THEORY AND PRACTICE IN EXPERIMENTAL BACTERIOLOGY

G. G. MEYNELL
M.D.
Guinness-Lister Research Unit
Lister Institute of Preventive Medicine

ELINOR MEYNELL
M.B., DIP. BACT.
Medical Research Council Microbial Genetics
Research Unit, Hammersmith Hospital

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PREFACE

In the past, the techniques of experimental bacteriology have, perhaps, received less emphasis in textbooks than those used in medical and public health laboratories. Although all branches of bacteriology overlap to some extent, the day-to-day problems encountered in experiments are not of a kind that usually disturb routine tests, and we have therefore tried to provide a documented guide to the basic bacteriological techniques, including some theory as well as working rules. We have also included a number of tables that greatly lessen the computation involved in standard procedures like viable counts by the dilution method. All the techniques described here are relatively simple, but others can be traced from the references which have been chosen largely for their bibliographies and not on grounds of priority. Much of this material is applicable to micro-organisms of all kinds, and, although the main emphasis is on bacteria, many of the examples concern viruses and yeasts.

A book of this sort inevitably relies heavily on methods devised by others, and we would like to express our gratitude to the following authors and manufacturers, and to the publishers and editors of the journals cited below, for giving us permission to reproduce their work:

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G. G. M.
E. W. M.

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MEASUREMENT OF BACTERIAL MASS AND NUMBER

Many experimental techniques depend on some measure of bacterial growth. This may be the total mass of bacterial protoplasm/ml. culture, estimated either directly, as dry weight, or indirectly from the concentration of bacterial nitrogen or the light-scattering power of the culture. In other experiments, it is more important to know the number of individual organisms/ml. than their total mass, and so either the total number of cells or the number of living cells is then measured by a total or by a viable count respectively.

GENERAL POINTS

Mass and number. It is the rule for mass and number to vary independently during bacterial growth. Clearly, an individual cell must enlarge before it divides. A bacterial culture often increases in mass without changing in number during the lag phase; while the opposite occurs at the end of exponential growth, when the cells become smaller (Hershey & Bronfenbrenner, 1938). Similar changes occur on altering the culture medium of an exponentially growing culture (see Maaløe, 1960). The ratio, mass:number, increases even more in damaged cells, which sometimes enlarge without dividing to produce long filaments, as on exposure to u.v.-irradiation or to metabolic analogues which block DNA but not protein synthesis.

Mass and number have therefore to be treated separately in measuring bacterial growth.

Heterogeneity in bacterial cultures. The usual methods for estimating bacterial mass and number tend to obscure the fact that all bacterial cultures are grossly heterogeneous. The estimates are necessarily averages, derived from individuals which, although they may well be genetically identical, nevertheless differ in many characteristics like length and breadth (Henrici, 1923), antibiotic resistance (Hughes, 1956), cell wall composition (Pennington, 1950; Cole & Hahn, 1962), and doubling time and age (Powell, 1955, 1956*a*). The extent of these differences which almost certainly arise from non-genetic causes can sometimes be determined

directly, as in measuring the size of bacteria by light microscopy (p. 134) or the mass of individual yeast cells by interference microscopy (Mitchison, Passano & Smith, 1956). Other differences can only be determined retrospectively. One example is antibiotic resistance. Another is the varying number of phage particles released by individual infected bacteria which differ in a way that cannot be determined before lysis, for example from the distribution of cell volumes in the culture (Delbrück, 1945). Some differences, as in size, are eliminated if cell division is synchronized, but others, like differences in composition, may follow from the nature of bacterial growth.

Viable counts. Definitions of viability. A viable count ostensibly measures the concentration of living organisms, but its significance necessarily depends on what is meant by 'living'. No satisfactory definition has ever been provided, with the result that a multitude of working definitions has been proposed in the past (see Wilson, 1922; Postgate, Crumpton & Hunter, 1961). Some depend on a characteristic, like the ability to form a colony visible to the naked eye, which would be universally accepted as a criterion of viability. Other definitions depend on less obvious characteristics, such as motility or fermentative ability (see Wilson, 1922), resistance to staining (see Knaysi, 1935), appearance by electron microscopy (McFadzean & Valentine, 1959), ability to reduce tetrazolium salts (see, however, Stille, 1953; Parker, 1955), or the formation of elongated cells during incubation on nutrient agar containing urea (Valentine & Bradfield, 1954). None of these alternatives has been widely accepted, presumably because they rest on functions which, at present, are generally felt to be only distantly connected with our intuitive preconceptions of 'viability'. The usual working definition, accepted here, equates 'viability' with the power to form a macroscopic colony on nutrient agar or to produce visible turbidity in broth in a dilution count (p. 21).

The limitations of this definition should be appreciated. The qualification, 'macroscopic', is important, as instances of limited multiplication are known where cells divide a few times to produce a microcolony and then stop, as with irradiated yeast (p. 237) or bacteria treated with ferrous ions (Catlin, 1956). Furthermore, organisms which are dead according to this definition may still possess functions characteristic of viable cells, including the ability to support the growth of virulent phage (Benzer, 1952) and to form β-galactosidase upon exposure to an inducer (Spiegelman, Baron & Quastler, 1951). It is also the rule in bacteriology to find that the viable count is higher on some media than on others, especially when the organ-

isms have been exposed to a bactericidal agent—whether it be radiation (Norman, 1953), heat (Olsen & Scott, 1946), or a chemical (see Harris, 1963). Higher counts are usually obtained on 'rich' media, like digest agar, than on simple defined media, like glucose-salts medium, although the reverse was true of a u.v.-resistant mutant of *Escherichia coli* exposed to ultra-violet irradiation (Alper & Gillies, 1960). It follows that no viable count has any absolute significance, for its value often depends as much on the experimental conditions as on the state of the organisms.

The following sections describe the various methods for determining bacterial mass, total and viable counts, their precision, and the effect of technical errors. Sampling errors arising, for example, from the random distribution of organisms in suspension, are discussed more fully in chapter 6.

BACTERIAL MASS

The relation between dry weight, chemical composition, and light-scattering power of a culture necessarily depends considerably on the species of organism and the conditions in which it is grown. However, as a guide, 10^9 cells of a Gram-negative organism like *Escherichia coli* or *Pseudomonas pyocyanea* have a dry weight of *ca.* 0·32 mg.; contain *ca.* 56 μg. nitrogen and 8 μg. DNA; and have an extinction of *ca.* 0·4 with a 1 cm. path at 450 mμ (Schaechter, Maaløe & Kjeldgaard, 1958; Gaby, Logan & Whitaker, 1962; Cohn & Horibata, 1959).

Dry weight

A sample of the culture is treated with formalin at a final concentration of 1 %, v/v, and centrifuged in a weighed tube to deposit the cells which are then washed once with 0·85 %, w/v, saline + 1 % formalin, and once with 0·05 % saline or with distilled water. The deposit is dried to constant weight in an oven at 105°. Before each weighing, the tube is placed in a desiccator over P_2O_5 until it has cooled to room temperature.

Chemical estimates

Estimates of bacterial nitrogen or protein are often used as indices of bacterial mass. Although this is satisfactory for many experiments, other estimates are sometimes more useful on occasions when a sizeable fraction of the cell nitrogen is a structure whose formation varies independently of cytoplasmic growth, like the polyglutamic acid capsule of *Bacillus* species. Two alternatives are to measure bacterial phosphorus (Bennett & Williams,

1957) or bacterial DNA, which also estimates the number of genetically independent individuals and may be especially useful for organisms growing in clumps or chains.

Nitrogen estimations. The Kjeldahl method is widely used. The nitrogen in organic material is converted to ammonium sulphate by digestion with H_2SO_4 in the presence of a catalyst (see Kirk, 1947; Steyermark, 1961). The ammonia can then be estimated:

(*a*) by volumetric titration following distillation, for example in the Markham still which measures 20μg. N $\pm$ 1 % (Markham, 1942), or in a Conway micro-diffusion unit which measures 0·5μg. N (Parker, 1961); or

(*b*) colorimetrically, after addition of Nessler's reagent, which measures 1μg. N $\pm$ 4–5 %.

The second method is quicker and simpler, and is given here. It is obvious that the more sensitive the test, the more important it becomes to avoid contamination by extraneous nitrogen contributed by glassware, reagents, and the atmosphere, including tobacco smoke.

The colorimetric method requires the following reagents (Macfarlane & Gray, personal communication):

(1) Nessler's reagent.

(2) Digestion mixture. Equal volumes of:

(i) 1 ml. conc. H_2SO_4 (analytical or N-free grades) + 5 ml. glass-distilled water;

(ii) 10 ml. 72 % perchloric acid (analytical grade) + 5·5 ml. glass-distilled water.

(3) Standard nitrogen solution, e.g. NH_4Cl.

(4) Gum ghatti solution (to prevent turbidity of Nessler's reagent). Cover 1 g. gum ghatti + 1 g. benzoic acid with 500 ml. glass-distilled water for 3–4 days at room temperature. Then dilute to 1 l. with glass-distilled water saturated with benzoic acid, and filter through cotton wool to remove undissolved solids.

Reagents 1, 3, and 4 are available from commercial sources.

The method is as follows:

(1) Measure the sample ($\leqslant$ 1 ml.) into a narrow Pyrex boiling tube or into a 10 ml. ground glass stoppered tube graduated at 5 ml. (ext. diameter 16 mm., length 100 mm.). Also put up 3 samples of the standard solution containing 5, 10, and 20μg. N respectively, and set aside 2 tubes for blanks. The tubes must be long enough for acid to condense in their upper part during heating in stage 3.

(2) Add 0·2 ml. digestion mixture to each tube.

(3) Heat the tubes at 200° on a digestion rack or in a heated block for 1 hr. in a fume cupboard.

(4) Remove the tubes from the heater and stand on the bench to cool to room temperature. As soon as possible, add *ca.* 1 ml. glass-distilled water and again cool to room temperature.

(5) Add 1·0 ml. gum ghatti solution and mix well.

(6) Add 1·5 ml. Nessler's reagent: if the stock solution is turbid, centrifuge and use the supernatant. Add sufficient glass-distilled water to bring the total volume to 5 ml.

(7) Measure the extinction at 425 mμ, using a 1 cm. path. The intensity of colour due to N = extinction of the sample minus the mean extinction of the blanks.

The extinction is proportional to N concentration over the range, 5–40μg. N.

Estimation of DNA. The method given here depends on measurement of the blue colour formed by deoxysugars, like deoxyribose, with diphenylamine.

The following reagents are needed:

(1) Perchloric acid ($HClO_4$).

(2) Diphenylamine reagent (Burton, 1956). Dissolve 1·5 g. steam-distilled diphenylamine in 100 ml. analytical grade acetic acid and add 1·5 ml. conc. H_2SO_4. Store in the dark. If a blue colour develops during storage, redistilled acetic acid should be used. Add 0·1 ml. of 1·6 %, w/v, aqueous acetaldehyde to every 20 ml. stock solution on the day it is to be used.

Add to the sample 2·5–12 N-$HClO_4$ to a final concentration of 0·25 N, chill for 30 min., and then centrifuge. Discard the supernatant. Stir the deposit with 0·5 ml. of 0·5 N-$HClO_4$. After adding another 3·5 ml. 0·5 N-$HClO_4$, heat at 70° for 15 min., with occasional stirring. Centrifuge a second time. Tip the supernatant into a 10 ml. graduated tube. The precipitate is extracted again with 3 ml. 0·5 N-$HClO_4$, the mixture centrifuged, and both supernatants pooled and their total volume measured (Burton, 1956, using *Escherichia coli*).

Alternatively, freeze the sample of culture quickly in a bath of ethanol and solid CO_2, thaw, and centrifuge in the cold for 30 min. to deposit the cells. Take up the deposit in 2·5 ml. chilled 0·85 %, w/v, saline and transfer 2 ml. to 0·1 ml., 70 %, w/v, $HClO_4$. Heat at 70° for 30 min., centrifuge, discard the deposit and retain the supernatant for assay (Schaecter *et al.* 1958, using *Salmonella typhi-murium*).

Mix 1–2 ml. supernatant with 2 ml. diphenylamine reagent. Incubate at 30° for 16–20 hr. Measure the extinction of the colour that develops at 600 mμ.

Also include blanks containing $HClO_4$ but no DNA, and standard DNA or deoxyribose preparations.

Light-scattering

The amount of light scattered by a bacterial suspension may be proportional to its concentration expressed as mass or number, or to mean cell length, depending on the way in which the measurements are taken. The relationships observed in practice are best discussed after the optical theory which, though difficult in detail (see West, 1960; Oster, 1960; Koch, 1961; Powell, 1963), is fairly simple in principle.

Consider a culture placed in a beam of parallel monochromatic light. The proportion of incident light failing to traverse the culture unchanged represents the sum of light absorbed and of light scattered by the organisms. Absorption of visible light is usually negligible, since most species are unpigmented and almost transparent (p. 126). Changes in transmission are therefore determined by scattering, due to reflection at the surface of the organisms and to diffraction within them. Consequently, there is no scattering if the refractive index of the organisms and their suspending medium is the same. The intensity of the light deviated at any particular angle to the incident rays may be plotted against the corresponding angle to show how scattering occurs. Such graphs reveal that, as this angle increases, the intensity does not fall smoothly but shows peaks and depressions caused by interference between rays arising from different points in the suspension (Fig. 5.3). With large transparent particles, like bacteria illuminated by white light, the greatest intensity is immediately to each side of the emergent undeviated light at an angle of less than 90° to the axis (Fig. 1.1; Mestre, 1935; Koga & Fujita, 1962; Powell, 1963).

The total amount of light scattered increases directly with the ratio, particle size: wavelength of incident light (cp. Taysum, 1956; Koch, 1961). It follows that scattering will be greater (*a*) at a given wavelength, the larger the organisms, and (*b*) the shorter the wavelength used with a given organism. The shortest wavelength commonly used is 450 mμ as measurements in the ultra-violet introduce technical complications. 650 mμ is preferable for broth cultures as the extinction of broth is then minimal.

In estimating bacterial concentration, either scattered light or undeviated light can be measured. The former is the more sensitive method at

low cell concentrations. The receiver, whether it is the observer's eye or a photocell, will then be detecting the difference above zero intensity; whereas the amount of undeviated light differs from the blank reading by only a small amount that may be more difficult to measure. The reverse is true at high cell concentrations where measurements of undeviated light should be more sensitive. In practice, measurements of undeviated light necessarily include some scattered light as all receivers accept light over an angle, a factor which necessarily leads to a decrease in sensitivity (see Powell, 1963, Table 1).

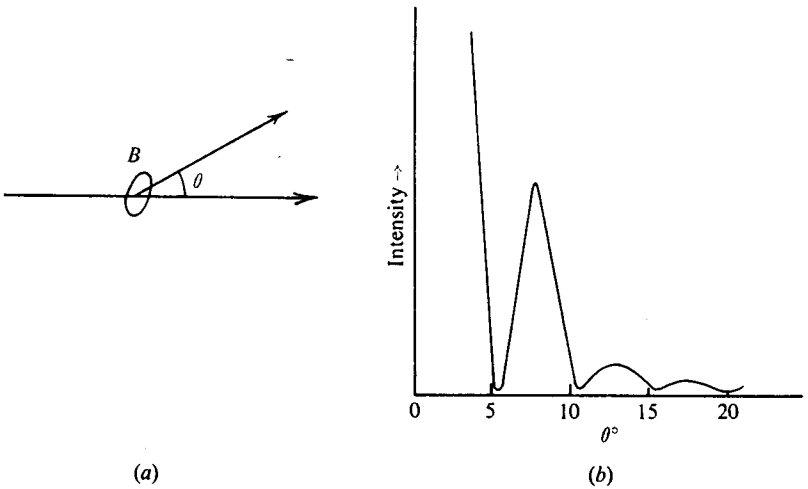

Fig. 1.1. Light-scattering by a bacterial suspension. (*a*) A ray encountering the bacterium, *B*, is deflected from its original path through the angle θ. (*b*) Angular distribution of scattered light. Ordinate: intensity at a given angle θ. Abscissa: values of the angle θ. (After Koga & Fujita, 1962.)

The numerous methods and types of apparatus are described by Snell & Snell (1948), West (1960), and Oster (1960), and only a few of the most common are mentioned in the following sections.

Measurement of undeviated light. The incident light, I_0, and the transmitted light, I (that is, the light *not* scattered) are often related at low bacterial concentrations by the Lambert–Beer law:

$$I = I_0 10^{-\epsilon lc},$$

where ϵ is the extinction coefficient, l the depth of the suspension, and c the bacterial concentration.

$$\log (I/I_0) = -\epsilon lc \quad \text{or} \quad \log (I_0/I) = \epsilon lc.$$