Turbidimetric Assays: the Antibiotic Dose-Response Line

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Received for publication 19 February 1968

Several antibiotics reduced growth of bacteria without killing them when present in the range of concentrations of significance in assay ing. The reduction in growth rate was linearly related to concentration of antibiotic. From this fact may be derived an equation of the form \( \log N = A - BC \) where \( N \) is the concentration of bacteria at the end of the incubation period, \( C \) is the concentration of antibiotic, and \( A \) and \( B \) are constants. This equation is used to guide the selection of the best range of concentrations of unknown for assay and to show the large influence of variations of temperature upon an assay.

The photometric (or turbidimetric) method of assay for antibiotic substances, in contrast to the diffusion method, has a rudimentary and inadequate theoretical basis. The log-probability plot, as introduced by Treffers (7) in 1956, provides an empirical relationship between concentration of antibiotic substance and observed growth of the organism, as measured by uncorrected optical-density units. A slight modification of this plot is valuable in characterizing antibiotics and in investigating the factors influencing antibacterial activity. Families of antibiotics, e.g., the penicillins, form parallel lines with spacings inversely proportional to the specific activities. Examples of the log-probability plot for several antibiotics are given in an earlier publication (6). The expression is more useful for characterizing than for routine assay. Lack of a theoretical basis derived from the kinetics of the interaction of organism and antibiotic is a defect in the log-probability relation. An equation with a better foundation is needed to guide the development and evaluation of turbidimetric assays.

Photometric measurements (5) indicated that a penicillin and erythromycin reduced the growth rate of *Staphylococcus aureus* in proportion to concentration of antibiotic. Hopps et al. (4), who used optical density as a measure of growth, stated that the slope of the growth curve of *Escherichia coli* obtained in the presence of chloramphenicol bears an inverse relation to chloramphenicol concentration. The elegant work of Garrett and students (1-3), who followed by direct counts the growth of *E. coli* in the presence of chloramphenicol and tetracycline, showed that the generation rate constant was linearly dependent upon antibiotic concentration for ranges considerably larger than those encountered in assay ing. They also showed that the viable count and total count (Coulter counter) were the same.

The following assumptions will be made in devising a new equation relating turbidity and concentration of antibiotic substance: (i) The lag period is zero or the same for all concentrations of antibiotic. (ii) The incubation conditions are identical for all tubes in an assay. (iii) The bacteria in all tubes are growing in the log phase when incubation is terminated, and the generation rate constant in the individual tube is constant throughout the incubation. (iv) The apparent growth rate constant is a linear function of antibiotic concentration.

Increase in numbers of bacteria in the log phase may be represented by

\[ N = N_0 e^{kt} \]  

where \( N_0 \) is the concentration of cells at the beginning of log-phase growth which is assumed to start at the time of \( t = 0 \). In the presence of an antibiotic of concentration \( C, k = k_0 - k_aC \) where \( k_0 \) is the generation rate constant in the absence of the antibiotic, and \( k_a \) is an inhibitory coefficient. In assay work, \( C \) is small enough so that \( k \) is positive, i.e., no kill.

Therefore

\[ N = N_0 e^{(k_0-k_aC)t} \]  

or

\[ \log N - \log N_0 = 0.4343(k_0 - k_aC)t \]  

Since \( N_0 \) is constant, a graph of \( \log N \) against \( C \) will be a straight line with a slope of \(-0.4343k_a\).
Because the bacteria in all of the tubes are killed at the same time, \( t \) is constant (assumption 2, above). The direct counts of Garrett and Miller (2) fit equation 3, and numbers derived from photometric measurements approximate equation 3 (Fig. 1). The photometric data do not give a perfect fit because assumption 3 is violated, and even photometric measurements corrected for nonlinear response of the photometer are not necessarily strictly proportional to the concentration of bacteria. A graph of log (optical density) against amount of antibiotic added to the tube is used in routine assaying; correction of the data for instrument response is unnecessary. Investigative work is better done with the numbers obtained after converting photometer response to relative concentration of bacteria.

There is a limit in practice to the value of \( N \) when \( C = 0 \) that should not be exceeded if the linear relation is to hold. This limit is determined by the nutritive qualities of the medium, including the buffer capacity. There seems to be a lower limit to the incubation time that gives an acceptable assay for certain antibiotics. The incubation time of a penicillin assay should afford at least three generations of the bacteria in the zero (no antibiotic) tube. Usually the inoculum level is chosen so that the incubation period is 3.5 to 4 hr at 37.5 C. Doubling the inoculum will reduce the maximal time by about one generation time. Since the generation times are different in the zero tube and antibiotic tube, the inoculum must be the same in all tubes if the assays are to be of acceptable accuracy. The same argument applies to time and temperature of incubation.

In the usual assay procedure, \( N \) is not measured. Its value may be computed from measurements of turbidity by means of a calibration curve. The values of \( N \) in the following discussions were calculated from turbidity. For both investigative and routine assays, \( N_0 \) is not usually obtained and may be eliminated by computing growth relative to the zero tube (no antibiotic).

The growth constant, \( k_0 \), in the absence of antibiotic and the inhibitory coefficient, \( k_a \), follow typical Arrhenius equations for dependence of reaction rates upon temperature. The equation holds for the linear portion of the plot extending from 25 to 38 C (3). Although the Arrhenius equation can be used to compute the error in an assay caused by a difference of temperature between standard and sample, I preferred to plot \( k_0 \) and \( k_a \) as a function of \( 1/T \) and interpolate for the values at 36.5 C and then use equation 1 to compute the cell populations after 4 hr of incubation for tetracycline as the antibiotic (3; Table 1).

The last column of Table 1 gives the ratio of cell populations for the temperatures of 36.5 and 37.5 C. The ratio is large and indicates that any assay system is very sensitive to small temperature inhomogeneities. Tubes with and without antibiotic have different ratios, as do tubes with different concentrations of antibiotic. A constant temperature difference of just 0.1 C causes an intolerably large error of 3.5% in the zero tube. If the tubes of broth are not all at the same temperature when placed in the water bath and if they do not heat at exactly the same rate to the same final temperature and stay at that temperature within \( \pm 0.02 \) C, there will be appreciable errors in the test.

Then equation 3, when the cell population, \( P \), is reported as percentage of that of the zero tube, may be rewritten as

\[
\log P = 2 - BCt
\]

(4)

where \( B = 0.4343 \) \( k_a \).

An error in incubation time of \( \Delta t \) causes an error of

\[
\Delta P/P = 2.3 B \Delta t
\]

(5)
An error in \( t \) (between standards and samples) causes an error in \( P \) which is reflected as an error in \( C \). An example will show the size of the error to be expected in \( C \) when the error in \( t \) is only 1 min. The equation for an erythromycin assay was

\[
\log P = 2 - 0.218 C t
\]

where \( t \) is hr and \( C = \mu g/\text{tube} \). In \( P = 2.3 \log P = 4.6 - 0.502 C t \) and \( \Delta P/P = -0.502 C \Delta t = -0.0067 \) for \( C = 0.8 \mu g/\text{tube} \) and \( \Delta t = 1 \) min. To find the error in \( C \), \( \Delta P/P = -0.502 t \Delta C = -0.502(4) \Delta C = -0.0067 \) or \( \Delta C = 0.0033 \mu g/\text{tube} \). This error is 0.4% in \( C \) at 0.8 \( \mu g/\text{tube} \) for a time error of 1 min in 4 hr of incubation. A deviation in the time of growth of the zero tubes causes an error of about 1.5% of \( P \) for each minute.

The calculations concerning time show that all tubes of standard and samples must be handled as nearly alike as is possible in a batch process. One of the advantages of a continuous operation is the identity of the time course of events.

The availability of an equation with a theoretical basis permits rational selection of the best region of the dose-response curve to use in assaying unknowns and in computation of errors caused by errors in measuring turbidity.

In an assay, \( N_0 \), \( t \), \( k_o \), and \( k_a \) are constants. Therefore equation 2 may be written in the natural logarithmic form

\[
\ln N = A - BC
\]

where \( C \) is the amount of antibiotic per tube or some other convenient expression of antibiotic concentration in the assay broth and \( B \) is a constant. An equation relating the error, \( \Delta C \), in \( C \) caused by an error \( \Delta N \) may be derived from equation 6 by standard methods to be

\[
\Delta C/C = [-1/(A - \ln N)]\Delta N/N
\]

Thus the relative error in \( C \) is independent of the slope, \( B \), of the dose response line and is nearly proportional to the relative error in \( N \). For a fixed error in \( N \), the error in \( C \) will decrease with increase in the value of \( N \). Errors in measuring \( N \) can be a constant error or a proportional error and in an actual measurement is an unknown mixture of the two types. Therefore, the two types of errors must be considered separately.

A standard curve (Fig. 1) for erythromycin could be written as \( \ln N = 3.989 - 1.44 C \) for values of \( C \) between 0.1 and 0.8 \( \mu g/\text{tube} \) (10 ml total volume).

Relative errors in values of \( C \) computed from equation 7 for the two types of errors are given in Table 2. The values of \( N \) are in terms of concentration of bacteria and not as instrument readings in optical density.

The error equation has a minimum value at \( A - \ln N = 1 \) when the error in measuring \( N \) is constant. This minimum occurs near the upper limit of concentration of antibiotic in the usual assay system. A constant proportional error in \( N \) gives decreasing error in \( C \) as \( N \) decreases. Both expressions indicate that the unknown is assayed with least measuring error when its concentration is near the upper limit of concentration and not when the turbidity is high—the intuitive assumption usually made.

This emphasis upon measuring turbidity with rather small error (±0.5%) is not misplaced because simple and relatively inexpensive equipment is available for measuring turbidity of assay tubes with an instrumental uncertainty of 0.2% of full scale. Measuring errors can be and should be made insignificant in an assay.

Conformity of a dose-response line to the one described by equation 2 should not be taken to indicate that the kinetics of interaction of antibiotic and bacteria are the same as those underlying equation 2. For example, an equation of the same form results if the antibiotic killed bacteria in proportion to its concentration but did not affect growth rate—an unlikely occurrence.

The antibiotics assayed with \( S. aureus \) that follow equation 4 approximately are cephalosporins, erythromycin, penicillins, tylosin, and streptomycin. Other systems that follow equation 4 are \( E. coli \) and tetracycline or chloramphenicol (1–3) and tetracycline and \( Aerobacter aerogenes \) growing in a synthetic medium (8). Not all assay systems, streptomycin assayed with \( Klebsiella pneumoniae \), for example, conform to equation 4.

**Table 2. Errors in concentration caused by errors in measuring bacterial population**

<table>
<thead>
<tr>
<th>( C (\mu g/\text{tube}) )</th>
<th>( N )</th>
<th>( \ln N )</th>
<th>( A - \ln N )</th>
<th>( \Delta N = -0.5 )</th>
<th>( \Delta N/N ) = -1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>1.154</td>
<td>2.835</td>
<td>0.094</td>
<td>7.25</td>
<td>6.75</td>
</tr>
<tr>
<td>0.2</td>
<td>0.290</td>
<td>3.969</td>
<td>0.290</td>
<td>4.26</td>
<td>3.45</td>
</tr>
<tr>
<td>0.3</td>
<td>0.434</td>
<td>3.665</td>
<td>0.334</td>
<td>3.30</td>
<td>2.31</td>
</tr>
<tr>
<td>0.4</td>
<td>0.578</td>
<td>3.382</td>
<td>0.458</td>
<td>2.86</td>
<td>1.73</td>
</tr>
<tr>
<td>0.5</td>
<td>0.727</td>
<td>3.262</td>
<td>0.427</td>
<td>2.62</td>
<td>1.38</td>
</tr>
<tr>
<td>0.6</td>
<td>0.867</td>
<td>3.122</td>
<td>0.332</td>
<td>2.54</td>
<td>1.15</td>
</tr>
<tr>
<td>0.7</td>
<td>1.009</td>
<td>3.029</td>
<td>0.309</td>
<td>2.33</td>
<td>0.99</td>
</tr>
<tr>
<td>0.8</td>
<td>1.154</td>
<td>3.170</td>
<td>0.220</td>
<td>2.55</td>
<td>0.87</td>
</tr>
</tbody>
</table>

**LITERATURE CITED**


and mechanisms of action of antibiotics on microorganisms. III. Inhibitory action of tetracycline and chloramphenicol on *Escherichia coli* established by total and viable counts. J. Pharm. Sci. **54**:427–431.


