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Experimental and Mathematical Techniques for Kinetic Studies of Larval Fruit Fly Growth[1]

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Abstract. Techniques have been developed in our laboratory which permit the study of growth patterns under a variety of experimental conditions. The experimental subject under investigation is the larva of the common fruit fly *Drosophilia melanogaster.* Studies involving exposure of the organism to thermal, gravitational, electromagnetic, and magnetic agents have already been conducted. A particular advantage of employing the larvae is that they possess a relatively rapid and simple growth pattern from which experimental constants can be obtained and statistically analyzed. During their 96-hour growth period, the approximate time from the hatching of the egg to pupae formation, the larvae need only be interrupted for about one-half hour each day for purposes of measurement. Growth patterns have been fitted to curves predicted by Compound-Interest and Verhulst-Reed-Pearl Equations. During the early growth phase excellent fit can be obtained to the exponential curve predicted by the former equation.

In this paper a fairly simple means is presented by which one may study the phenomenon of growth under various experimental conditions. Larvae of the common fruit fly, *Drosophila melanogaster,* are used as the experimental subject. Because of their relatively simple and rapid growth pattern, kinetic analysis may be made with considerable statistical significance. Their size and relative ease of handling permit chronic exposure of the organisms to many physical agents. Investigations conducted in our laboratory using techniques outlined in this text have concerned growth studies influenced by thermal (1), gravitational (2), electro-magnetic (3), and magnetic agents. A detailed description of our techniques was not considered appropriate in the aforementioned publications. The primary purpose of this paper, therefore, is to elucidate the principles of our technique, thereby providing an adequate foundation for studies of this type by other investigators.

GROWTH

The definition of growth has been, and still is, a subject of extensive discussion and controversy. Mathematical analyses concerning innumerable factors which influence this dynamic process have been formulated. It is not the purpose of this paper to redefine growth nor to criticize the existing definitions.

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Proceedings of the Iowa Academy of Science, Vol. 68 [1961], No. 1, Art. 84

but merely to present a useful tool for the study of this phenomenon.

Growth, according to Wetzel (4), is considered to be that fundamental property of living things concerned only with their changes in size or number. In a similar manner, Thompson (5) defines growth to be variations in magnitudes taken over successive intervals of time. Essentially, then, growth may be thought of as a rate of change in the amount of living material expressed mathematically as

$$\frac{dv}{dt} \qquad \text{(i)}$$

where v is the amount of living material and t is time. Julian Huxely (6) proceeds to state that growth is a self multiplication of living substance. Thus, the following relationship may be applied

$$\frac{dv}{dt} = kv \qquad \text{(ii)}$$

in which k is the well-known growth constant. This constant or growth index represents the relative rate of growth

$$k = \frac{1}{v}\frac{dv}{dt} \qquad \text{(iii)}$$

or

$$k = \frac{\triangle \ln v}{\triangle t} \qquad \text{(iv)}$$

The dependent variable may be mass, volume, or any other characteristic dimension. Equation (ii) is a geometrical progression which applies only to the simplest considerations of growth so long as living conditions are optimum. It is commonly referred to as the Compound-Interest Equation for growth.

A slightly more complex formulation, yet seemingly practical, is the Verhulst-Reed-Pearl Equation

$$v = \frac{V}{Ce^{-pVt} + 1} \qquad \text{(v)}$$

where v is the volume at any time, t, V the limiting or final volume of the organism, and C and p are constants. When the value, v, is sufficiently smaller than V, pV is similar to the growth constant, k, and the equation reduces to the Compound-Interest form. Equation (v) is characterized by its sigmoid shape and predicts the well-known logistic curve found in many growth analyses (7, 8).

Although it does not describe the course of larval growth perfectly, it was felt that the errors in this method would be smaller than those in the initial slope method of the Compound-Interest Equation. However, upon applying both equations to data acquired from a group of experimental and control animals, one finds the initial slope method of equation (ii) to have the best fit during the initial growth stages. By rewriting equation (v) in the form

$$-\ln\left(\frac{V}{v} - 1\right) = p\ Vt + C \qquad \text{(vi)}$$

one can readily see that pV is really an index of how rapidly v approaches V (Figure 1). It therefore seems more reasonable to compare the absolute increase in total protoplasm per unit of protoplasm than the increase relative to the final size; so the relative growth rate index of equation (iii) has been adopted.

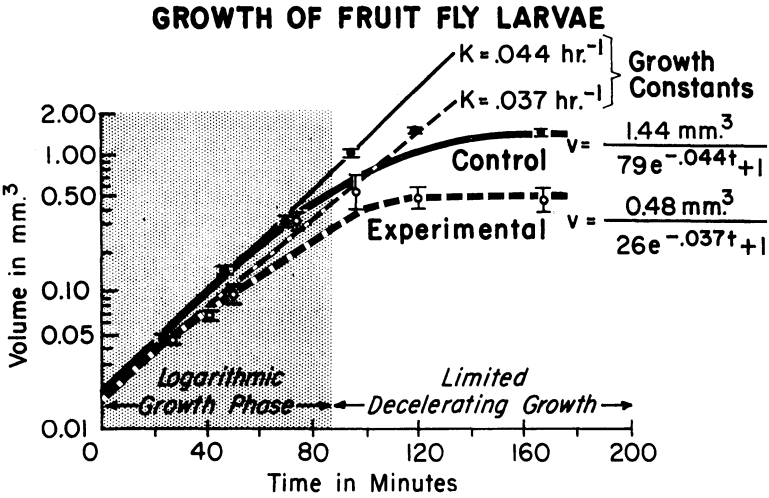


Figure 1. The thin lines are predicted by the Compound-Interest Equation, and heavier curves by the Verhulst-Reed-Pearl Equation. Notice particularly the better fit provided by the linear curves during the logarithmic growth phase than that provided by the Verhulst-Reed-Pearl Equation. In this investigation experimental animals were centrifuged at 2200 G's at a temperature of 32°C.

Larva

Developmental Stages

To say a few words at this point about the time course of larval growth seems appropriate. Information presented in this section has been obtained largely from Demerec (9) and Wigglesworth (10); a more detailed description of larvae and larval growth can be acquired from these authors.

Proceedings of the Iowa Academy of Science, Vol. 68 [1961], No. 1, Art. 84

The time required from the hatching of the egg to the development of the pupa is approximately 96 hours at a temperature of 25°C. During this period the larvae moult twice and pass through three developmental stages, or so-called "instars". The first instar occurs between hatching and the first moult and has a time duration of approximately 25 hours; the second between the first and second moults, lasting about 23 hours, and the final or third instar between the second moult and pupa formation, about 48 hours. The cells of the organism do not all grow in the same manner. According to Demerec the purely larval organs grow by increases in cell size, while the presumptive imaginal organs grow by increases in cell number.

Studies conducted by Alpatov (11) on *Drosophila* larvae have concerned growth measurements for each of the three individual instars. He found the most rapid growth to occur in the third instar, a less rapid growth in the second, and the least rapid growth in the first. Measurements made by Alpatov involved killing the larvae by immersing them in boiling water, preserving them in 70 percent alcohol, and determining their dimensions under a microscope.

In our studies the entire growth of the three instars was studied as a unit and in no case was it necessary to kill the organisms for purposes of measurement.

Culturing Mechanisms

Rather than the commonly used milk bottle for larval culturing, a wooden box is employed for stock cultures of the *Drosophila* larvae (Figure 2). The use of the particular container immensely facilitates feeding, culturing, and handling of the organisms. Cultures which are to be kept under stable conditions may be maintained at room temperature, about 25°C and fed with mashed bananas. However, optimum larval growth rate is attained at a temperature of 28°C and on a nutrient medium consisting of a banana-agar preparation (12) described in Appendix II. This preparation, given about a week prior to an experimental culture, is conducive to a maximum yield. The nutrient media, the mashed bananas or the banana-agar preparation, should be kept moist. This may be accomplished by squirting water from a plastic water bottle through a small hole in the cellulose cover or side of the box. The hole may then be covered with a piece of ordinary masking tape or by cotton plugs.

Although some authors state otherwise, the method found best in our laboratory to collect eggs consists in first withhold-

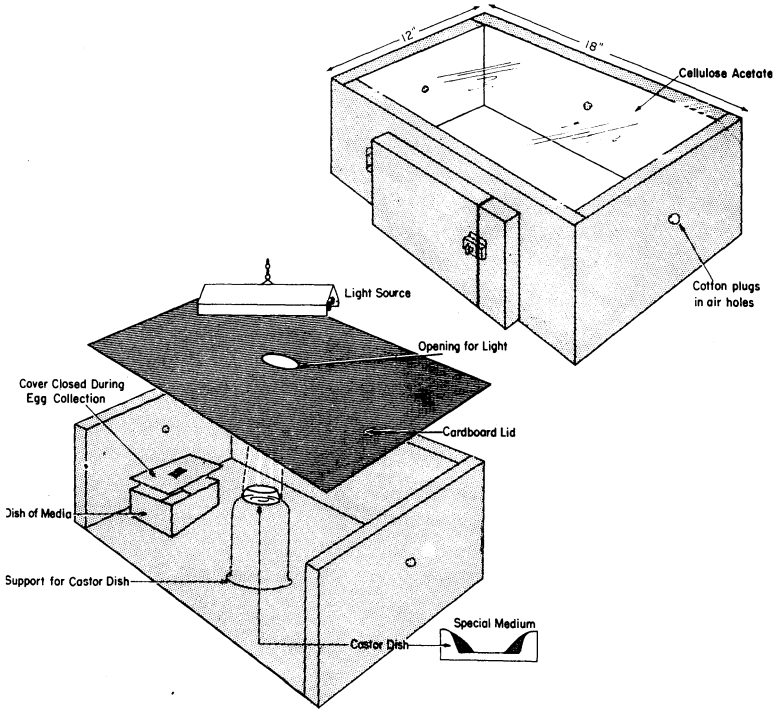


Figure 2. This figure is a schematic illustration of the material and means by which the larvae are cultured and collected. Masking tape can be employed to attach the cellulose acetate and to seal any cracks from which flies may escape.

ing the nutrient medium from the culture for a period of about five hours. Larger egg collections seem to result when the initial fasting takes place through the late morning or early afternoon hours. After the fasting period a castor dish containing a special medium (see Appendix II) is placed into the box. About four drops of formalin-acetic alcohol and a direct light source on the medium have been found to attract more flies (Figure 2). The castor dish is allowed to remain in the box for about five hours; this would be during the late afternoon or early evening hours. An egg collection period of longer than five hours gives scatter to the growth data resulting from a too great variation in larval size. Eggs collected during this time are allowed to incubate at an experimental temperature for 24 hours. During the incubation period each castor dish is placed into a separate covered beaker which has its bottom lined with a water-saturated paper towel. It is possible to collect over 1,000 eggs in a single harvest.

The newly hatched larvae are separated from the medium by gently squirting water down the incline formed by the medium

in the castor dishes. At this stage the larvae remain near the surface and are carried to the center of the dish by the water stream. Very slight strokes with a small, artist's brush will facilitate loosening of the larvae from the medium. They are then transferred with a medicine dropper to a small vial. In the vial the larvae settle to the bottom, thus permitting the removal of excess water by means of the dropper. This procedure is repeated until most of the medium and as much of the water as possible are removed.

Excess water may be removed from the larvae by one of two methods, the choice of which is left to the investigator. One method consists in pouring the larvae from the vial into a clean castor dish where the water may be removed by aspiration through a cotton wad with the medicine dropper. The alternate procedure is to place a piece of white filter paper over a castor dish. Larvae may then be removed from the vial with a dropper and transferred onto the filter paper where excess water is absorbed.

The organisms are then separated into the required number of experimental groups with the artist's brush. The groups are assigned numbers for identification and prepared for photographing.

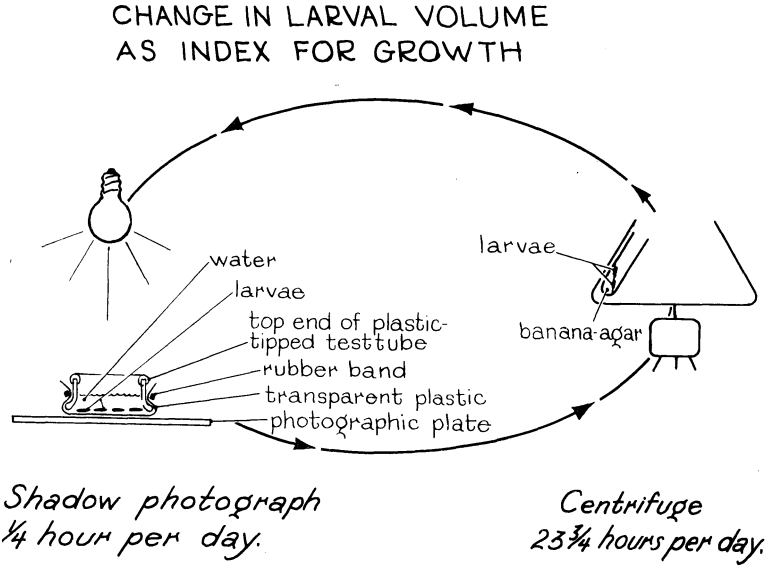
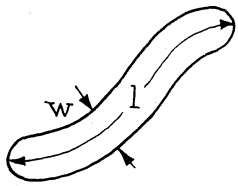


Figure 3. This diagram schematically illustrates how the larvae are photographed as a means of volume determination. Animals should be replaced as soon as possible under experimental conditions. The centrifuge is shown as an example of one of the experimental agents which can be employed.

Photographic Procedure

Initially, and preferrably every 24 hours during the course of experimentation, the larvae are photographed as a means of volume determination. Larvae that die or pupate are photographed separately; depending upon the type of investigation, dead larvae may or may not be included in the results (see Appendix I). Grouped larvae are placed into "camera dishes" which consist of a top end of a plastic centrifuge tube over which a piece of transparent plastic (Saran Wrap®) is stretched (Figure 3). Larvae are photographed under water, pupae dry. Glass photographic plates of medium contrast are used; results are a one to one shadow photograph. Plates should be developed immediately to examine for clarity of reproduction. Shortly after being photographed, the larvae are put into fresh cells and returned to experimental conditions.



$$V \cong \text{Volume of ellipsoid of revolution}$$

$$= \frac{\pi w^2 l}{6}$$

$$K = \text{growth constant} = \frac{dv}{vdt} = \frac{\Delta \log_e v}{\Delta t}$$

$$= \frac{\log_e v_2 - \log_e v_1}{\Delta t} \qquad \text{assuming exponential growth}$$

Figure 4. Larval volume is measured and determined by the method indicated in this figure. Growth constants can then be obtained.

Mathematical Methods

Larval Measurements

The length and width of the larvae are determined by projecting the shadow photographs to approximately a ten-fold magnification with a slide projector and tracing the length and width onto unlined paper. Small pieces of graduated millimeter paper, which can be bent to conform to the shape of the larvae, are used as measuring tapes. Assuming the larvae to take the

Proceedings of the Iowa Academy of Science, Vol. 68 [1961], No. 1, Art. 84

shape of an ellipsoid of revolution, one calculates the volume by the formula

$$v = \frac{\pi}{6} LW^2 \qquad \text{(vii)}$$

where v is volume, L is length, and W the width (Figure 4). A geometric mean of the daily volume of each group may then be found.

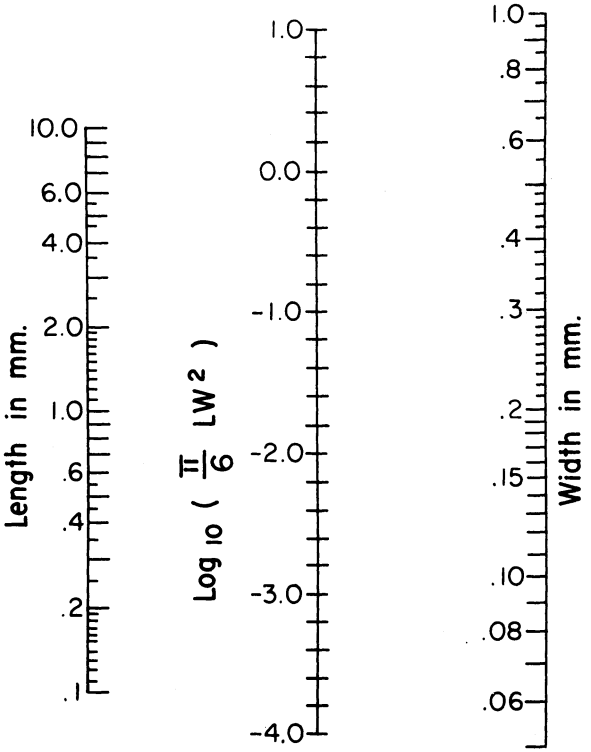


Figure 5. By knowing the length and width of the individual larvae, one can easily determine the logarithms of their respective volumes from this nomogram. These values are for the logarithm to the base of ten. Multiplication of such values by 2.30 gives the natural logarithm so that

$$k = \frac{\Delta \ln v}{\Delta t} = \frac{2.30 \log v}{\Delta t}.$$

Growth Indices

In computing growth indices the logarithms of the larval volumes are used. A nomogram whereby one may directly determine such values from the length and width is reproduced in Figure 5. Histograms of logarithmic values show a somewhat more bell-shaped distribution than do the absolute values of the

volumes (Figures 6 and 7). Therefore, it is assumed in the data analysis that the logarithms of the volumes are more symmetrically distributed than are the absolute volumes. Since larval growth is of the logarithmic type, this is not surprising. Thus standard statistical techniques which assume the logarithms of the volumes to be normally distributed may be employed.

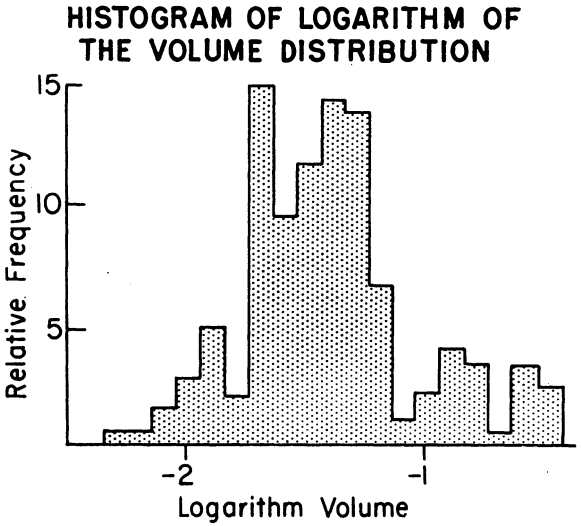


Figure 6. When compared to the distribution of actual volumes illustrated in Figure 7, this figure of the logarithms of the volumes will be shown to more closely approximate a normal distribution.

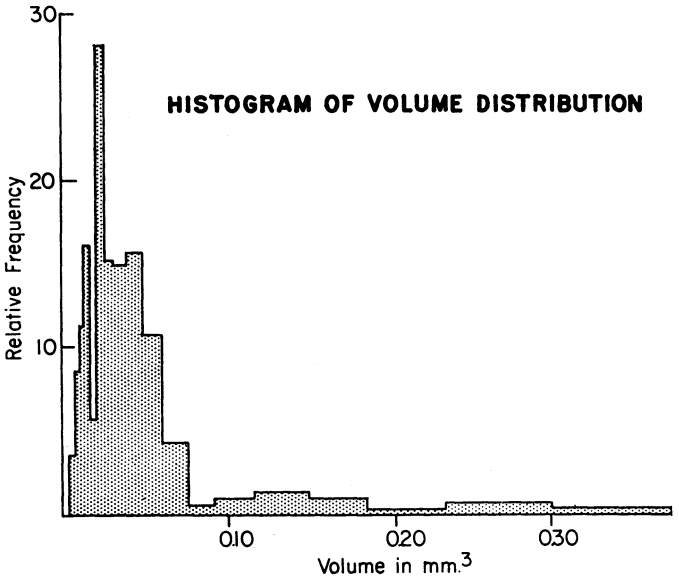


Figure 7. This figure illustrates a skewed distribution obtained from values of the absolute volume.

Proceedings of the Iowa Academy of Science, Vol. 68 [1961], No. 1, Art. 84

The growth constant is calculated from the equation (iv)

$$k = \frac{\triangle \ln v}{\triangle t}$$

Assuming exponential growth one may compute this constant during any phase of the linear growth period determined by $\triangle \ln v/\triangle t$ (Figure 1). The ratio

$$\frac{k_x}{k_c} = \frac{\triangle \ln v_x}{\triangle t_x} \cdot \frac{\triangle t_c}{\triangle \ln v_c} \qquad \text{(viii)}$$

where the subscripts, x and c, refer to experimental and control growth constants respectively, may be regarded as a relative growth index.

Statistical Analyses

An appropriate consideration would be a test of significance between experimental, x, and control, c, groups from time, t_1, to t_2. In the case of the experimental group, means, $\bar{x}_1$ and $\bar{x}_2$, and standard errors, $S.E._{\bar{x}_1}$ and $S.E._{\bar{x}_2}$, of the logarithmic values are determined at t_1 and t_2; thus

$$\bar{x}_1 = \overline{\ln v} = \frac{\Sigma \ln v}{n} \qquad \text{(ix)}$$

and

$$S.E._{\bar{x}_1} = \frac{\Sigma d^2}{n(n-1)} \qquad \text{(x)}$$

where n is the number of organisms and d the difference between individual values and the mean, $\bar{x}_1$. Determinations at time, t_2, are made in the same manner. The growth constant or index, k_X, is then the change in the average logarithm of the volume from t_1 to t_2, or $\frac{\triangle x}{\triangle t}$.

To compute the error for the constant, k, one employs the relationship

$$S.E._{k\bar{x}} = \sqrt{\frac{S.E.^2_{\bar{x}1} + S.E.^2_{\bar{x}2}}{\triangle t}} \qquad \text{(xi)}$$

where $S.E.^2_{X1}$ is the square of the standard error of the average logarithm of the volume at time, t_1, and $S.E.^2_{\bar{x}2}$ is the value calculated at t_2. A similar procedure is followed for the analysis of the control values.

The error of the relative growth index

$$k_r = \frac{k_X}{k_c} \qquad \text{(xii)}$$

is obtained from the equation

$$S.E._{kr} = k_r \sqrt{\frac{S.E.^2_{kx}}{k^2_x} + \frac{S.E.^2_{kc}}{k^2_c}} \qquad \text{(xiii)}$$

To test for a significant difference between the relative growth index and the control value of one, which would be the value if both the experimental and control groups grew at equal rates, one can then apply an ordinary T-Score to the data.

Appendix I

In Wunder's work (1) with gravity, dead larvae were included in the calculations over successive days. They were distributed in this manner in an attempt to eliminate the survival factor. However, this technique introduced an error into the results by including in the total protoplasm an amount incapable of self-multiplication. Because the physical agent employed by Wunder was, in itself, lethal, the effects of survival appeared to be a more important error to eliminate than the latter. In cases where larvae are exposed to non-lethal agents, i.e. agents whose properties are not directly involved in the death of the organism, it seems best not to include naturally dead organisms over successive days. By this means one eliminates the factor that one is no longer dealing with living material.

When it is appropriate to eliminate the survival factor, pupae and dead larvae are averaged in the results over successive days. The pupae are added, because they are still living, although not growing; the dead larvae are included to distribute the lethal effects. If in the eventuality larvae are lost, one can only assume on the basis of fair selection a corresponding loss of dead larvae and of pupae. As an example let us consider the calculations of combined means over days F, G, and H. Let us further assume that 9 larvae had pupated on day F, and that on day G, 95 larvae are living, 30 have pupated, 3 are dead, and 3 lost. Then, for day G

$$\bar{x}_G, \text{combined} = \frac{95\bar{x}_{G,l} + 30\bar{x}_{G,p} + 3\bar{x}_{G,d} + \frac{125}{128}(9\bar{x}_{F,p})}{95 + 30 + 3 + \frac{125}{128}(9)} \qquad \text{(xiv)}$$

where $\bar{x}$ denotes the average logarithm of the volume, the subscripts l, d, and p denote living and dead larvae and pupae respectively. If on day H there are 81 living larvae, 11 pupae, and 2 dead, the combined mean for this day will

Proceedings of the Iowa Academy of Science, Vol. 68 [1961], No. 1, Art. 84

$$\bar{X}_{H,\text{combined}} = \frac{81\bar{X}_{H,l} + 11\bar{X}_{H,p} + 2\bar{X}_{H,d} + \left[\frac{94}{95}\right]\left[3\bar{X}_{G,d}\right] + \left[\frac{94}{95}\right]\left[\frac{125}{128}\right]\left[9\bar{X}_{F,p}\right]}{81 + 11 + 2 + \left[\frac{94}{95}\right]\left[3\right] + \left[\frac{94}{95}\right]\left[\frac{125}{128}\right]\left[9\right]} \quad \text{(xv)}$$

Standard errors are computed in a manner similar to that previously mentioned. However, somewhat more cumbersome expressions must be employed in combining errors of the separate measurements. Combination of the errors may be made by the expression

$$\text{S.E.}_x = (\text{S.E. of y and z combined}) = \sqrt{\left[\frac{\partial x}{\partial y}\right]^2 \text{S.E.}^2_y + \left[\frac{\partial x}{\partial z}\right]^2 \text{S.E.}^2_z} \quad \text{(xvi)}$$

when $x = f(y,z)$. A description of this technique can be found in Worthing and Geffner (13).

Appendix II

The formula for the banana-agar preparation described in the Turtox leaflet is essentially as follows:

575 cc water
20 grams of agar-agar
35 grams of brewer's yeast
125 cc of white corn syrup
225 cc of homogenized (ripe) banana
1/2 gram of Turtox Mold Inhibitor (in 5 cc of alcohol)

The agar-agar should be added to the water and the mixture brought to a boil. Stir in the homogenized banana and mix thoroughly. Add the yeast, syrup, and mold inhibitor and boil for ten minutes. After boiling the contents pour it into containers, preferably small milk bottles, and allow it to gel. The containers may then be sealed with paraffin wax and refrigerated until ready for use.

The special medium is the same as that described above except that the seeds and pith are removed from the homogenized banana by differential centrifugation. This is desirable since the seeds are nearly the same size as the young larvae.

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