained through biweekly subculturing since arrival.

II. Culturing Techniques.

Three liquid media were used in this study. These were Loefer's (Loefer, Small, and Furgason, 1966), N.I.H. Thioglycollate, and Sabouraud (Difco Manual B-256 and B-382, 1953).

Loefer's medium was used for growing the stock and test cultures of *T. pyriformis*. The stock cultures were grown in 16 x 120 mm screw cap culture tubes containing 10 ml of medium. The cultures were placed in a BOD incubator at 15°C after inoculation. The test cultures were grown in 22 x 180 mm test tubes equipped with slip-on aluminum caps. The tubes contained 27 ml of medium and were placed in a BOD incubator at 25°C after inoculation. The medium was prepared, placed in culture tubes, autoclaved, and frozen. The tubes of medium were thawed as needed and held at room temperature for eight hours prior to use.

The other two media used in this study, N.I.H. Thioglycollate and Sabouraud, were used for sterility checks of the stock and test cultures. The media were obtained from Difco Laboratories, Inc., Detroit, Michigan, as a ready mixed powder, and required the addition of distilled water only. The media were used in the 16 x 120 mm culture...
tubes. The culture tubes contained 10 ml of medium. After inoculation, the tubes were placed in BOD incubator at 25°C for 24 hours. The two media were prepared, placed in culture tubes, autoclaved, and stored in the dark at room temperature. Preparation was carried out no more than one week prior to the intended date of use.

All distilled water used for the preparation of the various media was passed through an ion exchange demineralizer. This was done to reduce the possibility of copper contamination.

Subculturing of the stock cultures was done with a bacteriological loop. The contents of three loop transfers were considered a sufficient inoculum.

A specially prepared subculture was used to furnish the inocula for the test cultures. This preparation method was used to acclimate the protozoa to the 25°C temperature that was used for growing test cultures. A standard-sized stock subculture was prepared as described previously. The subculture was grown at 25°C for 5 days. A sterile pipette was used to withdraw 0.5 ml of medium from this subculture. The medium was used as the inoculum for a large culture tube of medium. Prior to inoculation 0.5 ml of sterile demineralized distilled water was added to the tube of medium. This was done to make the final culture volume 28 ml. This large subculture was grown for 5 days in a BOD incubator at 25°C. At the end of the 5 days this subculture was used to furnish inocula for the test cultures.

The test cultures were inoculated with 0.5 ml of medium withdrawn from the large subculture that was described above. Sterile pipettes
were used to withdraw the inocula. As for the subculture that furnished the inocula, the total volume of the test cultures was 28 ml.

The sterility-check cultures were inoculated with 0.1 ml of medium that was withdrawn with a sterile pipette from the culture which was to be checked. One tube of each check media was inoculated for each culture that was checked. The checks were made on all special stock subcultures and test cultures immediately after their final use in the study. Any cultures that were found to be contaminated were discarded.

III. Data Collection Techniques.

The data used to determine the regression line estimates of the growth curves for the test cultures were obtained by withdrawing 1 ml samples from the cultures every 24 hours after inoculation. The samples were removed with a sterile pipette and placed in individual glass vials. The vials contained 2 ml of a fixing solution composed of 0.7% NaCl and 5% formalin in distilled water. This solution served to make the counting process easier by killing the protozoa, lowering their concentration, and allowing the counts to be made at convenience. The saline fraction was used to prevent osmotic lysis. Prior to removal of the counting samples, this mixture was swirled to resuspend the protozoa.

Two 1 ml counting samples were removed from the above mixture and the number of protozoa in 30 fields was determined and recorded for each milliliter. The counts were made using the Sedgwick-Rafter plankton counting method as outlined in Standard Methods for the Examination of
Water and Wastewater, 1965. A "field" was the area enclosed by the outer lines on the Whipple-Hauser ocular micrometer that was used with this method. The sum of the protozoa counted in the 60 fields examined was the unconverted method estimate of the number in two-thirds of the sample from the culture. This sum is not the actual number of protozoa in the two-thirds of a milliliter (see the method description, loc. cit.). The number of protozoa found in the 60 fields examined was used in the calculations performed in this study. This was done because of the more manageable magnitude of these numbers.

The subcultures that were used to inoculate the test cultures were sampled and counted as described above. This was done to obtain an estimate of the number of protozoa in 60 fields from the test cultures immediately after inoculation. This estimate was obtained by multiplying the sum of the protozoa counted in 60 fields by the dilution factor (the inoculum volume divided by the final culture volume). This value was used as the Day 1 sum of the protozoa in 60 fields for the test cultures.

All cultures were swirled prior to sample or inoculum removal to obtain a uniform distribution of the protozoa in the medium.

IV. Test Cultures.

A group of 20 cultures was inoculated to obtain data for the determination of the regression line estimate of the mean growth curve. The data were obtained from the counts made on a sample withdrawn from each culture every 24 hours after inoculation. The cultures were sampled for 8 days after inoculation.
For the pilot assay, a group of 29 cultures was used. This group was subdivided into 1 set of 8 and 7 sets of 3 cultures. The set of 8 was the control and furnished the data that were used in the determination of the regression line estimate of the mean growth curve for the assay. This set also served as the link which would allow comparisons of the results of the assay with the original regression line estimate of the mean growth curve that was obtained using the first group of 20 cultures. The 7 sets of 3 cultures were the assay cultures that were used to determine the effects of daily sample removal. The effect of the reduction of the volume of the cultures by the daily removal of 1 ml samples was the primary concern. This determination was made using the data that were obtained from counts of samples which were removed on a staggered sampling schedule (Table 1).

TABLE 1
SCHEDULE OF SAMPLE REMOVAL FOR THE TEST OF SAMPLING EFFECT

<table>
<thead>
<tr>
<th>Day</th>
<th>Sample Set</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>s s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>s s s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>s s s s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>s s s s s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>s s s s s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>s s s s s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>s s s s s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
Set 1 contained 8 cultures, and all other sets contained 3 cultures. The "s" indicates a sample was removed from the cultures in a set on the day indicated.
The utility of the analysis of covariance was determined by pooling all of the data from the determination of the regression line estimate of the mean growth curve with the data from the pilot assay. These pooled data were tested in various groupings with the analysis of covariance. Cultures, which lacked data as the result of the staggered sample removals, were assigned 60 field sums of 0 for the days data were missing.

V. **Statistical Analysis.**

The method for the determination of counting accuracy was that of Moore (1952). This method determined the per cent standard deviation of the mean count per field for the counts that were obtained by the use of the Sedgwick-Rafter method. This analysis was used on all counts that were made in this study. A modification of this method was used to obtain an estimate of the error in pipette and counting chamber volumes (see Appendix).

The method of Dixon and Massey (p. 128, 1957) was used to determine the upper and lower confidence limits for the mean count per field. This analysis was used on all counts that were made in this study. The limits were determined at the 5% level of significance ($\alpha = .95$).

The weighted analysis method of Ostle (p. 127, 1963) was used to determine weighted regression line estimates of the mean growth curves for the various groups of cultures that were used in this study. This method also provided individual culture regression line estimates. The weights for the analysis were based on the total error. Total error consisted of the sums of the counting, pipette volume, and counting chamber
volume errors. The conversion table that was used for the determination of the weights is given in Table 2.

Ostle's analysis of covariance (p. 201, 1963) was adapted for use with a weighted regression analysis by Dr. H. E. Reinhardt (Department of Mathematics, University of Montana). This analysis was used to test the various regression line estimates of growth curves. The main question which this analysis answered was whether all of the lines in a group were estimates of one mean line. For a full description of the hypotheses tested, see the Appendix, Program Part 4B. This analysis was performed at the 5% level of significance ($\alpha = .95$).

The percentiles of the F distribution for the above analysis were determined by the method of Dixon and Massey (p. 402, 1957). Values which could not be determined accurately using this method were obtained from the tables in the same source.

**TABLE 2**

ERROR TO WEIGHT CONVERSION TABLE

<table>
<thead>
<tr>
<th>Total Error Range</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% - 3%</td>
<td>3.0</td>
</tr>
<tr>
<td>3% - 10%</td>
<td>5.0</td>
</tr>
<tr>
<td>10% - 20%</td>
<td>4.0</td>
</tr>
<tr>
<td>20% - 30%</td>
<td>3.0</td>
</tr>
<tr>
<td>30% - 40%</td>
<td>2.0</td>
</tr>
<tr>
<td>40% - 50%</td>
<td>1.0</td>
</tr>
<tr>
<td>50% - 100%</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Notes:

Total error was never less than 2%. This was the value for the combined pipette and counting chamber errors. Decimal parts over the maximum in any range placed that error into the next higher range.
The computer programs that were used for evaluating the above statistical analyses are listed in the Appendix.
RESULTS

Four of the 20 cultures grown for the determination of the regression line estimate of the mean growth curve were found to be contaminated and discarded. The remaining cultures were assigned the numbers 1-16. The starting number of protozoa in cultures 1-16 was calculated to be 5.

The equation of the regression line for cultures 1-16 is given and graphed in Figure 1. The ranges of the sums of the 60 fields that were counted each day are also given. The mean daily count per field on Day 5 for cultures 1-16 was 2.81 protozoa. The mean confidence limits for this value were 5.50 for the upper limit, and 0.11 for the lower limit. The analysis of covariance indicated that the regression lines for each culture were estimates of the mean regression line as given in Figure 1.

In the pilot assay, one of the control cultures was contaminated. This was discarded, and the remaining cultures assigned the numbers 17-23. None of the cultures in the assay sets were contaminated. The cultures in the assay sets were assigned numbers 24-44 as the sets were sampled. The starting number of protozoa in the cultures, which were used in the pilot assay, was 2 per culture.

The regression line estimate of the mean growth curve for cultures 17-23 is given in Figure 2. The mean daily count per field for the cultures was 2.28 protozoa. The mean upper confidence limit was 4.53 and the mean lower limit was 0.04.
The regression line estimate for cultures 24-44 was not calculated. The mean daily count per field for the cultures in this group was 2.32 protozoa. The confidence limits were 4.87 for the upper limit and -0.24 for the lower limit.

The analysis of covariance was tested by pooling the above data and grouping these data as follows:

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled Data Grouping</td>
</tr>
<tr>
<td>Group 1 -- Cultures 1-16</td>
</tr>
<tr>
<td>Group 2 -- Cultures 17-23</td>
</tr>
<tr>
<td>Group 3 -- Cultures 1-23</td>
</tr>
<tr>
<td>Group 4 -- Cultures 17-44</td>
</tr>
<tr>
<td>Group 5 -- Cultures 1-44</td>
</tr>
</tbody>
</table>

The slopes and intercepts calculated for the above groups are given in Table 4. The regression line estimate of the mean growth curve of cultures 1-23, with the range of the sums of the protozoa in 60 fields per day, is given and graphed in Figure 3. This line is the estimate of the pooled mean growth curve.

The analysis of covariance, which was performed on each group given in Table 3, indicated that the cultures in the above groups were estimates of one mean regression line.
Figure 1—the mean regression line for cultures 1-16.

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max.</td>
<td>5</td>
<td>39</td>
<td>91</td>
<td>137</td>
<td>183</td>
<td>195</td>
<td>260</td>
<td>279</td>
<td>313</td>
</tr>
<tr>
<td>Mean</td>
<td>5</td>
<td>31</td>
<td>76</td>
<td>121</td>
<td>147</td>
<td>168</td>
<td>211</td>
<td>241</td>
<td>280</td>
</tr>
<tr>
<td>Min.</td>
<td>5</td>
<td>21</td>
<td>64</td>
<td>97</td>
<td>104</td>
<td>153</td>
<td>182</td>
<td>213</td>
<td>250</td>
</tr>
</tbody>
</table>

Daily Ranges are in terms of the Sixty Field Totals. The value for Day 1 is the calculated starting number.
$Y = 32.41X - 28.01$
Figure 2—the mean regression line for cultures 17-23.

Daily Data Range

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max.</td>
<td>2</td>
<td>20</td>
<td>77</td>
<td>123</td>
<td>139</td>
<td>146</td>
<td>167</td>
<td>209</td>
<td>262</td>
</tr>
<tr>
<td>Mean</td>
<td>2</td>
<td>15</td>
<td>64</td>
<td>107</td>
<td>122</td>
<td>137</td>
<td>155</td>
<td>194</td>
<td>241</td>
</tr>
<tr>
<td>Min.</td>
<td>2</td>
<td>11</td>
<td>48</td>
<td>96</td>
<td>100</td>
<td>132</td>
<td>142</td>
<td>181</td>
<td>207</td>
</tr>
</tbody>
</table>

Daily Ranges are in terms of the Sixty Field Totals. The value for Day 1 is the calculated starting number.
$Y = 28.55X - 27.20$
<table>
<thead>
<tr>
<th>Value</th>
<th>Min.</th>
<th>2</th>
<th>48</th>
<th>96</th>
<th>100</th>
<th>132</th>
<th>142</th>
<th>178</th>
<th>207</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>3*</td>
<td>29</td>
<td>72</td>
<td>117</td>
<td>134</td>
<td>160</td>
<td>174</td>
<td>194</td>
<td>227</td>
</tr>
<tr>
<td>Max.</td>
<td>5</td>
<td>91</td>
<td>279</td>
<td>313</td>
<td>379</td>
<td>260</td>
<td>183</td>
<td>137</td>
<td>117</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
</table>

---

Values

The value for Day 1 is the mean of the calculated starting values. Daily ranges are in terms of the sixty field totals. Rounded to the nearest whole protozoa.

---

Daily Data Range

---

Figure 3: The mean regression line for cultures 1-23.
$Y = 34.09X - 28.37$
### TABLE 4
MEAN GROWTH CURVE REGRESSION LINE ESTIMATES

<table>
<thead>
<tr>
<th>Group</th>
<th>Cultures</th>
<th>Slope</th>
<th>Intercepts</th>
<th>Day 0</th>
<th>Day 1 Cultures</th>
<th>Day 1 Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-16</td>
<td>34.09</td>
<td>-28.37</td>
<td>5.00</td>
<td></td>
<td>5.62</td>
</tr>
<tr>
<td>2</td>
<td>17-23</td>
<td>28.55</td>
<td>-27.20</td>
<td>2.00</td>
<td></td>
<td>1.35</td>
</tr>
<tr>
<td>3</td>
<td>1-23</td>
<td>32.41</td>
<td>-28.01</td>
<td>3.00</td>
<td></td>
<td>4.40</td>
</tr>
<tr>
<td>4</td>
<td>17-44</td>
<td>26.39</td>
<td>-43.58</td>
<td>2.00</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1-44</td>
<td>29.14</td>
<td>-35.86</td>
<td>3.00</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

**Notes:**

The values listed under Day 1 are the values calculated as the inoculation day starting number in the cultures. The value of 3.00 is an average of the values contained in the group.

The counting error for all 44 of the cultures ranged from 4% to 35%. There was a definite mode at 9% for counts made between days 2 and 6.

The error in pipette and counting chamber volumes was found to be less than 2%. A constant value of 2% was used in this study for this error.
DISCUSSION

The original regression line estimate of the mean growth curve given in Figure 1 is a reasonable fit to the data. The intercept given with this line is the Day 0 Intercept given in Table 4. This is the mathematical intercept. This intercept was used with the slope to calculate mean daily values. The calculated values were compared with the mean daily values determined directly from the data to test the validity of the assumed model. The values for Day 1 are typical examples and are given in Table 4 under Intercepts - Day 1 Regression.

The assumed model, a linear relationship, is shown to be incorrect by the difference in the Day 1 intercepts within the various groups. The Day 1 Regression intercepts for Groups 4 and 5 (Table 4) were not calculated because the assigned data values of zero depressed the intercepts. The values for the Day 1 Intercepts are so close that the error associated with the use of the linear model probably makes very little difference. The magnitude of the error is such that the numerical results of the error will still fall well within the range of the natural variation of the culture populations. The main point is that the model be recognized as unreal, but still useful since a close approximation of reality is present. This approximation was considered sufficient to meet the requirements of the bioassay method.

The mean count per field on Day 5 for cultures 1-16 indicated that the value of 5 calculated for the starting number of protozoa was slightly high. However, this value is within the confidence range...
for 5 day old cultures. The difference was probably the result of natural variation in the cultures.

The significance of the analysis of covariance is that any deviations from the mean values are placed in their proper perspective. Differences that might appear to be significant when first encountered are examined within the range of the data and compared to the mean values. The analysis allowed the comparison of the individual regression lines that were calculated for each culture with each other, the mean regression line, and the range of the data. For a more complete discussion of covariance, see the appropriate sections in Dixon and Massey (1957) and Ostle (1963).

The analysis of covariance that was performed on the regression lines which were determined for cultures 1-16 indicated that the mean line in Figure 1 was being estimated by all of the 16 individual lines. The importance of this result is that a basis was established for recognizing natural growth and population levels. This is necessary before the effects of an assayed material can be determined.

The results of the pilot assay indicate that the daily removal of 1 ml samples had no effect on the attained population levels. This is indicated by the closeness of the mean count per field on Day 5 for the control and assay cultures, and by the overlap of the confidence ranges. The calculated starting number of protozoa in these cultures, 2, is very close to the Day 5 means for cultures 17-44, that is the pilot assay cultures.
The utility of the analysis of covariance for detecting natural variation was determined through a study of the results of the analyses performed on the groups in Table 3.

Group 1, Cultures 1-16, was found to be representable by one regression line (Figure 1). Group 2, Cultures 17-23, was found to be representable by one regression line (Figure 2). This mean line is a fair fit to the data. The fact that the line failed to pass through some of the data ranges is probably the result of the small number of cultures (7) used to determine this line. This is indicated by the results of the analysis of Group 3, which is this small group pooled with Group 1.

Group 3 was the pooled group that was composed of the data from Groups 1 and 2. The regression line estimate of the mean growth curve for this group is given in Figure 3. The analysis of covariance indicates that this line was estimated by the individual lines. The mean regression line for Group 3 was a better estimate of the universal mean growth curve for cultures of T. pyriformis, and was used subsequently for this purpose.

The manipulation of the data made the results of the analyses of covariance performed on Groups 4 and 5 difficult to interpret. The regression lines for the cultures in these groups were indicated to be estimates of the mean regression lines. The expectation was that the assigned data values of zero would cause some of the lines to be estimates of a different mean. The reason this was not the case may
be that the data, when obtained, was within the acceptable range for estimation of the one mean.

The above results indicate that the analysis of covariance is a sufficiently good tool for detecting similarities and differences in population levels by an analysis of the regression lines that are based on these population levels.

The tentative bioassay based on the results of this study would be set up as follows:

Part 1. A group of cultures would be grown and sampled each day for 8 days. Three separate groups of 20 each are suggested. The data from the cultures would be used to estimate the mean natural growth curve by the calculation of the regression line.

Part 2. A second group of cultures would be grown with various concentrations of a chemical in them. Various techniques will allow the aseptic addition of the chemical with the 0.5 ml of demineralized distilled water added to bring the volumes of the cultures to 28 ml. The technique would depend on the properties of the selected chemical. Three assay cultures and 1 control culture per concentration are recommended. The control cultures would be used to furnish the natural growth regression line for the assay. Each of the 3 cultures per chemical concentration would furnish one mean line that would be used to determine the effects of the chemical at that concentration. If significant differences were found to exist between the mean regression line for the control cultures and the mean regression line for
an assay triplet when an analysis of covariance was performed, the mean lines would then be tested against the line determined in Part 1. The control line would be compared in any case. If no difference is found to exist between the line from Part 1 and the control line, but the assay triplet mean line is still different, the difference is probably real and can be accepted. If the control culture line is found to differ from the line determined in Part 1 the results of either Part 1 or 2 will have to be discarded and redetermined.

Part 3. Subsequent use of the assay would be done similar to Part 2. The only difference would be that the mean regression line, with which the assay control culture regression line would be compared, would be the result of pooling the data from Part 1 with the control culture data from Part 2. This pooled estimate would be a more accurate estimate of the universal mean. This pooling technique can be used as long as the data pool is manageable.

If significant differences exist when the data from the assay are analyzed with the analysis of covariance, the slope of the regression line that is indicated to be different is checked against the list of slopes determined for the various concentrations of the chemical standard in Part 2. When a match is found, the assayed chemical is reported as being similar in effect to a given concentration of the standard.

The chemical standard is used in place of a comparison that is based directly on the mean natural growth curve, since the former
would be more meaningful to workers that are studying the effects of chemicals on living organisms. If a worker was interested in the effects of compound y on another organism, and he knew the effect of compound x, a bioassay reporting the effects of y in terms of x would be more useful than a knowledge of the effects on the natural growth curve.

This assay is tentative and untested. There are several problems which remain to be solved prior to any extensive utilization of an assay based on the above outline. The time required to obtain the data by the direct count technique which was used in this study, would be prohibitive in actual assay work. There are several alternatives to this method that should be explored.

The computer programs used with this study to perform the statistical calculations require approximately 2 hours of machine time to run. This can be reduced by revision of the program. The manipulation of the cultures for inoculations and sample removal require approximately 2 hours for 20 cultures. This can be reduced if reasonably good approximations of the mean growth curves can be obtained by basing the regression analysis on the samples that are removed on the last three days of the eight the cultures are grown. This will require careful study.

The approach does seem to have enough merit to justify time spent on the solution of the above problems.
The significance of the results of this particular study is that the base for actual assay work has been determined. The definition of the regression line estimate of the mean growth curve for the universe composed of curves from naturally growing cultures of \textit{T. pyriformis} will allow future work to be done directly on the bioassay. The defined curve, Figure 3, when combined with the determined statistical analysis for recognition of real differences in the population levels as outlined in the tentative assay, has placed a quantitative bioassay within reach.
SUMMARY

A regression line estimate of the mean growth curve of axenic cultures of *Tetrahymena pyriformis* grown under certain conditions was determined when a linear relationship was assumed.

The removal of daily samples from the cultures had no significant effect on the population levels.

The use of an analysis of covariance for the separation of apparent and real differences is feasible.

A tentative bioassay, based on the above results of this study, is outlined.

Extensive testing of the proposed bioassay and the utility of the analysis of covariance remains to be done before this assay can become a standard technique.
LITERATURE CITED


APPENDIX
The computer program which follows is not offered as the most efficient possible. The major consideration was not the efficiency of the program, but whether it would do the intended job. This was necessitated by the fact that I had never touched a computer until January, 1968. This caused my attitude concerning the program to be, "If it works and the answers are correct, leave it alone." These programs will be revised prior to any future work.

The following description of operation assumes a familiarity with the IBM 1620-1622 Data Processing System. A copy of the program source decks are on file at the University of Montana Computer Center.

The overall program is broken up into two accessory programs and eight main subprograms. They were used as described below:

Accessory Program 1: The data for this program were obtained by weighing the volume of distilled water that was held by a random selection of the pipettes which were used in this experiment. The weighing was done with an analytical balance. Five weighings were obtained for each of the five pipettes that were tested. Five weighings were also made for the counting chamber that was used in this study. The weights were punched on cards and read into the programmed machine. Note that the last card is the chamber data card. The answers obtained are:

- PEM - The percent error for the pipettes.
- CEM - The percent error for the counting chamber.
- T - The total error (PEM + CEM).
If the total error is over 2%, the value for "z" in the Program Part 1 and 2B must be changed accordingly. This analysis is based on Moore (1952).

Accessory Program 2: This program is used to obtain the F distribution values used in Part 4B. \( V_1 \) and \( V_2 \) are the degrees of freedom in the numerator and denominator respectively of the F ratios that were used in testing the various hypotheses. Beta is here used as the indicator of significance in place of the more usual Alpha. Care must be taken to enter the data in the same order the tests are performed in Part 4B. For tests where the value of either \( V_1 \) or \( V_2 \) is less than 10, an F table should be consulted and the values punched out and the cards placed in the correct position in the deck.

Thesis Data Analysis Actual Program: Part 1: This program is used to obtain various answers concerning the inoculation culture counts. The X variable is the symbol for any one of the 60 fields which were counted. As this is simply a short version of 2A and 2B combined, the answers are the same as for 2A and 2B and will be covered in the discussion of these two programs. The answers are punched out directly from this program. In this program Y is the value for the sum of 60 fields for the cultures inoculated from the appropriate inoculation culture.

Part 2A: In this program, the individual 60 field counts are stored as integer numbers, but are used as floating point numbers. The answers obtained are:
D - The mean count per field,
B - The sum of the square of the individual field counts,
XX - The sum of the 60 fields counted for each culture.

For this program the above cards had to be separated into the three groups for use in the next program. This program was run a total of four times to accommodate all of the data. The separation of the answer cards can be done by observing the perforations in the first column of the cards. Do not allow these card sets to intermix as the data groups are run. The cards can be sorted by using the number punched 10 columns to the right of the answers, but this is time consuming. As the sets come out of the machine, separate into the three answer groups and place the appropriate answer group in back of its earlier run answer group. Thus all D's from the first data run will be in front of one answer deck, and the D's from the last data run will be in back of the same card deck. This will also be true for the B's and the XX's.

Part 2B: The answers from 2A are read into this program. The answers obtained are:

P - Moore's percent error (Moore, 1952)
MU - Upper confidence limit for D (Dixon and Massey, 1959)
ML - Lower confidence limit for D (Dixon and Massey, 1959)
AM - Sum of D for various data groupings
MN - Mean D for various data groupings
MXX - Mean XX for various data groupings
(There is no MXX for Program Part 2)

TE - is the total error (P+Z)

AM, MN, and MXX are values used to check the program and the answers it is calculating. These values are easy to calculate from the data and were used only as a check. TE cards should be removed from the back of the answer deck.

Part 2C: The weighting desired is decided on, and Switch 1 is turned on or off. If an unweighted regression line is desired, use the W = 1 setup. The TE cards from Part 2A, with an appropriate TE card from Part 1 inserted every eight cards starting from the front of the deck, are read in and the weights are read out on a one for one basis. The TE cards from Part 1 are used to enter the accuracy of the count made on the inoculation cultures.

To this point the only cause for a "Checkstop" would be improper data format. For wrong answers check the data input sequence.

Part 3AA: This program is the start of the linear regression analysis. The data read in is:

Y - The XX's from Part 2A with one card in every eight, starting from the front of the deck, that has the appropriate Y from Part 1 punched on it.

W - The weights obtained from Part 2C.

The answers A, B, C, D, and E will be punched out in order and will amount to one box of cards. Do not allow these to become mixed or out of the order in which the machine puts them. It would be a long process
to sort these cards on the letter and then on the (44, 9) code. These are the results of the first operation on the data.

Part 3AB: This reads the answers from 3AA and puts out the intermediate calculation answers AC, BC, CC, DC, EC. This deck of answers must be sorted. Use the sorter set on column one. One pass will do the job. The answers are then picked up AC, BC, CC, DC, and EC so that the machine will read AC first.

Part 3B: This part of the program reads in the sorted answers from 3AB and calculates the slope and intercepts of the regression lines for cultures 1-44 and the mean slopes and intercepts for groups 1-16 (45), 17-23 (46), 24-44 (47), 17-44 (48), 1-44 (49), and 1-23 (50), the number in parentheses being the code number for the indicated group.

The last 24 cards of the deck are removed and, starting from the top (card face side up) of the deck of 24, sort one card at a time into four piles placed face down. These four piles are DYX (45-50), XB (45-50), YIN (45-50), XC (45-50). Insert these into the rest of the deck with DYX's following the first 44 DYX's and the rest on the back of the deck in the order YIN, XB, and XC. These last two are values used in the analysis of covariance.

The only sources of error in the operation to this point are failure to have placed the Y's from Part 1 in the input deck of Y's (XX's) for part 3AA or the TE's from Part 1 in the input deck which calculated weights, Part 2C. This is indicated by a Reader No Feed
Stop on the machine when all cards are read through. Mixing of 3AA output or failure to sort 3AB output will cause wrong answers.

Part 4A: This is the sums of squares calculator for the regression analysis (Ostle, 1963). The Y's, W's, DYX's, YIN's, XB's and XX's are read in and AAA, BBB, CCC and DDD are punched out. XMU is a variable calculated from XB and XC for use in determining the sums of squares. Notice that appropriate header cards are punched for the group being tested. A special color card duplicate should be substituted for these to facilitate finding them.

Part 4B: This performs the F test evaluation and comparison. The AAA's, BBB's CCC's, and DDD's from 4A are read in and also the answers, (FT), from Accessory Program 2. Care must be taken to remove the header cards after obtaining a printout, and sort the cards into four decks of AAA, BBB, CCC, and DDD. Sort on Column 1 as before and assemble the deck so that AAA reads in first.

Answer cards are printed if the hypothesis being tested is true. The hypotheses tested are:

Hypothesis 1 - One mean regression line is determined by the data.

Hypothesis 2 - The within-the-groups regression line slopes are the same.

Hypothesis 3 - The regression line for the group means is linear.

Hypothesis 4 - The regression line slopes for the means and pooled within-groups slopes are the same.
If Hypothesis 1 is not true, Hypotheses 2, 3, and 4 are tested. The latter three hypotheses indicate why Hypothesis 1 was not true. The answers for Hypotheses 3 and 4 are meaningful only if the preceding hypothesis is true. Hypotheses 3 and 4 may be stated as being true; however, if Hypothesis 2 is not, the answers are meaningless and Hypothesis 2 is the reason that Hypothesis 1 was not true.
THESIS DATA ANALYSIS. ACCESSORY PROGRAM 1.

ERROR ANALYSIS FOR PIPETTES AND COUNTING CELL.

INTERNAL AND EXTERNAL CONSISTANCY.

W IS THE WEIGHT IN GRAMS OF 1 ML. OF WATER AT 25 DEGREES CELCIUS. DATA FROM C.R.C. HANDBOOK.

LENGTH 15560

DIMENSION X(6,5),D(6)*B(6),P(6)

READ 12*((X(I,J),J=1,5),I=1,6)

12 FORMAT (5(5X,F7.5))

DO 5 I=1,6
DO 5 J=1,5
D(I)=0.

5 B(I)=0.

DO 115 I=1,6
DO 115 J=1,5
D(I)=D(I)+X(I,J)/5.

115 B(I)=B(I)+X(I,J)**2

DO 16 I=1,6
16 PUNCH 111,I,B(I),I,D(I)

111 FORMAT (2HB(I2.4H) = ,F10.4,5X.2HD(I2.4H) = .F10.4)

DO 10 I=1,6

CV=SQRTF((B(I)/D(I)**2-5.)/4.)

P(I)=((100.*CV)/SQRTF(5.))

10 PUNCH 101,I,P(I)

101 FORMAT (2HP(I2.4H) = .F10.4)

DM = 0.

BM = 0.

DO 15 I=1,5

DM = DM+D(I)/5.

15 BM = BM+B(I)/5.

CV = SQRTF((BM/DM**2-5.)/4.)

PM=((100.*CV)/SQRTF(5.))

PUNCH 102,PM

102 FORMAT (5HPM = .F10.4)

Z=0.

DO 20 I=1,5

DO 20 J=1,5

20 Z=Z+X(I,J)/25.

W=0.9971

PEM = (Z-W)*100.

PUNCH 103,PEM,Z

103 FORMAT (6HPEM = .F10.4,5X,4HZ = .F10.4)

Y=0.

DO 25 J=1,5

25 Y=Y+X(6,J)/5.

CEM =(W-Y)*100.

PUNCH 104,CEM,Y

104 FORMAT (6HCEM = .F10.4,5X,4HY = .F10.4)

DO 30 I=6,6

30 T=PM+P(6)
IF (2.-T)40,45,50
40 PUNCH 105,T
45 PUNCH 106,T
50 PUNCH 107,T
105 FORMAT (17HT OVER 2 PERCENT.,5X,4HT = ,F10.4)
106 FORMAT (19HT EQUALS 2 PERCENT.,5X,4HT = ,F10.4)
107 FORMAT (18HT UNDER 2 PERCENT.,5X,4HT = ,F10.4)
GO TO 1
END

THESIS DATA ANALYSIS ACCESSORY PROGRAM 2.
APPROXIMATION OF F DISTRIBUTION PERCENTILES.
DO NOT USE FOR SAMPLES OF LESS THAN (10,10).
AFTER DIXON AND MASSEY (1957), PG 402.
LENGTH 14382

DIMENSION ALPHA(9),A(9),B(9),C(9),DC(6),DD(6)
1 READ 9,V1,V2,BETA
9 FORMAT (7X,F5.0,7X,F5.0,7X,F5.0,7X,F6.5)
ALPHA(1) = 0.50
ALPHA(2) = 0.75
ALPHA(3) = 0.90
ALPHA(4) = 0.95
ALPHA(5) = 0.975
ALPHA(6) = 0.99
ALPHA(7) = 0.995
ALPHA(8) = 0.999
ALPHA(9) = 0.9995
A(1) = 0.0
A(2) = 0.5859
A(3) = 1.1131
A(4) = 1.4287
A(5) = 1.7023
A(6) = 2.0206
A(7) = 2.373
A(8) = 2.6841
A(9) = 2.8580
B(1) = 0.0
B(2) = 0.58
B(3) = 0.77
B(4) = 0.95
B(5) = 1.14
B(6) = 1.40
B(7) = 1.61
B(8) = 2.09
B(9) = 2.30
C(1) = 0.29
C(2) = 0.355
C(3) = 0.527
C(4) = 0.681

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C(5) = 0.846
C(6) = 1.073
C(7) = 1.25
C(8) = 1.672
C(9) = 1.857
XM= 2.30258
DO 2 I=1,9
2 DC(I)=V1-10.
   IF (V1-10.)12,23,23
23 DO 3 I=1,9
3 DD(I)=V2-10.
   IF(V2-10.)13,4,4
12 PUNCH 102
13 PUNCH 103
102 FORMAT (13HV1 TOO SMALL*)
103 FORMAT (13HV2 TOO SMALL*)
4 DO 5 I=1,9
   IF (ALPHA(I)-BETA) 5,10,5
10 H=(2.*V1*V2/(V1+V2))
   G=(V2-V1)/(V2*V1)
   FTC=(A(I)/SQRTF(H-B(I)))-C(I)*G
   EFTC=FTC*XM
   PFTC=EXPF(EFTC)
   PUNCH 101,PFTC
101 FORMAT (7HPFTC = ,F12.5)
5 CONTINUE
GO TO 1
END

C  THESIS DATA ANALYSIS ACTUAL PROGRAM.
C  PART 1. INNOCULATION CULTURE ANALYSIS.
C W AND G ARE T TEST VALUES FOR ALPHA .95 AND FOR
C  60 DEGREES OF FREEDOM.
C LENGTH 16572
C
C
DIMENSION D(2),XX(2),B(2),X(2,60),TE(2),XMU(2),
1XMLC2),P(2),V(2),Y(2)
1 READ 12*((X(I,J),J=1,60),I=1,2)
12 FORMAT (20F3.0)
   DO 5 I=1,2
      B(I)=0.
      D(I)=0.
      TE(I)=0.
      Y(I)=0.
5 XX(I)=0.
   G=1.614
   H=-1.614
   Z=2.*0
   DO 10 I=1,2
   DO 10 J=1,60

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D(I) = D(I) + X(I,J)/60.
B(I) = B(I) + X(I,J)**2

10 XX(I) = XX(I) + X(I,J)
DO 15 I = 1, 2
E = D(I)**2
CV = SQRTF((B(I)/E - 60.)/59.)
P(I) = ((100.*CV)/SQRTF(60.*I))
S = SQRTF((B(I)-60.*E)/59.)
XMU(I) = D(I) + S*G
XML(I) = D(I) + S*H
DO 20 I = 1, 2
20 Y(I) = XX(I)/56.
DO 25 I = 1, 2
PUNCH 101, I, TE(I)
PUNCH 102, I, XX(I)
PUNCH 103, I, D(I), I, B(I)
PUNCH 104, I, P(I)
PUNCH 105, I, XMU(I), I, XML(I)
25 PUNCH 106, I, Y(I)
101 FORMAT (4X, 3HTE(, I14H = , F10.4)
102 FORMAT (4X, 3HXX(, I14H = , F10.4)
103 FORMAT (2HD(, I14H = , F10.4)
104 FORMAT (2HP(, I14H = , F10.4)
105 FORMAT (3HMU(, I14H = , F10.4)
106 FORMAT (2HY(, I14H = , F10.4)
GO TO 1
15 TE(I) = P(I) + Z
END

C PART 2A THESIS DATA ANALYSIS.
C SEGMENT FOR D(I,J), B(I,J), AND XX(I,J).
C LENGTH 39644
C
DIMENSION NX(I1, 8, 60)
NZ = 0.
1 READ 12, ((NX(I,J,K), K = 1, 60), J = 1, 8), I = 1, 11)
12 FORMAT (20I3)
DO 10 I = 1, 11
DO 10 J = 1, 8
DO 5 K = 1, 60
D = 0.
X = NX(I, J, K)
5 D = D + X/60.
NZ = NZ + 1
10 PUNCH 101, I, J, D, NZ
101 FORMAT (1X, 2HD(, I21H, I24H) = , F10.4, 10X, I4)
DO 20 I = 1, 11
DO 20 J = 1, 8
B = 0.
DO 15 K = 1, 60

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X = NX(I, J, K)

15 B = B + X**2
NZ = NZ + 1

20 PUNCH 103, I, J, B, NZ
103 FORMAT (1X, 2HB(*I2, 1H*, I2, 4H) =, F10.4, 10X, I4)
DO 30 I = 1, 11
DO 30 J = 1, 8
XX = 0.
DO 25 K = 1, 60
X = NX(I, J, K)
25 XX = XX + X
NZ = NZ + 1
30 PUNCH 105, I, J, XX, NZ
105 FORMAT (3HXX(*I2, 1H*, I2, 4H) =, F10.4, 10X, I4)
GO TO 1
END

C PART 2B THESIS DATA ANALYSIS.
C SEGMENT FOR P, Mu, ML, AM, MN, AND TE.
C LENGTH 32128

C

DIMENSION D(44, 8), B(44, 8), XX(44, 8), P(44, 8)
1 READ ?, (D(I, J), J = 1, 8), (B(I, J), J = 1, 8), I = 1, 44),
1 (XX(I, J), J = 1, 8), I = 1, 44)
2 FORMAT (12X, F10.4)
DO 5 I = 1, 44
DO 5 J = 1, 8
P(I, J) = 0.
IF (D(I, J)) 51, 5, 51
51 IF (B(I, J)/D(I, J)**2 - 60.) 5, 5, 52
52 CV = SORTF((B(I, J)/D(I, J)**2-60.)/59.)
P(I, J) = ((100.*CV)/SORTF(60.))
PUNCH 101, I, J, P(I, J)
101 FORMAT (1X, 2H P(*I2, 1H*, I2, 4H) =, F10.4)
5 CONTINUE
G = 1.614
H = -1.614
DO 10 I = 1, 44
DO 10 J = 1, 8
E = D(I, J)**2
S = SQRTF((B(I, J) - 60.*E)/59.)
XMU = D(I, J) + S*G
XML = D(I, J) + S*H
10 PUNCH 103, I, J, XMU, XML
103 FORMAT (3HXMU(*I2, 1H*, I2, 4H) =, F10.4, 5X, 5HML =, F10.4)
XMN = 0.
DO 20 J = 1, 8
AM = 0.
XMXX = 0.
DO 15 I = 1, 16

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\[ XMXX = XMXX + \left( X_{I,J} \right) / 16 \]

15 \[ AM = AM + D(I,J) \]
\[ XMN = AM / 16 \]

20 PUNCH 105, J, AM, XMN, XMXX

105 FORMAT (3HAM(, I2, 4H) = , F10.4, 5X, 5HMN = , F10.4, 5X,
16HMXX = , F10.4)
\[ XMN = 0 \]
DO 30 J = 1, 8
\[ AM = 0 \]
\[ XMXX = 0 \]
DO 25 I = 17, 23
\[ XMXX = XMXX + \left( X_{I,J} \right) / 7 \]

25 \[ AM = AM + D(I,J) \]
\[ XMN = AM / 7 \]

30 PUNCH 107, J, AM, XMN, XMXX

107 FORMAT (3HAM(, I2, 4H) = , F10.4, 5X, 5HMN = , F10.4, 5X,
16HMXX = , F10.4)
\[ XMN = 0 \]
DO 40 J = 1, 8
\[ AM = 0 \]
\[ XMXX = 0 \]
DO 35 I = 1, 23
\[ XMXX = XMXX + \left( X_{I,J} \right) / 23 \]

35 \[ AM = AM + D(I,J) \]
\[ XMN = AM / 23 \]

40 PUNCH 109, J, AM, XMN, XMXX

109 FORMAT (3HAM(, I2, 4H) = , F10.4, 5X, 5HMN = , F10.4, 5X,
16HMXX = , F10.4)
\[ XMN = 0 \]
DO 50 J = 1, 8
\[ AM = 0 \]
\[ XMXX = 0 \]
DO 45 I = 17, 44
\[ DMXX = DMXX + X_{I,J} \]

45 \[ AM = AM + D(I,J) \]
\[ XI = J \]
\[ T = 4 + 3 \times XI \]
\[ XMN = AM / T \]
\[ XMXX = DMXX / T \]

50 PUNCH 111, J, AM, XMN, XMXX

111 FORMAT (3HAM(, I2, 4H) = , F10.4, 5X, 5HMN = , F10.4, 5X,
16HMXX = , F10.4)
\[ XMN = 0 \]
DO 60 J = 1, 8
\[ AM = 0 \]
\[ XMXX = 0 \]
DO 55 I = 1, 44
\[ DMXX = DMXX + X_{I,J} \]

55 \[ AM = AM + D(I,J) \]
\[ XI = J \]
\[ U = 21 + 3 \times X \]
\[ X_{MN} = AM / U \]
\[ X_{MXX} = DMXX / U \]

60 PUNCH 113, J, AM, XMN, XMXX
113 FORMAT (3HAM(*I2*4H) = *F10.4,5X,5HMN = *F10.4,5X,
16HMXX = *F10.4)

Z = 2.0
DO 65 I = 1, 44
DO 65 J = 1, 8
TE = P(I, J) + Z
65 PUNCH 115, I, J, TE
115 FORMAT (3HTE(*I2*1H*,I2*4H) = ,F10.4)
GO TO 1
END

C PART 2C THESIS DATA ANALYSIS.
C WEIGHT CALCULATOR.
C LENGTH 12682
C
C WEIGHTS VARIOUS--SWITCH 1 ON.
C WEIGHTS ALL = 1.0--SWITCH 1 OFF.
C

KK = 0.
IF (SENSE SWITCH 1) 1, 50
1 KK = KK + 1
READ 99, TE
99 FORMAT (12X, F10.4)
A = 3.0
B = 5.0
C = 4.0
D = 3.0
E = 2.0
F = 1.0
G = 0.5
IF(W-3.) 101, 101, 2
2 IF(W-10.) 102, 102, 3
3 IF(W-20.) 103, 103, 4
4 IF(W-30.) 104, 104, 5
5 IF(W-40.) 105, 105, 6
6 IF(W-50.) 106, 106, 107
101 PUNCH 110, A, KK
GO TO 1
102 PUNCH 110, B, KK
GO TO 1
103 PUNCH 110, C, KK
GO TO 1
104 PUNCH 110, D, KK
GO TO 1
105 PUNCH 110, E, KK
GO TO 1
106 PUNCH 110,F,KK
GO TO 1
107 PUNCH 110,G,KK
110 FORMAT (11X,F10.4,10X,I4)
GO TO 1
50 READ 999,TE
999 FORMAT (12X,F10.4)
XYZ = 1.0
PUNCH 109, XYZ
109 FORMAT (11X,F10.4)
GO TO 50
END

C PART 3AA THFSIS DATA ANALYSIS*
C DETERMINATION OF REGRESSION LINES*
C DETERMINATION OF A,B,C,D,E*
C LENGTH 21856

C DIMENSION Y(44,9),W(44,9)
1 READ 99,((Y(I,J),J=1,9),I=1,44)
READ 999,((W(I,J),J=1,9),I=1,44)
99 FORMAT (12X,F10.4)
999 FORMAT (11X,F10.4)
XI=1.
A=0.
B=0.
C=0.
D=0.
E=0.
DO 5 I=1,44
DO 5 J=1,9
XJ=J
A=(XI*W(I,J)*XJ)
5 PUNCH 101,I,J,A
101 FORMAT (2HA(,I2,1H,,I2,4H) = ,F12.5)
DO 6 I=1,44
DO 6 J=1,9
B=(W(I,J)*Y(I,J))
6 PUNCH 102,I,J,B
102 FORMAT (2HB(,I2,IH, ,I2,4H) = ,F12.5)
DO 7 I=1,44
DO 7 J=1,9
C=(XI*W(I,J))
7 PUNCH 103,I,J,C
103 FORMAT (2HC(,I2,1H,,I2,4H) = ,F12.5)
DO 8 I=1,44
DO 8 J=1,9
XJ=J
D=(W(I,J)*XJ*Y(I,J))
8 PUNCH 104,I,J,D
PART 3AB THESIS DATA ANALYSIS.
DETERMINATION OF REGRESSION LINES.
DETERMINATION OF AC, BC, CC, DC, EC.
LENGTH 36558

DIMENSION A(44,9), B(44,9), C(44,9), D(44,9), E(44,9),
1AC(44), BC(44), CC(44), DC(44), EC(44)
1 READ 9*((A(I,J), J=1,9), I=1,44), (B(I,J), J=1,9), I=1,44),
1(C(I,J), J=1,9), I=1,44), (D(I,J), J=1,9), I=1,44),
1(F(I,J), J=1,9), I=1,44)
9 FORMAT (11X,F12.5)
DO 20 I=1,44
AC(I)=0.
BC(I)=0.
CC(I)=0.
DC(I)=0.
20 EC(I)=0.
DO 30 I=1,44
DO 25 J=1,9
AC(I)=AC(I)+A(I,J)
BC(I)=BC(I)+B(I,J)
CC(I)=CC(I)+C(I,J)
DC(I)=DC(I)+D(I,J)
25 EC(I)=EC(I)+E(I,J)
PUNCH 101, I, AC(I)
PUNCH 102, I, BC(I)
PUNCH 103, I, CC(I)
PUNCH 104, I, DC(I)
30 PUNCH 105, I, EC(I)
101 FORMAT (3HAC(*I2,4H) = *F12.5)
102 FORMAT (3HBC(*I2,4H) = *F12.5)
103 FORMAT (3HCC(*I2,4H) = *F12.5)
104 FORMAT (3HDC(*I2,4H) = *F12.5)
105 FORMAT (3HEC(*I2,4H) = *F12.5)
GO TO 1
END
DETERMINATION OF B(0) AND B(1).

LENGTH 27306

DIMENSION AC(44),BC(44),CC(44),DC(44),EC(44),DYX(50),
YIN(50)

READ 9999, (AC(I), I=1,44), (BC(I), I=1,44), (CC(I), I=1,44),
(1,DC(I), I=1,44), (EC(I), I=1,44)

9999 FORMAT (9X,F12.5)

DO 30 I=1,44

DYX(I)=(AC(I)*BC(I)-CC(I)*DC(I))/(AC(I)**2-CC(I)*EC(I))

30 PUNCH 101,I,DYX(I)

DO 10 1=1,44

YIN(I)=(AC(I)*DC(I)-EC(I)*BC(I))/(AC(I)**2-CC(I)*EC(I))

10 PUNCH 103,1,YIN(I)

XA=0.
XB=0.
XC=0.
XD=0.
XE=0.

DO 40 I=1,16

XA=XA+AC(I)/16.
XB=XB+BC(I)/16.
XC=XC+CC(I)/16.
XD=XD+DC(I)/16.

40 XE=XE+EC(I)/16.

I=45

DYX(I)=0.

YIN(I)=0.

DYX(I)=(XA*XB-XC*XD)/(XA**2-XC*XE)

YIN(I)=(XA*XD-XE*XB)/(XA**2-XC*XE)

PUNCH 105, I, DYX(I)

PUNCH 106, XB, I

PUNCH 107, XC, I

PUNCH 108, XE, I

105 FORMAT (4HDYX(,I2,4H) =  ,F12.5)

106 FORMAT (5HXB =  ,F12.5, 5X,I2)

107 FORMAT (4HYIN(,I2,4H) =  ,F12.5)

108 FORMAT (5HXC =  ,F12.5, 5X,I2)

XA=0.
XB=0.
XC=0.
XD=0.
XE=0.

DO 50 I=17,23

XA=XA+AC(I)/7.
XB=XB+BC(I)/7.
XC=XC+CC(I)/7.
XD=XD+DC(I)/7.

50 XE=XE+EC(I)/7.

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\[ I = 46 \]
\[
DYX(I) = 0. \\
YIN(I) = 0. \\
DYX(I) = \frac{XA \times XB - XC \times XD}{XA^2 - XC \times XF} \\
YIN(I) = \frac{XA \times XD - XD \times XB}{XA^2 - XC \times XE} \\
PUNCH 109, I, DYX(I) \\
PUNCH 110, XB, I \\
PUNCH 111, I, YIN(I) \\
PUNCH 112, XC, I \\
109 FORMAT (4HDYX(I2,4H) = , F12.5) \\
110 FORMAT (5HXB = , F12.5, 5X, I2) \\
111 FORMAT (4HYIN(I2,4H) = , F12.5) \\
112 FORMAT (5HXC = , F12.5, 5X, I2) \\
\]

\[ XA = 0. \\
XB = 0. \\
XC = 0. \\
XD = 0. \\
XE = 0. \\
DO 60 I = 24, 44 \\
XA = XA + AC(I)/21. \\
XB = XB + BC(I)/21. \\
XC = XC + CC(I)/21. \\
XD = XD + DC(I)/21. \\
60 XF = XE + EC(I)/21. \\
I = 47 \\
DYX(I) = 0. \\
YIN(I) = 0. \\
DYX(I) = \frac{XA \times XB - XC \times XD}{XA^2 - XC \times XF} \\
YIN(I) = \frac{XA \times XD - XD \times XB}{XA^2 - XC \times XE} \\
PUNCH 113, I, DYX(I) \\
PUNCH 114, XB, I \\
PUNCH 115, I, YIN(I) \\
PUNCH 116, XC, I \\
113 FORMAT (4HDYX(I2,4H) = , F12.5) \\
114 FORMAT (5HXB = , F12.5, 5X, I2) \\
115 FORMAT (4HYIN(I2,4H) = , F12.5) \\
116 FORMAT (5HXC = , F12.5, 5X, I2) \\
\]

\[ XA = 0. \\
XB = 0. \\
XC = 0. \\
XD = 0. \\
XE = 0. \\
DO 70 I = 17, 44 \\
XA = XA + AC(I)/28. \\
XB = XB + BC(I)/28. \\
XC = XC + CC(I)/28. \\
XD = XD + DC(I)/28. \\
70 XF = XE + EC(I)/28. \\
I = 48 \\
DYX(I) = 0. \\
YIN(I) = 0. \\
DYX(I) = \frac{XA \times XB - XC \times XD}{XA^2 - XC \times XF} \]
\[ \text{YIN}(I) = \frac{(X_A*XD - X_E*XB)}{(X_A**2 - X_C*XE)} \]

PUNCH 117, I, DYX(I)
PUNCH 118, I, XE
PUNCH 119, I, YIN(I)
PUNCH 120, I, XC

117 FORMAT (4H, I2, 4D) = ,F12.5)
118 FORMAT (5H, XE = ,F12.5, 5X, I2)
119 FORMAT (4H, YIN(I) = ,F12.5)
120 FORMAT (5H, XC = ,F12.5, 5X, I2)

\[ X_A = 0. \]
\[ X_B = 0. \]
\[ X_C = 0. \]
\[ X_E = 0. \]

DO 80 I=1,44
\[ X_A = X_A + \frac{AC(I)}{44}. \]
\[ X_B = X_B + \frac{BC(I)}{44}. \]
\[ X_C = X_C + \frac{CC(I)}{44}. \]
\[ X_E = X_E + \frac{EC(I)}{44}. \]

80 FORMAT (4H, I2, 4D)

I=49
\[ DYX(I) = 0. \]
\[ YIN(I) = 0. \]

PUNCH 121, I, DYX(I)
PUNCH 122, I, XE
PUNCH 123, I, YIN(I)
PUNCH 124, I, XC

121 FORMAT (4H, I2, 4D) = ,F12.5)
122 FORMAT (5H, XE = ,F12.5, 5X, I2)
123 FORMAT (4H, YIN(I) = ,F12.5)
124 FORMAT (5H, XC = ,F12.5, 5X, I2)

\[ X_A = 0. \]
\[ X_B = 0. \]
\[ X_C = 0. \]
\[ X_E = 0. \]

DO 90 I=1,23
\[ X_A = X_A + \frac{AC(I)}{23}. \]
\[ X_B = X_B + \frac{BC(I)}{23}. \]
\[ X_C = X_C + \frac{CC(I)}{23}. \]
\[ X_E = X_E + \frac{EC(I)}{23}. \]

90 FORMAT (4H, I2, 4D)

I=50
\[ DYX(I) = 0. \]
\[ YIN(I) = 0. \]

PUNCH 125, I, DYX(I)
PUNCH 126, XE
PUNCH 127, I, YIN(I)
PART 4A THESIS DATA ANALYSIS.
COVARIANCE ANALYSIS ON REGRESSION LINES.
DETERMINATION OF SUMS OF SQUARES.
LENGTH 33950

DIMENSION Y(44,9),W(44,9),DYX(50),YIN(50),XB(6),XC(6),XMU(6)
1 READ 9*((Y(I,J),J=1,9),I=1,44)
READ 99,((W(I,J),J=1,9),I=1,44)
READ 999,((DYX(I),I=1,50),YIN(I),I=1,50)
READ 9999,((XB(K),K=1,6),(XC(K),K=1,6))
9  FORMAT (12X,F10.4)
99 FORMAT (11X,F10.4)
999 FORMAT (10X,F12.5)
9999 FORMAT (5X,F12.5)
101 FORMAT (20HFIRST 16 SAME CURVE.)
A=0.
DO 10 J=1,9
10 A=A+(W(I,J)*((Y(I,J)-XMU(1))**2))
B=0.
DO 15 J=1,9
15 B = B+(W(I,J)*((Y(I,J)-YIN(I)-DYX(I)*XJ)**2))
AAA=A-B
C=0.
DO 20 J=1,9
20 C = C+(W(I,J)*((Y(I,J)-YIN(I)-DYX(I)*XJ)**2))
BBB=B-C
DDD=0.
DO 25 J=1,9
25 DDD=DDD+(W(I,J)*((Y(I,J)-YIN(I)-DYX(I)*XJ)**2))
CCC=C-DDD
PUNCH 102,AAA
PUNCH 103,BBB
PUNCH 104,CCC
PUNCH 105,DDD
102 FORMAT (6HAAA = E14.6)
103 FORMAT (6HBBB = E14.6)
104 FORMAT (6HCCCC = E14.6)
105 FORMAT (6HDDDD = E14.6)
AAA=0.
BBB=0.
CCC=0.
DDD=0.
PUNCH 106
106 FORMAT (20HFIRST 23 SAME CURVE.)
A=0.
DO 30 I=1,23
    DO 30 J=1,9
    A=A+(W(I,J)*((Y(I,J)-XMU(6)**2))
B=0.
    DO 35 I=1,23
    B=B+(W(I,J)*((Y(I,J)-YIN(50)-DYX(50)*XJ)**2))
35 XJ=J
AAA=A-B
C=0.
    DO 40 I=1,23
    C=C+(W(I,J)*((Y(I,J)-YIN(I)-DYX(I)*XJ)**2))
40 XJ=J
BBB=B-C
DO 45 I=1,23
45 DDD=DDD+(W(I,J)*((Y(I,J)-YIN(I)-DYX(I)*XJ)**2))
CCC=C-DDD
DO 50 I=1,23
PUNCH 107,AAA
PUNCH 108,BBB
PUNCH 109,CCC
PUNCH 110,DDD
107 FORMAT (6HAAA = E14.6)
108 FORMAT (6HBBB = E14.6)
109 FORMAT (6HCCCC = E14.6)
110 FORMAT (6HDDDD = E14.6)
AAA=0.
BBB=0.
CCC=0.
DDD=0.
PUNCH 111
111 FORMAT (19HLAST 28 SAME CURVE.)
A=0.
DO 50 I=17,44
    DO 50 J=1,9
    A=A+(W(I,J)*((Y(I,J)-XMU(4)**2))
50 A=A+(W(I,J)*((Y(I,J)-XMU(4)**2))

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B = 0.
DO 55 I = 17, 44
DO 55 J = 1, 9
XJ = J
55 B = B + (W(I, J) * ((Y(I, J) - YIN(48) - DYX(48) * XJ)**2))
AAA = A - B
C = 0.
DO 60 I = 17, 44
DO 60 J = 1, 9
XJ = J
60 C = C + (W(I, J) * ((Y(I, J) - YIN(I) - DYX(I) * XJ)**2))
BBB = B - C
DO 65 I = 17, 44
DO 65 J = 1, 9
XJ = J
65 DDD = DDD + (W(I, J) * ((Y(I, J) - YIN(I) - DYX(I) * XJ)**2))
CCC = C - DDD
PUNCH 112, AAA
PUNCH 113, BBB
PUNCH 114, CCC
PUNCH 115, DDD
112 FORMAT (6HAAA = 6F14.6)
113 FORMAT (6HBBB = 6F14.6)
114 FORMAT (6HCCC = 6F14.6)
115 FORMAT (6HDDDD = 6F14.6)
AAA = 0.
BBB = 0.
CCC = 0.
DDD = 0.
PUNCH 116
116 FORMAT (18HALL 44 SAME CURVE.)
A = 0.
DO 70 I = 1, 44
DO 70 J = 1, 9
70 A = A + (W(I, J) * ((Y(I, J) - XMU(5))**2))
B = 0.
DO 75 I = 1, 44
DO 75 J = 1, 9
XJ = J
75 B = B + (W(I, J) * ((Y(I, J) - YIN(49) - DYX(49) * XJ)**2))
AAA = A - B
C = 0.
DO 80 I = 1, 44
DO 80 J = 1, 9
XJ = J
80 C = C + (W(I, J) * ((Y(I, J) - YIN(I) - DYX(I) * XJ)**2))
BBB = B - C
DO 85 I = 1, 44
DO 85 J = 1, 9
XJ = J
85 DDD = DDD + (W(I, J) * ((Y(I, J) - YIN(I) - DYX(I) * XJ)**2))
CCC = C - DDD

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PUNCH 117,AAA
PUNCH 118,BBB
PUNCH 119,CCC
PUNCH 120,DDD

117 FORMAT (6HAAA = *E14.6)
118 FORMAT (6HBBB = *E14.6)
119 FORMAT (6HCCC = *E14.6)
120 FORMAT (6HDDD = *E14.6)
PUNCH 121

121 FORMAT (17H17-23 SAME CURVE.)
   A=0.
   DO 90 I=17,23
   DO 90 J=1,9
   90 A=A+(W(I,J)*((Y(I,J)-XMU(2))**2))
   B=0.
   DO 95 I=17,23
   DO 95 J=1,9
   XJ=J
   95 B=B+(W(I,J)*((Y(I,J)-YIN(46)-DYX(46)*XJ)**2))
   AAA=A-B
   C=0.
   DO 100 I=17,23
   DO 100 J=1,9
   XJ=J
   100 C=C+(W(I,J)*((Y(I,J)-YIN(I)-DYX(46)*XJ)**2))
   BBB=B-C
   DDD=0.
   DO 500 I=17,23
   DO 500 J=1,9
   XJ=J
   500 DDD=DDD+(W(I,J)*((Y(I,J)-YIN(I)-DYX(I)*XJ)**2))
   CCC=C-DDD
PUNCH 122,AAA
PUNCH 123,BBB
PUNCH 124,CCC
PUNCH 125,DDD

122 FORMAT (6HAAA = *E14.6)
123 FORMAT (6HBBB = *E14.6)
124 FORMAT (6HCCC = *E14.6)
125 FORMAT (6HDDD = *E14.6)
PRINT 126

126 FORMAT (15HEND OF PROGRAM.)
GO TO 1
END

PART 4B THESIS DATA ANALYSIS.
COVARIANCE ANALYSIS ON REGRESSION LINES.
HYPOTHESIS TESTS.
LENGTH 15296

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DIMENSION AAA(5), RBB(5), CCC(5), DDD(5), FT(5,4), FC(5,4)
1 READ 9, (AAA(I), I=1,5), (RBB(I), I=1,5), (CCC(I), I=1,5),
1 (DDD(I), I=1,5)
2 READ 99, (FT(I,J), I=1,5, J=1,4)
9 FORMAT (6X*E14.6)
99 FORMAT (7X*F12.5)
DO 5 I=1,5
FC(I,1) = ((AAA(I)+RBB(I)+CCC(I))/DDD(I))
FC(I,2) = (RBB(I)/DDD(I))
FC(I,3) = (CCC(I)/(DDD(I)+RBB(I)))
5 FC(I,4) = (AAA(I)/(DDD(I)+RBB(I)))
DO 45 I=1,5
IF (FC(I,1)-FT(I,1)) 10,15,15
10 PUNCH 101, I
101 FORMAT (39HHYPOTHESIS ONE HOLDS FOR CULTURE GROUP ,I1)
GO TO 45
15 IF (FC(I,2)-FT(I,2)) 20,25,25
20 PUNCH 102, I
102 FORMAT (39HHYPOTHESIS TWO HOLDS FOR CULTURE GROUP ,I1)
25 IF (FC(I,3)-FT(I,3)) 30,35,35
30 PUNCH 103, I
103 FORMAT (41HHYPOTHESIS THREE HOLDS FOR CULTURE GROUP ,I1)
35 IF (FC(I,4)-FT(I,4)) 40,45,45
40 PUNCH 104, I
104 FORMAT (40HHYPOTHESIS FOUR HOLDS FOR CULTURE GROUP ,I1)
45 CONTINUE
GO TO 1
END