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III. Turbidimetric Methods

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PRINCIPLE

In these methods, graded concentrations of the substance to be assayed are added to a series of test tubes or flasks containing liquid nutrient medium. This medium is inoculated with the test organism and incubated for a suitable length of time. The response of the test organism, as evidenced by the development of turbidity, is measured by either visual observation of an end point (all-or-none response) or by use of a photoelectric photometer (graded response). By comparing the response of the test organism to the substance being assayed, with that of the test organism to a known standard preparation, the potency of the sample may be determined.

TYPES OF TURBIDIMETRIC METHODS

Turbidimetric assay procedures may be classified in either of two categories based on the type of growth response obtained. In the first group are methods in which the test organism gives an all-or-none response. The second group consists of methods in which the test organism gives a graded response. Methods of the first type are qualitative, while those of the second are quantitative.

Qualitative Methods

In qualitative methods, the response of the test organism is measured between two concentrations of a dilution series. A fixed volume of culture medium is placed in each tube of the series and sterilized. Various dilutions of the substance to be assayed are then added to the series of tubes. The tubes are inoculated with the test organism, a predetermined constant inoculum, and incubated for a period of time. They are observed for growth as evidenced by the development of turbidity in the individual tubes. The end point lies between the concentration at which growth occurs and the concentration at which there is no growth. If dilutions of a known standard preparation are assayed at the same time and under the same conditions, an estimation of the potency of the sample may be obtained.

The degree of precision obtained is dependent upon the concentration of the substance in the dilution series. The narrower the increments, the greater the precision becomes. A twofold dilution series will give a more precise estimation of the concentration of the sample than will a tenfold scale. The series of increments is chosen by the individual analyst in the light of his particular requirements.

The advantages of the qualitative methods are:

(1) They are more sensitive than other methods. Low concentrations of active material can be detected.

(2) They can be used to evaluate materials when there are no standards available. They are valuable tools in the preliminary screening of compounds for various types of activity.

(3) An index of activity, for a compound or a series of compounds, for a large number of microorganisms can readily be obtained in a relatively short period of time.

The disadvantages are:

(1) Measurement is limited to a point between two concentrations of a dilution series. The precision is thus related to the range of concentrations used in the series.

(2) The end point may shift upon repeated assay, even under identical test conditions.

(3) Turbid or highly colored solutions interfere with the determination of the response.

(4) Samples must be sterile. The presence of microorganisms not affected by the material being assayed would cause the development of turbidity which would invalidate the results obtained.

(5) Substances in sample solutions other than the compound being assayed, may interfere with the assay. This occurs, particularly, when samples of natural products are tested.

Quantitative Methods

In quantitative methods, under the conditions selected for each assay, the response of the test organism is a direct function of the concentration of the compound being assayed. The growth response is graded in proportion to the dose concentration. As this response is also proportional to the optical density changes in the various tubes of the series, it may be

measured by means of a suitable photoelectric photometer. By including a known standard preparation, assayed at the same time and under the same conditions, the potency of the sample is determined. The turbidity values of the tubes in the standard series are plotted against their respective dose concentration. Interpolation of the turbidity values of the tubes of the sample series on this curve permits the concentration of the sample to be calculated.

Quantitative methods may be subdivided into two classes. In one class are the methods in which total growth is measured. In the other are the methods in which the rate of growth is compared. When total growth is measured, the incubation period is such that maximum turbidity, for the appropriate concentration range, is obtained. Methods based on the measurement of the rate of growth of the test organism have short, predetermined incubation periods or incubation is terminated when a definite turbidity value is reached in a control tube. Methods measuring the rate of growth are more susceptible to the influence of the various factors involved in microbiological assay procedures. They are more affected by substances likely to be present in crude samples than are those methods measuring total growth. Snell (1950) stated that it is usually more difficult to devise a short term assay than to devise a longer term assay of equal accuracy.

The advantages of quantitative assays are:

(1) The methods are more sensitive to low dilutions than diffusion methods.

(2) As results are obtained at several concentration levels, the methods lend themselves readily to statistical analysis.

(3) The methods are adaptable to rapid assays. Results can be obtained in short periods of time.

The disadvantages are:

(1) Turbid or highly colored solutions interfere with the determination of the response.

(2) Samples should be sterile. When rapid assay procedures (that is, 3 to 4 hr incubation) are used, samples need not be sterile.

(3) The presence of solvents or other substances are more likely to interfere with these methods than with diffusion methods.

(4) A questionable disadvantage is the necessity of including a standard curve each time an assay is run. In many cases, using diffusion methods, a composite standard curve can be prepared utilizing data from a series of assays. After this curve is prepared, it is not necessary to include a standard series for each assay. On occasion, the slope and accuracy of the master curve is checked. However, McMahan (1944) pointed out that even the precision of the Oxford plate method is increased by the use of a daily standard curve instead of a master composite standard curve.

Measurement of Turbidity

The development of turbidity in the assay tubes may be measured by either visual inspection or the use of a suitable instrument. End point determinations, where only the presence or absence of growth is noted, are usually visual observations. The graded response procedures utilize some type of instrument for assessing the amount of growth at each assay level. It is obvious that comments are not necessary regarding visual observations. Only instrumental measurements will be considered.

The use of the adjective "turbidimetric," in connection with these methods, is technically incorrect, as is pointed out by Treffers (1956). The designation, "photometric," is correct. However, as almost any photoelectric photometer will serve as a turbidimeter without any change in the instrument (Willard, *et al.*, 1949), this distinction will not be made here.

A variety of instruments, both filter photometers and spectrophotometers, have been employed in measuring growth response. They may be classified as either one-cell or two-cell instruments. For example, of the filter photometers in common use, the Evelyn is a one-cell instrument while the Klett-Summerson, the Lumetron and the Fischer Electrophotometer are two-cell instruments. Among the spectrophotometers, the Coleman and the Beckman are one-cell instruments.

These photometers measure the amount of trans mitted light relative to incident light. The basic difference between the filter photometer and the spectrophotometer is that the former uses a color filter for isolating the desired wavelength from the light source while the latter employs a monochromator. The spectrophotometer is capable of isolating a much narrower band of light than the filter photometer and as the purity of light available is better than that from filters, the per cent transmittance is determined more precisely.

The filter isolates a band of light, 30 to 50 μ wide. This band should be centered as close as possible to the minimum transmittancy of the sample. In microbiological procedures, the correct filter should be chosen for each culture medium used. The ideal filter is the one where the medium shows the minimum absorption This means that the filter selected for use with a particular culture medium is that which minimizes the effects of the color of the solution (Hirschberg, 1950). For spectrophotometers, the wavelength at which this minimum absorption takes place is chosen.

With one-cell instruments, it is necessary to maintain a constant light source while the readings of the standard and the unknown are being taken. If the circuits in a two-cell instrument are properly chosen, variations in light intensity are cancelled out. This is a definite

advantage. In addition, the null point method of balancing the cells against eath other is said to eliminate errors due to temperature changes and cell fatigue (Hirschberg, 1950). Certain of these two-cell instruments may be balanced so that the total range of turbidity is spread over the entire scale of the instrument.

Instrumental errors are similiar in filter photometers and spectrophotometers. The instrument, itself, should not be a serious source of error as carefully designed instruments are capable of greater precision of measurement than the reproducibility of the turbidities to be measured. Willard *et al.* (1949) list the significant instrumental errors as:

(1) Nonlinearity of response of the light sensitive devices and associated measuring circuits.

(2) Variations in the intensity of the light source.

(3) Stray light striking cells.

(4) Temperature rise in measuring photocell.

(5) Dust, scratches and imperfections in the optical system.

Toennies and Gallant (1948) evaluated the mechanical factors affecting the precision of photometric readings in microbiological assay. Their results (table 1) indicate that, in terms of optical density units, the errors due to the instrument, the human factor, and the individual tube corresponds to a standard deviation of less than ±2. They also found that variations of the glass of the individual tubes did not exceed the basic reading error. Variation in the diameter of the tubes contributed a small, but definite additional error. Finally, their results showed that measurement of bacterial suspensions appear somewhat less precise than those of transparent media.

TABLE 1. *Statistical analysis of mechanical factors in photometric readings**

Series No.	Number of Tubes	Medium in Optical Path	Variations Measured	Mean Reading	Standard Deviation
1	88	H_2O	Difference between second and first determination for each tube	0	±1.8
2	21	$CuSO_4$	Difference between second and first determination for each tube	488	±1.3
3	69	H_2O	Difference between readings of individual tubes and mean value of all tubes	0	±1.5
4	59	$CuSO_4$	Difference between readings of individual tubes and mean value of all tubes	487	±2.3
5	59	Bacterial suspension	Difference between readings of individual tubes and mean value of all tubes	486	±2.9
6	59	$CuSO_4$ or bacterial suspension	Difference between readings of individual tubes with $CuSO_4$ and bacterial suspension	487	±1.8

* Toennies and Gallant (1948).

The errors which might be due to differences in tubes can be eliminated by the use of a drain tube adapter which remains in the instrument. This allows all measurements to be made in the same tube. The adapters are commercially available for certain instruments and are easily fabricated for others.

Instruments vary in sensitivity and this affects the precision of the assay (Loy *et al.*, 1956). Joslyn and Gailbraith (1950) noted the difference in per cent transmission between a filter photometer and a spectrophotometer at the same wavelength. The 1953 Association of Official Agricultural Chemists Collaborative Assay of vitamin B_{12} had definite limits for the allowable assay range in terms of per cent transmission for each instrument used (Krieger, 1954). This was further modified in the 1954 study (Krieger, 1955) by use of a dried cell weight technique for establishing limits. By this technique any instrument may be calibrated to determine the minimum turbidity range for a given assay.

The data obtained in turbidity measurements have been reported in many ways. The readings have ranged from optical density units to galvanometer deflections. Some of these readings are only arbitrary units of turbidity with no relation to bacterial density or cell concentration. A variety of plots have been used; arithmetic dose-arithmetic response, log dose-arithmetic response, log dose-log response; all with the express purpose of obtaining a straight line relationship between dose and response. Both Barton-Wright (1953) and Hoff-Jørgensen (1954) stated that, in the vast majority of assays, there is no linear portion of the standard curve as it is curvilinear throughout its length. Any transformation that could be affected for such curves so that they would yield linear graphs would have manifest advantages. This would afford a sound mathematical basis for computation which is a desirable consideration for any analytical method.

Theoretically, optical density, when plotted versus concentration should give a straight line directly, if the substance follows Beer's law. As the light transmitted by a turbid solution does not always obey this law precisely, some deviation may be expected. With certain instruments, if the optical density does not surpass about 0.4, turbid bacterial suspensions obey Beer's law

(Snell, 1950). Monod (1949) in his discussion[1] on the growth of bacteria stated, in practice, the best instruments for the measurement of bacterial turbidity are those calibrated in terms of optical density. In general, with well dispersed cultures, the optical density remains proportional to bacterial density throughout the positive phases of growth of the culture. This had been previously noted in vitamin assay when Roberts and Snell (1946) constructed calibration curves relating galvanometer readings of an instrument to the dry weight of the cells of the test organism. They found that the optical density or the galvanometer readings could be used directly. Boyd *et al.* (1948) demonstrated that turbidity and optical density are proportional to cell count. With yeasts, light absorption is approximately a linear function of concentration (Atkin *et al.*, 1942). Treffers (1956) preferred the use of optical density units over per cent transmission in his graphical treatment of antibiotic assays. He stated that optical density is directly proportional to the microbial mass and, under standardized conditions to microbial numbers, in the appropriate concentration ranges.

Actually, the majority of the individuals in this field record turbidity measurements in terms of the instrument available to them. The activity is represented by plotting the data in the manner which gives the best linear relationship.

Factors which Influence Turbidimetric Methods

The factors which influence turbidimetric methods are, as with other types of methods, many and varied. Certain of these factors concern all procedures, while others affect only a single type. For example, in every method it is necessary to consider the effect of pH on the test organism and on the activity of the substance being assayed. However, the color of the test solution and its effect on the assay will be of particular importance in turbidimetric procedures, while the diffusion rate of a compound will affect only diffusion methods.

The following discussion is concerned with the general factors which have an effect on turbidimetric procedures. In addition, in order to develop a satisfactory assay procedure, it is necessary to have a knowledge of the physical and chemical properties of the material to be assayed and to be familiar with the growth characteristics of the test organism.

Substance to Be Assayed

In order to use a turbidimetric procedure for the assay of a particular substance, the material must meet certain requirements. These are:

(1) It should affect the growth response of some microorganism in a manner which is reflected by changes in the turbidity of the culture medium;

(2) It should be soluble in water, or some solvent miscible with water which will not interfere with the assay in the concentration used;

(3) It should not cause turbidity in the test solution or form a precipitate when in contact with the culture medium, (if filtration or centrifugation does not aid in the clarification of the sample solution, another procedure must be chosen);

(4) It should not cause the test solution to become darkly colored, (in certain cases, color interference can be eliminated by use of small volumes of test solution, 0.02 to 1.0 ml; by selection of a colorimeter filter in a range where the color of the test solution will not register (Toennies and Gallant, 1948; Hawk *et al.*, 1954); or, when a limited number of tubes are involved, removal of the colored culture medium by centrifugation of the cells and subsequent resuspension in saline before the turbidity values are obtained (Hopkins and Pennington, 1947); the color may also be compensated for by the use of color blanks);

(5) It should be sterile (this is not a problem in the assay of heat stable substances or in assays of short duration, that is, incubation periods of from 90 min to 6 hr).

If the above requirements are not fulfilled or compensated for as noted, another type of method must be chosen.

Culture Medium and Sample Solutions

Of the factors related to the successful performance of a turbidimetric assay, the culture medium is of greatest importance. This is partially due to the incorporation of the sample solution as an intrinsic part of the culture medium. The sample solution may be responsible for:

(1) The introduction of extraneous materials which change the composition of the basal medium and/or affect the growth response of the test organism;

(2) The alteration of the clarity and color of the basal medium;

(3) A decrease in the buffering capacity of the basal medium;

(4) A change in the salt concentration of the basal medium.

In highly purified mixtures, such as antibiotic dosage forms and vitamin mixtures, the problem is not great as the exact formulation is generally known. Natural products present a more formidable challenge. In some cases, there is little or no information concerning the composition of the material or its effect on test organisms.

The prime factor is the introduction of extraneous materials which might change the composition of the basal medium. The addition of inhibitory or stimula-

[1] Adelberg and Rabinovitz (1956) recommend careful study of Monod's essay by anyone intending to apply quantitative microbiology to biochemical problems.

tory substances, other than that being assayed for, will, of course, result in invalid assays. Adequate extraction procedures must be employed, in preparing such samples for assay, to minimize undesirable effects.

With high potency materials, dilution alone is effective in eliminating interfering materials. Other techniques such as solvent extraction, inactivation of compounds known to interfere with a specific assay, or choice of a test organism highly sensitive to the substances being assayed and relatively insensitive to other active substances (Kramer and Kirschbaum, 1955; Grove and Randall, 1955) might be used.

Dunn (1947), in the assay of amino acids in foodstuffs, assumed that basal medium adjusted on the basis of the nutritional requirements of the test organism would nullify almost completely the potential influence of any substance likely to be present in the added sample. He was able to improve certain assays by adjusting the composition of the culture media in accordance with his observations as to the optimal balance of nutrients needed for a constant response to a particular amino acid.

Sterilization of the culture medium can have an adverse effect on turbidimetric assays. Heat sterilization may cause major changes, such as color formation, destruction of certain nutrients, and the lowering of the pH, in the test medium. These undesirable changes may be due, in part, to the amino acid-sugar reaction described by Friedman and Kline (1950a, b). They found when proteins and amino acids are heated in the presence of glucose, the material was browned with a corresponding decrease in the nutritional value as measured by microbiological assay.

The solution to this problem, in certain cases, is shorter periods of exposure of the culture medium to heat. This has been done by several investigators (Roberts and Snell, 1946; Boyd *et al.*, 1948; Barton-Wright, 1953; Parrish *et al.*, 1955). In many assay procedures, the conditions used may eliminate the need for complete sterilization. For example, in a short term assay, sterilization may not be needed due to the rapid growth of the test organism in a short period of time. Or the incubation temperature used with certain test organisms might preclude rigid heat treatment.

Stokstad *et al.* (1956) have stated that assays with lactobacilli are almost immune to interference by contaminants because of the rapidity of growth, the immediate acidification of the medium and essentially anaerobic conditions. This combination represses practically all contaminants except lactobacilli.

There are other cases where complete sterilization is necessary. When slow growing organisms, such as *Tetrahymena* with an incubation period as long as six to eight days, are used, precautions must be taken to avoid contamination. Separate sterilization of glucose solutions or reduction of the glucose content of the culture medium (The Difco Manual, 1953) will aid in overcoming the problem of color changes.

Proper buffering of the culture medium before sterilization will prevent any substantial drop in its pH. Care must be taken in the selection of the type and quantity of buffer used, as it will depend, in part, on its effect on the test organism. An advantage of the turbidimetric procedure over the acidimetric method is that the medium can be buffered to a greater extent and allow heavier growth of acid producing microorganisms before pH levels, which are inhibitory to these organisms, are reached.

The effect of sterilization seems to be a particular problem with growth factor assays rather than with antimicrobial activity assays. With antimicrobial assay media, there is some color change and a lowering of pH, but this commonly occurs when most culture media are autoclaved. Destruction of nutrients might take place, but not to the apparent detriment of the assay procedure. This difference may be attributed to the composition of the media. Media for the assay of antimicrobial substances are composed of natural materials while growth factor assay media are synthetic or semi-synthetic and thus more susceptible to heat.

The color and clarity of the medium may be affected by:

(1) Sterilization, as indicated above;

(2) Incorporation of components which (a) are turbid or highly colored, (b) precipitate or cause precipitation when added to the medium, or (c) react under the conditions of the assay to produce solutions that are not suitable for optical measurements;

(3) Incubation conditions;

(4) The test organism, itself.

Sterilization and incubation of the culture medium cause a certain amount of darkening. If the color of the basal medium is quite dark when it is prepared, subsequent darking will make it unsuitable for turbidimetric measurement. Although turbidity values may be obtained, the assay range is limited and inconsistent. Invalid assays result from use of optically dense culture medium.

Certain ingredients of the medium may not dissolve readily. Other components, such as the "Tweens," when present in high concentrations, cause considerable turbidity. Care must be taken in the preparation of culture medium to produce a medium suitable for use in a sensitive optical system.

The test organism, itself, may affect the color of the culture medium. Toennies and Gallant (1948) found, after centrifugation, noninoculated tubes showed more color than inoculated ones. They regarded this as evidence that the test organism absorbs or consumes some of the color of the test medium. This should not be of

particular concern as the tubes of the standard series should reflect similar changes with increasing numbers of cells.

Inoculation of the Assay Tubes

There are three methods by which the assay tubes may be inoculated. These are:

(1) By seeding each assay tube with one or two drops of the inoculum suspension, using a capillary pipette or a syringe;

(2) By seeding each assay tube with a definite volume of the inoculum suspension;

(3) By seeding the test medium in bulk with a definite volume of the inoculum suspension, then dispensing a predetermined volume of the seeded test medium into each assay tube.

When the dropwise method is used there is some variation in the distribution of the test organism to the tubes. However, Snell (1948) has indicated, in most cases, the concentration of the limiting factor present, and not the fluctuation in drop size, is responsible for the final amount of growth obtained. This may be true in assays where the total growth response is determined, but in short-term assays, where the rate of growth is compared, such variation in the inoculum could affect the reproducibility of the results.

The first two methods also suffer the disadvantage of being tedious and time consuming.

The third method is preferred as it insures equal inoculation for each tube of the assay series and is less tedious than the other methods. In addition, considerable time can be saved when it is necessary to assay several samples.

Incubation

The temperature of incubation is dependent upon the test organism and the nature of the substance to be assayed. It should be close to the optimum temperature for the growth of the test organism or in a range that will permit a good growth response.

The actual temperature which is selected for any given assay procedure is not as critical as the maintenance of a constant temperature. Variability in different sections of the incubator may be a serious source of discrepancy between replicate tubes as each tube represents a separate population. This is of major importance in short-term assays as rigid control of all assay conditions is essential. If valid assays are to be obtained, each tube in the series should be subjected to identical environmental conditions.

Much of the variation in temperature conditions can be eliminated by use of a circulating water bath incubator. The water bath must be designed to minimize vibration as it has been shown (Lewis *et al.*, 1947) that variation in growth response can be attributed to this factor.

The length of the incubation period, although somewhat arbitrary, is also conditioned by the test organism and the material being assayed. Turbidimetric procedures have been developed in which the incubation time, using a bacterium as the test organism, is as short as 90 minutes (Lee *et al.*, 1944). On the other hand, using a protozoon as the test organism, an assay may take as long as 6 to 8 days (Hoff-Jørgensen, 1954).

In the assay of chlortetracycline, Beigelman (1949) reported that the error of assay is diminished when the incubation period is less than 12 hours. Lewis *et al.* (1947) found more variation, in the assay of subtilin, with an 18 hr incubation period than when a 4 hr period was used. When thiamine is assayed, using *Lactobacillus fermenti*, the pyrimidine and thiazole moieties show no growth promoting activity if the incubation period is 18 hr. With longer incubation periods, some activity from these products is apparent (Snell, 1950).

In the majority of vitamin and amino acid assays, the length of incubation is of minor importance. The concentration of the growth factor in each assay tube limits the response of the test organism. Growth, as evidenced by turbidity, ceases when the supply of the nutrient being assayed is exhausted. No significant change in the turbidity of the tube occurs when this point is reached.

There are certain exceptions where it is necessary to control the length of incubation. When the rate of growth rather than the total growth is determined the dosage levels of nutrient will be in a range where, under the particular assay conditions, a delineation of growth between the various tubes of the series will be noted. However, if the conditions set for the incubation period are exceeded, a graded response may not occur. Such assays must be terminated when a specific condition, either an arbitrary turbidity value or a definite period of time, is reached. The growth response may be stopped by the addition of a bactericidal agent (such as formaldehyde) to each assay tube, by chilling the tubes at low temperatures, by steaming, or by autoclaving the tubes. Autoclaving is not recommended as the turbidity of cultures sterilized by steam pressure may be markedly changed.

If a procedure is based on growth inhibition, the length of the incubation period will depend upon whether the substance is bactericidal or bacteriostatic. In the former case, assay results should not be affected by this factor. However, as noted previously for growth factors, when rapid assay procedures are used, it becomes important. The substance being assayed although bactericidal in nature, may not exert a bactericidal effect in the range of dosage levels chosen for the assay. It might only be bacteriostatic, thus, when the incubation period is extended over a longer period of time, bacteriostasis ceases and inhibition is not noted. Steps, such as those indicated above for short-term growth

factor assays, must be taken to stop growth at the proper time.

In the case of bacteriostatic substances, the inhibition effect is limited, in general, to a certain time period. Any extension beyond that limit will result in invalid assays as the end point shifts on prolonged incubation. This makes the length of the incubation period critical for comparison of results from assay to assay.

Oxygen Relationships

Certain test organisms, for example, protozoa, yeast and strict aerobic bacteria require oxygen to yield a good growth response. The work of Rahn and Richardson (1941) indicated that the rate of oxygen diffusion in stationary cultures is not sufficient to provide an entire culture of an aerobic organism with oxygen. When this is considered in the light of analytical techniques, it means that a factor, other than the one being assayed, might limit the growth of the test organism.

In fact, when *Tetrahymena gelii* is used as a test organism, death of the culture occurs when the tubes are not slanted to provide aeration (Dewey *et al.*, 1950). In the assay of thiocytic acid, poorly aerated cultures of *Tetrahymena pyriformis* give erratic results (Stokstad *et al.*, 1956).

Snell (1950) has stated that although yeast will grow anaerobically, a heavier growth response is obtained when the cultures are aerated. Aerobic bacteria, such as *Proteus vulgaris*, *Acetobacter suboxydans*, and *Corynebacterium bovis* will only grow when sufficient oxygen is present.

Aeration of cultures may be accomplished by mechanical shaking or by providing a favorable ratio of surface to assay medium volume. The use of flasks or slanted test tubes provides a large surface area with a minimum depth so that oxygen diffuses readily throughout the test medium.

Interaction of the constituents of the culture medium during autoclaving generally results in a favorable oxidation-reduction potential for microaerophilic and facultative aerobic bacteria (Snell, 1950; Lees and Toothill, 1952). In certain cases, reducing agents may be added to the medium to control this variable. The slope of the dose response curve for microaerophilic bacteria may be improved by the use of narrow test tubes in order to increase the depth of the medium and create more favorable conditions (Lees and Toothill, 1952). Use of large volumes of test medium in test tubes 18 by 200 mm will achieve the same effect.

When anaerobic test organisms are used, conditions for their growth may be established by the routine methods for cultivating these microorganisms, that is, use of anaerobic jars. As most of these methods are cumbersome, it is easier to incorporate reducing agents into the assay medium to control the potential.

Glassware

A major, but sometimes overlooked, factor in the successful performance of turbidimetric methods is the cleanliness of the glassware used in the procedures. Kersey and Fink (1954), in their discussion of the assay of antibiotics in biologic materials, stated that contamination of glassware may well invalidate conclusions drawn in an otherwise satisfactory manner. Loy *et al.* (1950) indicated that lack of recognition of this factor, as the cause of erratic results was an important deterrent in the development of a suitable assay for vitamin B_{12}.

Cleaning of glassware may be accomplished in many ways. Soaps and detergents, dichromate cleaning solution and acid washes, followed by rinsing and heating have been used in various combinations. A successful method of preparing glassware for the determination of one substance may not be applicable to all assay procedures. For example, Stapert and Stubberfield (1954) found that while tubes cleaned with detergent could be used for most vitamin assays, it was necessary to use 65 per cent vol/vol sulfuric acid solution to clean glassware used in the assay of vitamin B_{12}.

Thorough rinsing of glassware must follow the use of chemical agents in cleaning procedures. Many common laboratory cleansers can affect the test organism. In polymyxin assays Reese and Eisenberg (1949) found that less than 0.05 per cent Alconox in the culture broth will inhibit *Brucella bronchiseptica*. Soap has the opposite effect in these assays (Bliss *et al.*, 1949). The occurrence of "skipping" (false positive tubes) was found to be due to inactivation of polymyxin by soap. When special precautions were taken in the rinsing of the laboratory glassware, the frequency of "skipping" was greatly reduced. From these examples, it can be seen that either the test organism or the substance being assayed may be affected. In order to obtain valid and consistent turbidimetric assays, all glassware should be chemically and biologically clean.

Accuracy of Results

The limits of error reported in the literature for turbidimetric assays have ranged from ± 2 per cent (Kersey, 1950) to ± 20 per cent (Darker *et al.*, 1948). The majority of the reports indicate that limits of ± 10 per cent is about average for this type of procedure. However, an experienced analyst exercising rigid control of the factors which contribute to within day and between day variability, should be able to approach ± 5 per cent in quantitative turbidimetric methods.

The use of replicate tubes in these assays should increase the precision of measurement and aid in estimating the assay error. At a symposium on analytical microbiology (Gavin, 1956) Gaunt stated that replication increases the precision of measurement by a factor which is the square root of the number of replicates.

A pitfall in the use of replicates has been pointed out by Bliss (1952). He stated that unless the assay is arranged in randomized groups, the variation between duplicates may underestimate the assay error with a discrepancy as much as tenfold. Variation in replicate tubes will only indicate the error of assay to the degree that randomization has been used in the preparation of the tubes, their positioning in the autoclave and incubator, and in the reading of results.

Control of the various factors discussed above, aided by an assay design which minimizes the influence of bias in the set-up of the assay, should lead to reliable and reproducible results. The application of statistical analysis makes it possible to determine the validity of an assay, the contribution to over-all error made by a particular variable and the degree of reliability of the assay results obtained. Thus each analyst is able to evaluate any of the procedures he might use.

Advantages and Disadvantages of the Turbidimetric Type Assay

The advantages of the turbidimetric type assay are:

(1) The method is more sensitive to low concentrations of active material than other methods of assay (that is, diffusion, acidimetric and so forth).

(2) The method can be adapted to give accurate results in short periods of time.

(3) The method can be used to evaluate materials for which a standard is not available.

(4) By proper application of statistical procedures, both the precision and the accuracy of the method can be determined.

(5) The method can be used to obtain an index of activity for a variety of compounds against several organisms in a relatively short period of time.

The disadvantages of the turbidimetric type assay are:

(1) Turbid or highly colored solutions cannot be assayed by this method.

(2) In most cases, the sample being assayed must be sterile.

(3) The method requires more manipulation and is more tedious than diffusion methods.

(4) The method is more susceptible to variations in environmental conditions than diffusion methods.

(5) The method is more susceptible to the influence of organic solvents and extraneous substances in sample preparations than diffusion methods.

REFERENCES

ADELBERG, E. A. AND RABINOVITZ M. 1956 Metabolism of amino acids and proteins. Ann Rev. Biochem., **25,** 349–367.

ATKIN, L., SCHULTZ, A. S., AND FREY, C. N. 1942 Measurement of yeast growth. Arch. Biochem., **1,** 9–16.

BARTON-WRIGHT, E. C. 1953 *The microbiological assay of the vitamin B complex and amino acids.* Pitman Publishing Corporation, New York, N. Y.

BEIGELMAN, P. M. 1949 *In vitro* determination of bacterial sensitivity to Aureomycin. Proc. Soc. Exptl. Biol Med., **72,** 89–92.

BLISS, C. I. 1952 *The statistics of bioassay.* Academic Press, Inc., New York, N. Y.

BLISS, E. A., CHANDLER, C. A., AND SCHOENBACK, E. B. 1949 *In vitro* studies of polymyxin. Ann. N. Y. Acad. Sci., **51,** 944–951.

BOYD, M. J., LOGAN, M. A., AND TYTELL, A. A. 1948 A microbiological procedure for the assay of amino acids with *Clostridium perfringens* (*Welchii*) BP6K. J. Biol. Chem., **174,** 1027–1035.

DARKER, G. D., BROWN, H. B., FREE, A. H., BIRO, B., AND GOORLEY, J. T. 1948 The assay of bacitracin. J. Am. Pharm. Assn., Sci. Ed., **37,** 156–160.

DEWEY, V. C., PARKS, R. E., JR., AND KIDDER, G. W. 1950 Growth responses of *Tetrahymena gelii* to changes in the basal media. Arch. Biochem., **29,** 281–290.

Difco Manual of Dehydrated Culture Media and Reagents, 1953 Media for the assay of vitamins and amino acids. p. 214 9th ed. Difco Laboratories, Inc., Detroit, Michigan.

DUNN, M. S. 1947 Amino acids in food and analytical methods for their determination. Food Technol., **1,** 269–286.

FRIEDMAN, L. AND KLINE, O. L. 1950a The amino acid-sugar reaction. J. Biol. Chem., **184,** 599–606.

FRIEDMAN, L. AND KLINE, O. L. 1950b The relation of the amino acid-sugar reaction to the nutritive value of protein hydrolysates. J. Nutrition, **40,** 298–307.

GAVIN, J. J. 1956 Symposium on analytical microbiology. Bact. Reviews, **20,** 201–204.

GROVE, D. C. AND RANDALL, W. A. 1955 *Assay methods of antibiotics. A laboratory manual.* p. 82. Medical Encyclopedia, Inc., New York, New York.

HAWK, P. B., OSER, B. L., AND SUMMERSON, W. H. 1954 *Practical physiological chemistry.* 13th ed. p. 1186. The Blakiston Company, Inc., New York, New York.

HIRSCHBERG, E. 1950 The use of optical instruments in vitamin assay. *Vitamin Methods* (ed. by P. György) Vol. **1,** 507–537. Academic Press, New York, New York.

HOFF-JØRGENSEN, E. 1954 Microbiological assay of vitamin B_{12}. *Methods of biochemical analysis* (ed. by D. Glick) Vol. **1,** 81–113. Interscience Publisher, Inc., New York, New York.

HOPKINS, R. H. AND PENNINGTON, R. J. 1947 The assay of the vitamin B_6 complex. Biochem. J., **41,** 110–114.

JOSLYN, D. A. AND GAILBRAITH, M. 1950 A turbidimetric method for the assay of antibiotics. J. Bacteriol., **59,** 711–716.

KERSEY, R. C. 1950 A turbidimetric assay for Terramycin. J. Am. Pharm. Assn., Sci. Ed., **39,** 252–253.

KERSEY, R. C. AND FINK, F. C. 1954 Microbiological assay of antibiotics. *Methods of biochemical analysis* (ed. by D. Glick), Vol. **1,** 53–79. Interscience Publishers, Inc., New York, New York.

KRAMER, J. AND KIRSCHBAUM, A. 1955 A turbidimetric assay for Tyrothricin. Antibiotics and Chemotherapy, **5,** 561–565.

KRIEGER, C. H. 1954 Report on vitamin B_{12} (Microbiological method). J. Assoc. Offic. Agr. Chemists, **37,** 781–792.

KRIEGER, C. H. 1955 Report on vitamin B_{12} (Microbiological method). J. Assoc. Offic. Agr. Chemists, **38,** 711–721.

LEE, S. W., FOLEY, E. J., EPSTEIN, J., AND WALLACE, J. H., JR. 1944 Improvements in the turbidimetric assay for penicillin. J. Biol. Chem., **152,** 485–486.

LEES, K. A. AND TOOTHILL, P. R. 1952 The assay of vitamin B_{12}. Relationship between growth response of *Lactobacillus*

leichmannii 313 in tubes and diffusion of oxygen into the medium. Biochem. J., **50,** 455–459.

LEWIS, J. C., HUMPHREYS, E. M., THOMPSON, P. A., DIMICK, K. P., BENEDICT, R. G., LANGLYKKE, A. F., AND LIGHTBODY, H. D. 1947 The microbiological assay of subtilin. Arch. Biochem., **14,** 437–450.

LOY, H. W., JR., HAGGERTY, J. F., AND KLINE, O. L. 1950 A cause of variation in the microbiological assay for vitamin B_{12}. Arch. Biochem., **29,** 451.

LOY, H. W., JR., PARRISH, W. P., AND SCHIAFFINO, S. S. 1956 The use of turbidity measurements in microbiological methods for vitamins. J. Assoc. Offic. Agr. Chemists, **39,** 172–179.

MCMAHAN, J. R. 1944 An improved short term turbidimetric assay for penicillin. J. Biol. Chem., **153,** 249–258.

MONOD, J. 1949 The growth of bacterial cultures. Ann. Rev. Microbiol., **3,** 371–394.

PARRISH, W. P., LOY, H. W., AND KLINE, O. L. 1955 A study of the yeast method for vitamin B_6. J. Assoc. Offic. Agr. Chemists, **38,** 506–513.

RAHN, O. AND RICHARDSON, G. L. 1941 Oxygen demand and oxygen supply. J. Bacteriol., **41,** 225–249.

REESE, E. T. AND EISENBERG, G. M. 1949 Polymyxin: assay procedures. Ann. N. Y. Acad. Sci., **51,** 968–979.

ROBERTS, E. C. AND SNELL, E. E. 1946 Improved medium for microbiological assay with *Lactobacillus casei*. J. Biol. Chem., **163,** 499–509.

SNELL, E. E. 1948 Use of microorganisms for assay of vitamins. Physiol. Rev. **28,** 255–282.

SNELL, E. E. 1950 Microbiological methods in vitamin research. *Vitamin methods* (ed. by P. György) Vol. **1,** 327–505. Academic Press, Inc., New York, New York.

STAPERT, E. M. AND STUBBERFIELD, L. 1954 A note on cleaning culture tubes for the USP vitamin B_{12} microbiological assay. J. Am. Pharm. Assn., Sci. Ed., **43,** 382.

STOKSTAD, E. L. R., SEAMAN, G. R., DAVIS, R. T., AND HUTNER, S. H. 1956 Assay of thioctic acid. *Methods of biochemical analysis* (ed. by D. Glick) Vol. **3,** 23–47. Interscience Publishers, Inc., New York, New York.

TOENNIES, G. AND GALLANT, D. L. 1948 Bacterimetric studies. I. Factors affecting the precision of bacterial growth responses and their measurement. J. Biol. Chem., **174,** 451–463.

TREFFERS, H. P. 1956 The linear representation of dosage response curves in microbial antibiotic assays. J. Bacteriol., **72,** 108–114.

WILLARD, H. H., MERRITT, L. L., JR., AND DEAN, J. A. 1949 *Instrumental methods of analysis*. pp. 10–44. D. Van Nostrand Company, Inc., New York, New York.

The Nonlogarithmic Rate of Thermal Destruction of Spores of *Bacillus coagulans*[1, 2]

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Many of the data concerning the destruction of bacteria and their spores by deleterious agents have been presented with the assumption that the "logarithmic order of death of bacteria" has been adequately validated (Bigelow, 1921; Esty and Williams, 1924; Rahn, 1945a, b; Stumbo *et al.*, 1950; Lee, 1953; Schmidt *et al.*, 1955; El-Bisi and Ordal, 1956a, b). Some investigators, however, have reported data which do not agree with this point of view (Falk and Winslow, 1926; Williams, 1929; Davis and Williams, 1948; Reed *et al.*, 1951; Sugiyama, 1951; Reynolds and Lichtenstein, 1952; Kaplan *et al.*, 1953; La Baw and Desrosier, 1954). The concept of a logarithmic order of death has led to the evolution of a useful tool, the death rate constant and its reciprocal, D, the thermal resistance value (Stumbo, 1948a). This constant, D, or its equivalent, has been used to express the resistance of an organism under specified conditions and has been employed in the derivation of methods for thermal process evaluation and calculation (Stumbo, 1949, 1953; Tischer and Hurwicz, 1954; Levine, 1956).

This investigation was initiated as a result of observed inconstancy of the D values of a putrefactive anaerobe (PA) strain 3679 caused by changes in initial concentration and/or length of heating time. Because of difficulty in obtaining accurate colony counts of this gas forming anaerobe, *Bacillus coagulans* strain 43 P was chosen as the test organism for the rate of destruction studies. This organism exhibits no dormancy following heat treatment, thus survivors can be enumerated accurately by conventional plating procedures, and has fairly heat resistant spores. Furthermore, Youland and Stumbo (1953), using the "tube method," obtained constant D values with this strain of *B. coagulans*, for 4 initial concentrations at 4 temperatures. The thermo-

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