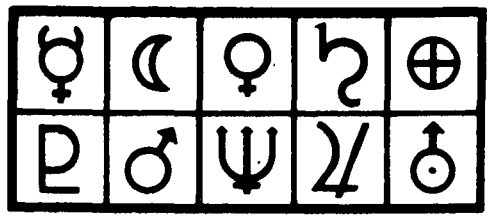
Research Report



PLANETARY QUARANTINE

SC-RR-71 0681
September 1971

A COMPUTERIZED PROGRAM FOR STATISTICAL TREATMENT OF BIOLOGICAL DATA

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NOTICE

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Completed - September 1971

Abstract

Biologists frequently conduct experiments which measure the patterns of inactivation of bacterial populations after exposure to a lethal environment. This document discusses a computer program which calculates many of the quantities that have proven to be useful in the analysis of such experimental data.

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Introduction

In the programs now underway in the Planetary Quarantine Department, it is frequently necessary to compare subtle changes in the destruction pattern of microorganisms. The use of standard pour plate techniques[1,2] for microbial assay during experimentation in some cases yields hundreds of data bits (plate counts). These must be reduced in a way that these successive samples taken during process application represent the destruction rate of microorganisms as a consequence of the process. This destruction rate is best described by a survivor curve since it relates the number of surviving organisms at any time to the sterilization process. The survivor curve is usually a y-axis plot of the logarithm of the number of organisms surviving the sterilization treatment versus the equivalent process time on the x-axis. This process time versus log of survivors or logarithmic model seems to be the most practical representation of data since essentially all thermoradiation and most heat and radiation sterilization has exhibited the logarithmic order of destruction. Consequently, the comparison of treatments can be made on the basis of the slope of the survivor curve or the D-value determined from the slope.

Based on this rationale, a computerized program has been developed to handle the statistical aspects of the data reduction. With plate counts of each successive sampling periods as an input, the program computes the mean value of the replicate plate counts, the variance, standard deviation, upper and lower .95 confidence intervals and the coefficient of variation for each sampling interval. Based on the coefficient of variation values for a sampling period, the dilution or data set exhibiting the best values are selected for each period. These best sets are then used in computing the survivor curve based on a least square fit of the logarithmic model.

Determination of Survivors

At any specific sampling period the procedure for assay is as follows: Four replicate samples are generally used for each sampling period. Aluminum foils or 0.020" thick square planchets are used as a substrate for the test organisms. After exposure to the sterilization treatment the substrate material is placed in a beaker with 10 ml sterile water and insonated for two minutes to suspend the organisms. From this base suspension, measured amounts of the inoculum are transferred to

petri dishes or additional dilution blanks[3] as required to result in plate counts between 30 and 300 colonies per plate. Within this range, the counts can be accurate, and the possibility of interference of the growth of an organism with that of another is minimized.

The determination of viable population from the resultant plate count is made as follows:

> Using the arrangement of dilution*, Figure 1, the inoculum from each of the four replicate samples for a single time period is plated in duplicate. Consequently, there are eight plates for each sampling period at a single level of dilution. Sometimes as many as three dilutions are plated out with the best set of data used as the surviving population at that sampling period.

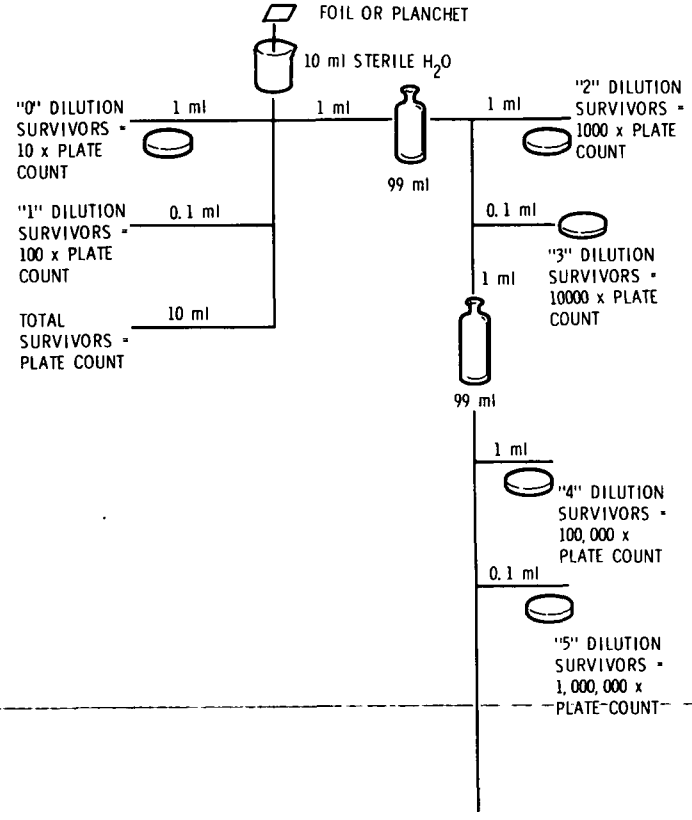

Figure 1. Sample Assay and Dilution Procedure

*For consistency in the input data, the total survivor plate counts will be assigned on order of dilution of "-1".

Statistical Methods

If we consider a single microorganism of a given type, we see that its loss of viability in a lethal environment is a random event. This fact has been explained in terms of natural variations between microorganisms brought about, in part, by their past history and by the hypothesis that loss of viability is due to the occurrence of chemical reactions[4]. In modeling the inactivation of microorganisms, researchers have usually attempted to derive expressions for the probability of single spore survival as a function of time of exposure to a given environment.

As we have pointed out earlier, instead of looking at the inactivation of a single spore, an experimenter considers the number of survivors in a given population as a function of time. We shall let the random variable $N(t)$ be the number of survivors at time t and let $p(t)$ represent the probability of single spore survival at time t. The model we shall assume defines the conditional probability as

$$\text{Prob.}\{N(t) = k | N(0) = N_o\} = \binom{N_o}{k} [p(t)]^k [1 - p(t)]^{N_o - k} . \tag{1}$$

Using the definition of conditional probabilities we have

$$\text{Prob.}\{N(t) = k\} = \sum_{N_o = 0}^{\infty} \text{Prob.}\{N(0) = N_o\} \ \text{Prob.}\{N(t) = k | N(0) = N_o\} . \tag{2}$$

Combining (1) and (2) yields

$$\text{Prob.}\{N(t) = k\} = \sum_{N_o = 0}^{\infty} \binom{N_o}{k} [p(t)]^k [1 - p(t)]^{N_o - k} \ \text{Prob.}\{N(0) = N_o\} . \tag{3}$$

We are usually interested in the expected value of the number of survivors as a function of time. Using the expression (3) it can be shown that

$$E(N(t)) = E(N(0))\, p(t) . \tag{4}$$

This is the basic expression for our model. In particular, the most widely used expression for the probability of single spore survival is provided by what is known

as the log model. Using this model we would have

$$p(t) = 10^{-t/D}$$

and thus (4) becomes

$$E(N(t)) = E(N(0))\ 10^{-t/D}. \tag{4'}$$

In this model, D is assumed to be a fixed parameter for a microorganism belonging to a homogeneous population.

Let us return for a moment to the experimental method used and consider the quantities we wish to compute for each dilution and each time period. Let us define

$x_{ij}(t_\ell)$ = number of colonies on plate i of dilution j at sampling period ℓ,

$\ell = 1, \ldots, M$

$j = 1, \ldots, K_\ell$

$i = 1, \ldots, N_{j\ell}$

where

M = number of sampling periods

K_ℓ = number of dilutions at sampling period ℓ

$N_{j\ell}$ = number of plates of dilution j for sampling period ℓ

and

t_ℓ = time of sampling period ℓ (in any units desired).

The mean of the plate counts for a particular dilution and sampling period is

$$\bar{x}_j(t_\ell) = \frac{1}{N_{j\ell}} \sum_{i=1}^{N_{j\ell}} x_{ij}(t_\ell)$$

while the variance of the distribution of plate counts can be approximated by the sample variance which is given by

$$s_j^2(t_\ell) = \frac{\sum_{i=1}^{N_{j\ell}} (\bar{x}_j(t_\ell) - x_{ij}(t_\ell))^2}{N_{j\ell} - 1}$$

$$= \frac{\sum_{i=1}^{N_{j\ell}} x_{ij}(t_\ell)^2 - N_{j\ell}\,\bar{x}_j(t_\ell)}{N_{j\ell} - 1} \quad . \tag{5}$$

Similarly, the standard deviation is approximated by the sample standard deviation which is given by

$$s_j(t_\ell) = \sqrt{s_j^2(t_\ell)} \quad . \tag{6}$$

To be more precise, let $\sigma_j^2(t_\ell)$ be the variance in the plate counts (this includes natural variation as well as any errors). The sample variance is a random variable which depends on the counts of the replicate plates. It can be shown that

$$\sigma_j^2(t_\ell) = E(s_j^2(t_\ell)) \quad .$$

Another desirable quantity for each dilution at each time interval is the confidence interval for the mean. This confidence interval is given for a particular time period by the expression

$$[L_j(t_\ell),\ U_j(t_\ell)] = \left[\bar{x}_j(t_\ell) - \frac{k s_j(t_\ell)}{\sqrt{N_{j\ell}}},\ \bar{x}_j(t_\ell) + \frac{k s_j(t_\ell)}{\sqrt{N_{j\ell}}}\right] \quad . \tag{7}$$

It is well known that as the number of samples, $N_{j\ell}$, becomes larger the parameter k for the α confidence limit should be chosen as the 100 $\alpha/2$ percentage point of the normal distribution. Thus, for the .95 confidence, k = 1.96 if $N_{j\ell}$ is large. Unfortunately, the number of plates of a given dilution at a given sampling time is usually small. In this case, the 100 $\alpha/2$ percentage point of the Student's t-distribution with $N_{j\ell} - 1$ degrees of freedom is more appropriate for k. Thus we can approximate k to sufficient accuracy by

$$k = \chi \left[1 + \frac{\chi^2 + 1}{4(N_{j\ell} - 1)} + \frac{(\chi^2 + 3)(5\chi^2 + 1)}{96(N_{j\ell} - 1)^2} \right] , \qquad (8)$$

where χ is the 100 $\alpha/2$ percentage point of the normal distribution. For our .95 confidence interval we let $\chi = 1.96$ in (8) to get our k for (7).

A good measure of the amount of spread in a particular set of data has been found to be the relative standard deviation[5]. This is more commonly known as the coefficient of variation. For each sampling period and each dilution it is defined to be

$$C_j(t_\ell) = \frac{s_j(t_\ell)}{\bar{x}_j(t_\ell)} .$$

In calculating the fit to the data of our "straight line" model, we wish to use the dilution at each time period which has the "tightest" data. We shall use the coefficient of variation as an index of the spread. Therefore, we let

$$X(t_\ell) = \bar{x}_J(t_\ell)$$

and

$$\sigma^2(t_\ell) = \sigma_J^2(t_\ell)$$

where J is chosen to minimize $C_j(t_\ell)$ for $j = 1, \ldots, K_\ell$. Let the order of this dilution (as defined in Figure 1) be d_ℓ.

We are now prepared to again consider the problem of applying our model to the data. Let

$$Y(t_\ell) = X(t_\ell) \times 10^{d_\ell + 1} . \qquad (9)$$

Then $Y(t_\ell)$ is an estimate of $E(N(t_\ell))$. In our model we wish to use $Y(t_\ell)$, $\ell = 1, \ldots, M$ to determine E(N(0)) and D as accurately as possible and to obtain some measurements of the statistical variations. Taking the natural log on both sides of (4') we obtain

$$\log E(N(t)) = \log (E(N(0)) + \gamma t , \qquad (10)$$

where

$$\gamma = \frac{2.303}{D} \quad . \tag{11}$$

With this model in mind, let us consider the equation

$$y(t) = \alpha + \beta t + \epsilon \tag{12}$$

where ϵ is a random variable representing the variation of the measured values about the line $\alpha + \beta t$.

Comparing (10) and (12) we see that we are assuming that

$$\alpha = \log (E(N(0))$$

or

$$E(N(0)) = e^{\alpha} \tag{13}$$

and

$$\beta = \frac{2.303}{D} = \gamma \quad . \tag{14}$$

The random variable ϵ in (12) represents the variation of the mean of the plate counts from the log model. This is assumed to be independent of time. This is consistent with assuming that the distribution of the variation in plate counts from the log model is independent of time. Let

$$y_{\ell} = \log Y(t_{\ell}) \quad .$$

Then y_{ℓ} is a sampled value of the random variable $\log E(N(t_{\ell}))$. Let us assume that ϵ is normally distributed and that

$$E(\epsilon) = 0.$$

For later convenience, let the variance of the distribution of ϵ be represented by σ_{ϵ}^2.

We are now prepared to calculate α and β. The following definitions will prove valuable:

1. $$\bar{t} = \frac{\sum_{\ell=1}^{M} t_\ell}{M} \tag{15}$$

2. $$\bar{y} = \frac{\sum_{\ell=1}^{M} y_\ell}{M} \tag{16}$$

3. $$b = \frac{\sum_{\ell=1}^{M} (t_\ell - \bar{t})(y_\ell - \bar{y})}{\sum_{\ell=1}^{M} (t_\ell - \bar{t})^2} = \frac{\sum_{\ell=1}^{M} (t_\ell - \bar{t})\, y_\ell}{\sum_{\ell=1}^{M} (t_\ell - \bar{t})^2} \tag{17}$$

4. $$a = \bar{y} - b\bar{t} \tag{18}$$

The quantities a and b depend on the samples used. The Gauss-Markoff theorem[6] tells us that a and b coincide with the maximum likelihood estimates of α and β and that they are unbiased, i.e.,

$$E(a) = \alpha$$

and

$$E(b) = \beta .$$

Letting

$$Z(t) = a + bt$$

and defining the standard error of estimate, $S_{y|t}$, by

$$S^2_{y|t} = \frac{\sum_{\ell=1}^{M} (Z(t_\ell) - y_\ell)^2}{M - 2}, \tag{19}$$

the Gauss-Markoff theorem also tells us that $S^2_{y|t}$ is an unbiased estimate of σ^2_ϵ, i.e.,[7]

$$E(S^2_{y|t}) = \sigma^2_\epsilon .$$

In addition to the Gauss-Markoff theorem, our assumptions on ϵ imply that a and b are also the minimum variance unbiased estimates of α and β respectively from among the class of all linear estimates. The standard deviation in b, which is given by

$$\sigma_b = \sqrt{\sigma_\epsilon^2 \Big/ \sum_{\ell=1}^{M} (t_\ell - \bar{t})^2}$$

can be approximated by the standard error in the slope,

$$S_b = \frac{S_{y|t}}{\sqrt{\sum_{\ell=1}^{M} (t_\ell - \bar{t})^2}} \quad .$$

Similarly, the standard deviation of the distribution of a,

$$\sigma_a = \sigma_\epsilon \sqrt{\frac{1}{M} + \frac{\bar{t}}{\sum_{\ell=1}^{M} (t_\ell - \bar{t})^2}}$$

can be approximated by the standard error in a*,

$$S_a = S_{y|t} \sqrt{\frac{1}{M} + \frac{\bar{t}}{\sum_{\ell=1}^{M} (t_\ell - \bar{t})^2}} \quad ,$$

where $S_{y|t}$ is given by (19).

It is also desirable in many applications to have a measure of how closely the variation in the log of the means of plate counts can be explained on the basis of only the variation of time in the lethal environment. The correlation coefficient, r, is defined by

*The quantity S_a shall be called the standard error in the estimated intercept.

$$r = \frac{\sum_{\ell=1}^{M} (t_\ell - \bar{t})(y_\ell - \bar{y})}{\sqrt{\sum_{\ell=1}^{M} (t_\ell - \bar{t})^2 \sum_{\ell=1}^{M} (y_\ell - \bar{y})^2}} = \frac{\sum_{\ell=1}^{M} t_\ell y_\ell}{\sqrt{\sum_{\ell=1}^{M} t_\ell^2 \sum_{\ell=1}^{M} y_\ell^2}}$$

Feller[8] proves the following statements can be made concerning r:

1. $|r| \leq 1$
2. $r = \pm 1$ implies that there exists constants ρ and θ such that $y = \rho t + \theta$ (except for a set of lines which have zero probability of occurring).

In addition, it can be shown that if y and t are independent, then $r = 0$. The converse of this statement is not true, however.

Let us return for a moment to the probability of single spore survival. Most microbiologists are interested in the D-value of the population. We have shown that we can approximate the D-value by

$$D = \frac{1}{b \log_{10} e} = \frac{2.303}{b} .$$

In addition, the standard error in the estimated D is given by

$$S_D = \frac{2.303\, S_b}{b^2} = \frac{D\, S_b}{b} .$$

Another feature which it is sometimes desirable to have available is the confidence band about the curve representing the model. This is also easily computed[6]. Let

$$S_{Z(t_\ell)} = S_{y|t} \sqrt{\frac{1}{M} + \frac{t_\ell - \bar{t}}{\sum_{\ell=1}^{M} (t_\ell - \bar{t})^2}}$$

Then the upper 95% confidence line is given by

$$Z_u(t_\ell) = a + b\, t_\ell + k\, S_{Z(t_\ell)}$$

and the lower by

$$Z_L(t_\ell) = a + b\,t_\ell - k\,S_{Z(t_\ell)}$$

where k is given by the Student's t-distribution of degree M-2. For the .95 confidence interval k is approximated by

$$k = \chi\left[1 + \frac{\chi^2 + 1}{4(M-2)} + \frac{(\chi^2 + 3)(5\chi^2 + 1)}{96(M-2)^2}\right]$$

where $\chi = 1.96$.

The Program

The flow chart for the program is given in Figure 2. This is self-explanatory. The input is prepared in the manner illustrated in Figure 3. The output is described in Figure 4 using the notation of the previous section.

Figure 5 provides an example of the input data while Figure 6 gives the output from the use of the progtam on this example.

Finally, a graphical representation of the data, the model, and the .95 confidence interval is shown graphically in Figure 7.

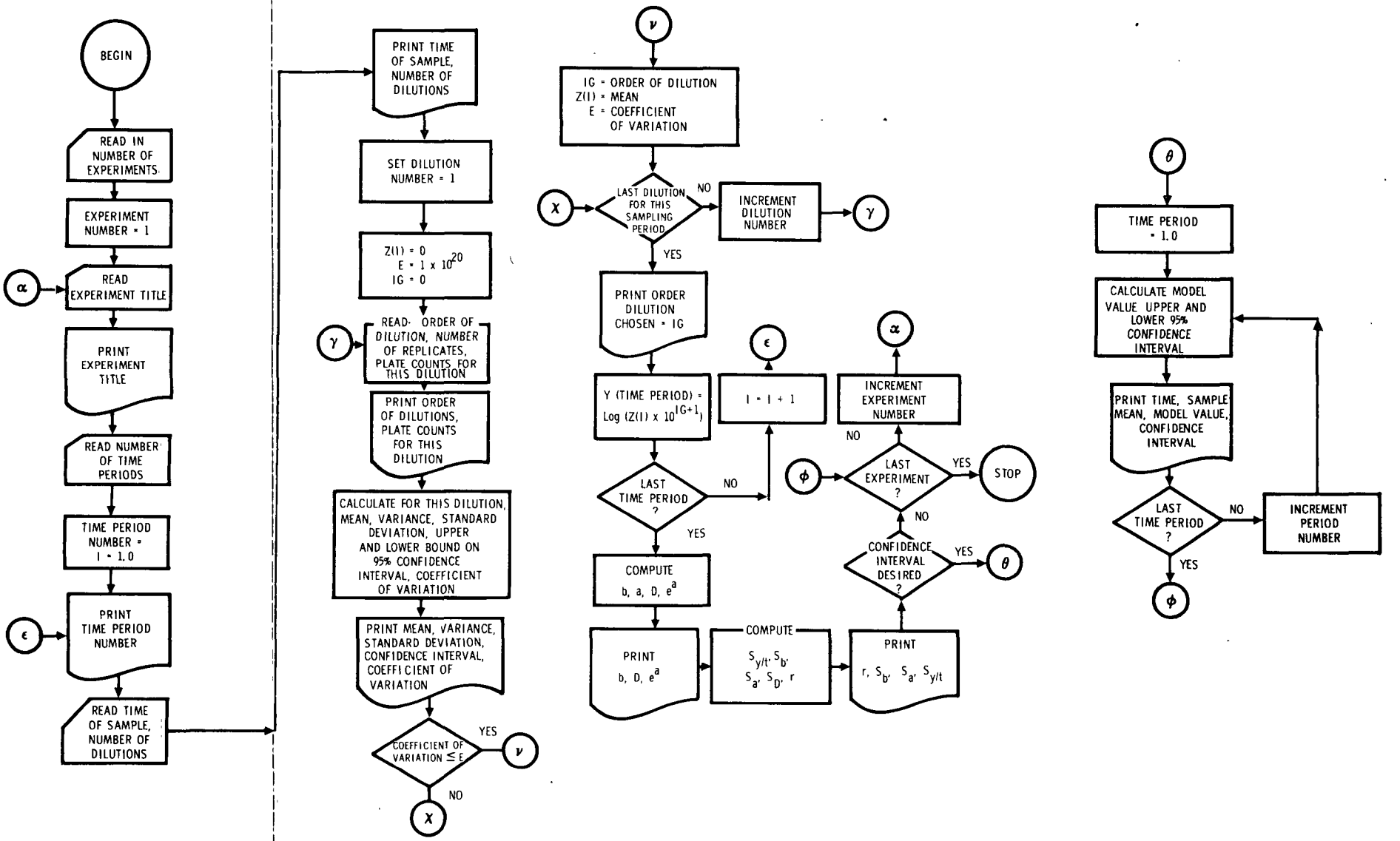

Figure 2. Program Flow Chart

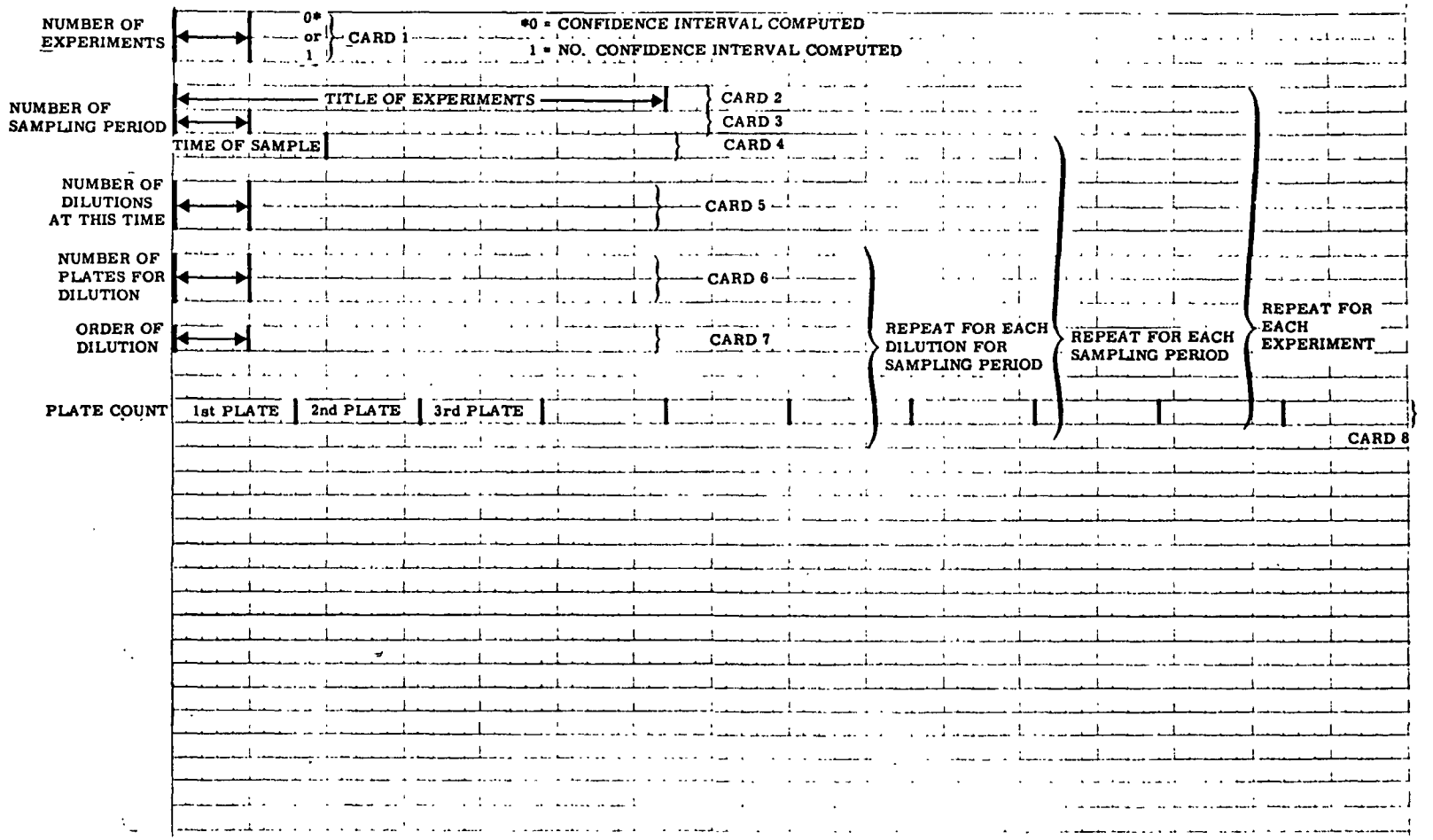

Figure 3. Input Format

TITLE OF EXPERIMENT

Repeat for each sampling time period:

Data Set = ____

Time = ____

Number of Dilutions = ____ (For this time period)

Repeat for each dilution:

Number of Data Points = ____ (Number of plate counts for this time period and dilution)

Order of Dilution = ____

Date (Plate Counts):

Mean = ____ Variance = ____ S.D. = ____ (Standard Deviation) Upper .95 C.I. = ____ (Upper limit of 95% Confidence Interval)(C.I.) Lower .95 C.I. = ____ C.V. = ____ (Coefficient of Variation)

Dilution Chosen = ____ (Dilution chosen for this time period on the basis of the coefficient of variation)

Slope = ____ (Slope of line of best fit to log of selected means) D-Value = ____ Intercept = ____ (Theoretical value of initial population*)

Corr. Coef. = ____ Stand. Err. in Est. Slope = ____ Stand. Err. of Est. = ____ Stand. Err. of Est. Inter. = ____

		.95 Conf. Interval (95% Confidence Interval about Model)		
T (Time)	SAMP (Data)	MODEL (Log Model Value)	Upper (Upper Limit of 95% Confidence Band)	Lower (Lower Limit of 95% Confidence Band)
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

*In computer output, E + 05 means 10^5 e.g., 2.31E + 06 represents 2.31×10^6

Figure 4. Output Format

```
    1
24 NOVEMBER 1970
    8
0.0
    1
    8
    3
265.     282.     299.     267.     250.     277.     265.     285.
3.0
    1
    8
    3
67.      58.      62.      57.      51.      75.      80.      72.
6.0
    1
    8
    2
58.      63.      80.      83.      62.      66.      75.      63.
9.0
    1
    8
    1
278.     212.     197.     201.     214.     255.     236.     231.
12.0
    1
    8
    1
48.      37.      37.      25.      25.      21.      42.      29.
15.0
    1
    8
    0
116.     135.     87.      62.      95.      82.      85.      84.
18.0
    2
    8
    0
26.      16.      12.      14.      11.      10.      18.      9.
    4
   -1
157.     117.     92.      162.
21.0
    1
    4
   -1
51.      50.      93.      89.
```

Figure 5. Example of Input Data

```
                                        24 NOVEMBER 1970

 DATA SET =     1
TIME=    0.000
 NO. DIL.=     1
NUMBER DATA POINTS=     8
 ORDER OF DIL. =     3
DATA
   265.00    282.00    292.00    267.00    250.00    277.00    265.00    285.00
MEAN= 272.875  VARIANCE=    135.0  S.D.=     13.6  UPPER .95 C.I.=    284.2  LOWER .95 C.I.=    261.5  CV =    .0498
 CIL. CHOSEN =     3

 DATA SET =     2
TIME=    3.000
 NO. DIL.=     1
NUMBER DATA POINTS=     8
 ORDER OF DIL. =     3
DATA
    67.00     58.00     62.00     57.00     51.00     75.00     80.00     72.00
MEAN=  65.250  VARIANCE=     99.4  S.D.=     10.0  UPPER .95 C.I.=     73.6  LOWER .95 C.I.=     56.9  CV =    .1528
 CIL. CHOSEN =     3

 DATA SET =     3
TIME=    6.000
 NO. DIL.=     1
NUMBER DATA POINTS=     8
 ORDER OF DIL. =     2
DATA
    58.00     63.00     80.00     83.00     62.00     66.00     75.00     63.00
MEAN=  68.750  VARIANCE=     86.2  S.D.=      9.3  UPPER .95 C.I.=     76.5  LOWER .95 C.I.=     61.0  CV =    .1351
 CIL. CHOSEN =     2

 DATA SET =     4
TIME=    9.000
 NO. CIL.=     1
NUMBER DATA POINTS=     8
 ORDER OF DIL. =     1
DATA
   278.00    212.00    197.00    201.00    214.00    255.00    236.00    231.00
MEAN= 228.000  VARIANCE=    777.7  S.D.=     27.9  UPPER .95 C.I.=    251.2  LOWER .95 C.I.=    204.8  CV =    .1223
 CIL. CHOSEN =     1

 DATA SET =     5
TIME=   12.000
 NO. DIL.=     1
NUMBER DATA POINTS=     8
 ORDER OF DIL. =     1
DATA
    48.00     37.00     37.00     25.00     25.00     21.00     42.00     29.00
MEAN=  33.000  VARIANCE=     99.4  S.D.=      9.5  UPPER .95 C.I.=     40.9  LOWER .95 C.I.=     25.1  CV =    .2866
 CIL. CHOSEN =     1

 DATA SET =     6
TIME=   15.000
 NO. DIL.=     1
NUMBER DATA POINTS=     8
 ORDER OF DIL. =     0
DATA
   116.00    135.00     87.00     62.00     95.00     82.00     85.00     84.00
MEAN=  93.250  VARIANCE=    508.5  S.D.=     22.5  UPPER .95 C.I.=    112.0  LOWER .95 C.I.=     74.5  CV =    .2418
 CIL. CHOSEN =     0

 DATA SET =     7
TIME=   18.000
 NO. DIL.=     2
NUMBER DATA POINTS=     8
 ORDER OF DIL. =     0
DATA
    26.00     16.00     12.00     14.00     11.00     10.00     18.00      9.00
MEAN=  14.500  VARIANCE=     30.9  S.D.=      5.6  UPPER .95 C.I.=     19.1  LOWER .95 C.I.=      9.9  CV =    .3831
NUMBER DATA POINTS=     4
 ORDER OF DIL. =    -1
DATA
   157.00    117.00     92.00    162.00
MEAN= 132.000  VARIANCE=   1116.7  S.D.=     33.4  UPPER .95 C.I.=    183.2  LOWER .95 C.I.=     80.8  CV =    .2532
 CIL. CHOSEN =    -1

 DATA SET =     8
TIME=   21.000
 NO. DIL.=     1
NUMBER DATA POINTS=     4
 ORDER OF DIL. =    -1
DATA
    51.00     50.00     93.00     89.00
MEAN=  70.750  VARIANCE=    549.6  S.D.=     23.4  UPPER .95 C.I.=    106.7  LOWER .95 C.I.=     34.8  CV =    .3314
 CIL. CHOSEN =    -1


SLOPE=    -.521       D VALUE=    4.421       INTERCEPT=     2.3104308639E+06
CORR. COEF.=     .98018 STAND. ERR. IN EST. SLOPE=     .01896 STAND. ERR. OF EST.=     .13595 STAND. ERR. IN EST. INTER.=     .19913
                                          .95 CONF. INTERVAL
         T                    SAMP                  MODEL                 UPPER                 LOWER
    0.                  2.7287500000E+06      2.3104308639E+06      4.1234704598E+06      1.2945626333E+06
    3.0000000000E+00    6.5250000000E+05      4.8409570870E+05      7.7422063309E+05      3.0268975685E+05
    6.0000000000E+00    6.8750000000E+04      1.0143071530E+05      1.4820424844E+05      6.3418995170E+04
    9.0000000000E+00    2.2800000000E+04      2.1252388364E+04      2.9406330336E+04      1.5359414317E+04
    1.2000000000E+01    3.3000000000E+03      4.4529313417E+03      6.1613954984E+03      3.2131991139E+03
    1.5000000000E+01    9.3250000000E+02      9.3300560831E+02      1.3632497272E+03      6.3854732390E+02
    1.8000000000E+01    1.3200000000E+02      1.9548908311E+02      3.1264826143E+02      1.2223314929E+02
    2.1000000000E+01    7.0750000000E+01      4.0960077062E+01      7.3102238390E+01      2.2950431476E+01
```

Figure 6. Example of Output Data

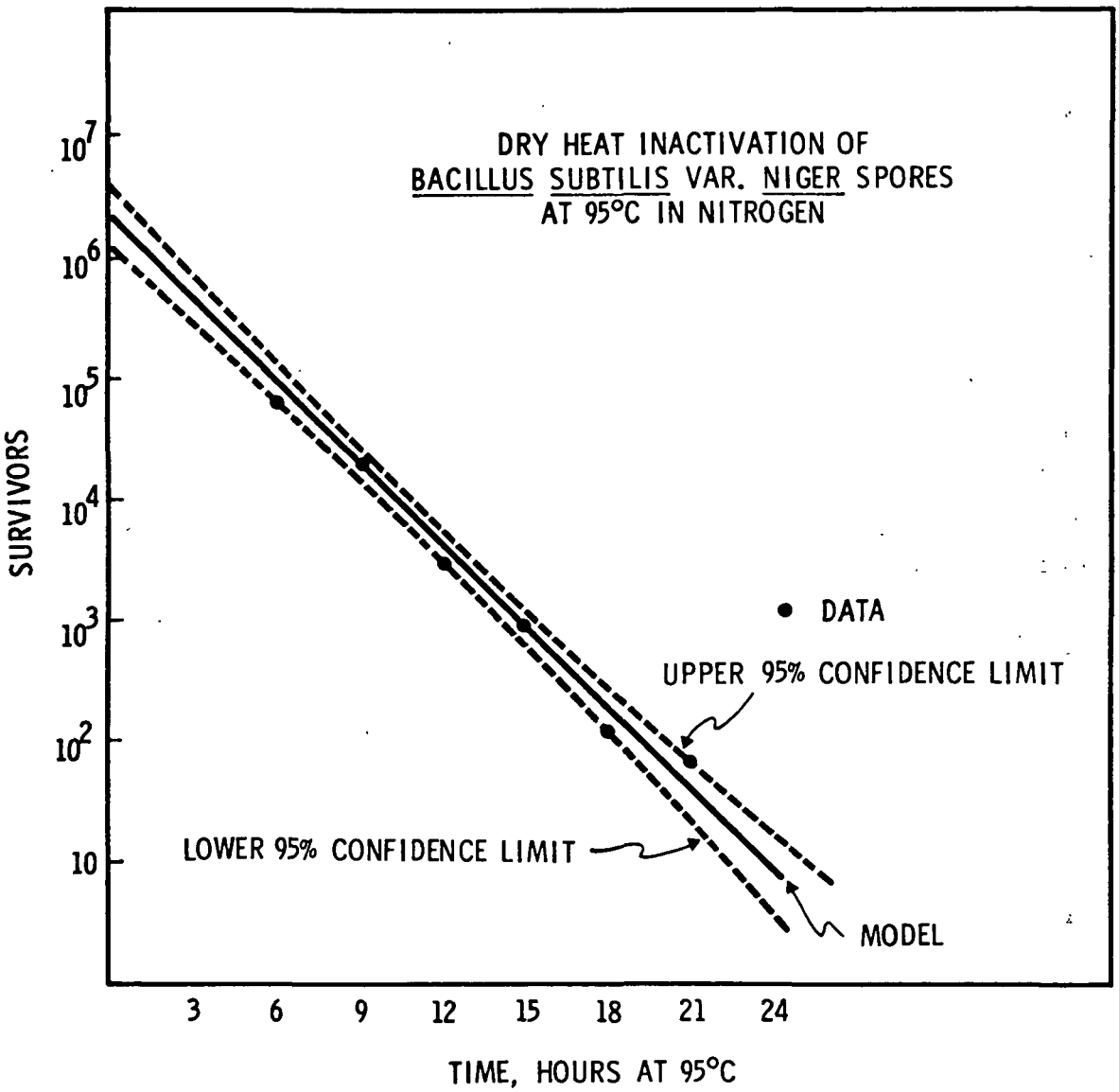

Figure 7. Graphical Representation of Program Output

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