DISTRIBUTION OF BACTERIA IN THE VELOCITY GRADIENT CENTRIFUGE

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ABSTRACT Cells in different parts of the cell cycle can be separated by brief centrifugation in a density stabilized gradient: the Mitchison-Vincent technique. The position of a cell in the tube depends upon its size, shape, and density, upon the gradients of density, viscosity, and centrifugal force through which it sediments, and upon time. A program to compute the velocities and integrate the velocity profile for particles of a particular size class is presented. Because enteric bacteria are a form intermediate between right cylinders and prolate ellipsoids of revolution, the program uses values for the frictional coefficient intermediate between those calculated for ellipsoids and for cylinders. The formula $f = 6\pi\eta b(a/b)^{1/2}$ possesses this property and because of its simplicity greatly speeds the calculations. A second program computes the distribution of masses and then of sedimentation constants for a bacterial population, expressed either as a frequency distribution or as total mass per s-class. The effect of the known variation in cell size at division is included in these calculations, which apply to organisms undergoing balanced, asynchronous growth in which mass increase is proportional to cell size. The two programs in conjunction compute the mass or cell-number profile in an arbitrary gradient. The programs have been used to design gradients to maximize the resolution of the technique.

INTRODUCTION

A variety of technical improvements have recently been made in the sizing of bacteria in balanced growth by use of the Mitchison and Vincent (1) velocity gradient centrifugation method (Blumberg and Koch, in preparation). In conjunction with this work we have developed the theoretical cell distributions appropriate to this technique. The present treatment will deal first with the formulation of the mass distribution for the simplest idealized case. Then, it will describe those modifications necessary to account satisfactorily for variation in cell size at division, deviation from exponential cell mass increase, and the application to arbitrary nonisokinetic gradients.

IDEALIZED SIZE DISTRIBUTION

Workers too numerous to mention[1] have independently derived the limiting law describing the distribution of ages in an asynchronous population of exponentially grow-

[1] 1952, The Mitotic Cycle: The Cytoplasm and Nucleus during Interphase and Mitosis, Butterworth's Scientific Publication, 89; 1954, *J. Exp. Biol.* **31**:8; 1955, Annual Report of Institute of Food Microbiology, **8**:75; 1955, *Biometrika.* **24**:16; 1957, *Acta Pathol. Microbiol. Scand.* **41**:161; 1960, *J. Biophys. Biochem. Cytol.*

ing cells where all cells have precisely the same doubling time. The earliest published paper with the seeds of this derivation appears to be that of J. G. Hoffman in 1949 (2).

The distribution which we shall call the canonical age distribution is shown in Fig. 1 as the solid line curve, and is mathematically expressed by:

$$\left.\begin{aligned} \varphi_N(A) &= 2\lambda e^{-\lambda A}; \qquad 0 \leq A \leq \ln 2/\lambda = \bar{\tau} \\ \varphi_N(A) &= 0 \qquad\quad ; \qquad A < 0; \bar{\tau} < A \end{aligned}\right\},$$

the canonical form of the age distribution by cell numbers, where A is the age of the cell, $\varphi_N(A)$ is the probability that a cell is of age A, λ is the specific growth rate constant and $\bar{\tau}$ is the doubling time. This distribution accounts for the fact that there are twice as many newborn cells as there are cells about to divide. Its exponential character is the consequence of the overall exponential increase in numbers of the population as a whole, and is independent of the rate of growth of the individual cell within the span of a single division cycle.

A parallel formulation has been derived by MacLean and Munson (3) and by Koch and Schaechter (4) for the distribution of cell masses for the case of precise growth and division in an asynchronous exponentially growing population. This distribution which we shall call the zero variation, or canonical mass distribution is shown in Fig. 2 by the curve marked 0% and is mathematically described by:

$$\left.\begin{aligned} \theta_N(m) &= C/m^2; \qquad \bar{c}/2 \leq m \leq \bar{c} \\ \theta_N(m) &= 0 \qquad\quad ; \qquad m < \bar{c}/2; \bar{c} < m \end{aligned}\right\},$$

the canonical form of the mass distribution by cell numbers, where $\theta_N(m)$ is the number of cells of a particular mass, m is the wet mass of a single cell, C is a normalization constant, and $\bar{c}$ is the mass at division. For the mass distribution, the physiology and the details of macromolecular synthesis are important and it was assumed in the derivation of this distribution that the mass, m, of each cell increases exponentially throughout the cell cycle with the same growth rate constant, λ, that characterizes all extensive increases in the population, such as, for example, cell number. In addition, it is assumed that the cell divides into equal halves—this usually seems to be the case for enteric bacteria.[2]

The shape of this distribution is such that there are four times as many cells in a size class just after division as there are in the class of cells about to divide. One factor of two arises because there are two daughter cells resulting from the division of one

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7:273; 1960, *Ann. N.Y. Acad. Sci.* **90**:550; 1960, *Biochim. Biophys. Acta*, **37**:406; 1960, *J. Protozool.* **7**:362; 1962, *Nature (Lond.)*, **193**:555; 1967, *J. Cell Biol.* **37**:729; 1968, *Exp. Cell Res.* **47**:581. This list presents clear evidence that biology as a discipline is markedly different than physics, where it would be inconceivable for example that a dozen Pauli's might discover, and publish as news, their exclusion principle in different journals over a 19-year period. The basic, more general principle that leads to this distribution was stated by Euler in 1760 (see 1970, *Theor. Pop. Biol.* **1**:307).

[2]This question is discussed in more detail in refs. 4, 22, 24.

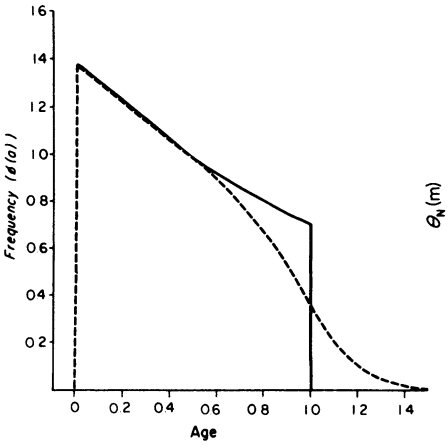

FIGURE 1

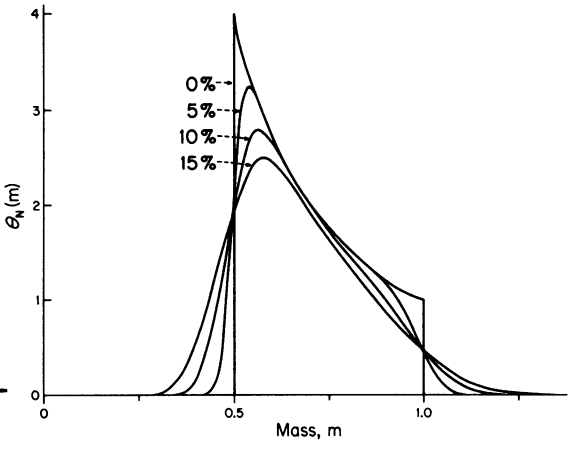

FIGURE 2

FIGURE 1 The age distribution by cell numbers. The solid line shows the age distribution by numbers for a balanced population when all cells divide at the same age, while the dashed line shows the corresponding distribution on the assumption that the distribution of ages in those cells in the act of division has a 20% coefficient of variation and is Gaussian.

FIGURE 2 The mass distribution by cell numbers. The curve marked 0% shows the canonical mass distribution, while the other curves correspond to various coefficients of variation for mass at division as indicated. The computation of the latter is described in the text.

mother cell. The second factor of two arises because it takes twice as long for a newly arisen cell to synthesize a given amount of protoplasm than it does a cell approaching cell division, and to therefore graduate from their respective size classes. Although this canonical mass distribution is not as well known as the canonical age distribution, it has been found useful in computing a variety of average properties of cells from balanced bacterial populations (4–7).

Strictly speaking, actual distributions should be used in which variations in the behavior of individual cells is accommodated. For actual populations (see ref. 4), it has been determined that typically the coefficient of variation is 10–15% for size at division while for age at division it has a considerably larger value of 20–25%. The actual age distributions observed by many workers do not look like the canonical age distribution, but usually resemble the distribution shown by the dashed line in Fig. 1. This latter distribution takes into account the biological variation in the cell division process. The particular example shown was calculated on the assumption that the variation in ages of cells at division is normally distributed with a coefficient of variation of 20%. These choices are consistent with the findings of Schaechter et al. (8).

For some purposes (such as gene mapping) the canonical age distribution has been used. Painter (9) argues that this procedure is justified even if the age at division varies, as long as the length of time required for DNA chromosome replication is constant. For most purposes the variation in age at division must be taken into account. But aside from replacing the 20% with a more precise value for each particular case, there is little need to take the higher moments of the age at division distribution into account

since they hardly effect the shape of $\varphi_N(A)$. Actually the variation of age at division, in much of the literature (10–12), has been observed to be somewhat non-normal and moderately positively skewed. In our work (8) the skewing was not significantly different from zero. The error involved in application of the canonical distribution of mass may be sufficiently small unless the property under consideration varies greatly throughout the cell cycle. Realistic cases correspond to the dashed line in Fig. 1 (coeff. var. = 20%) or to the curves marked 10% and 15% in Fig. 2.[3] An important conclusion from the present work is that considerable error does result if this variation is not taken into account in gradient centrifugation studies.

It is necessary to discuss briefly the assumption, specified above, that growth of each cell is exponential. The claim that cell length increases exponentially (8) was rejected by Errington et al. (13) as not describing all cells in the population. It was rejected by Harvey et al. (14) because it did not describe the properties deduced using the Collins and Richmond principle (15) of the very small and very large cells in the population. It was rejected by Kubitschek (16) who presented evidence that the volume of cells increased linearly. None of these four studies are unequivocal when compared with the autoradiographic experiments of Ecker and Kokaisl (17) which showed that the rate of protein and RNA accumulation was proportional to cell length. This study strongly implies exponential volume growth of *Escherichia coli* unless the protein content varies throughout the cycle. This latter possibility of variation in density will be considered further below.

THE SEDIMENTATION OF CELLS IN ISOKINETIC GRADIENTS

Let us first consider the canonical mass distribution of cell numbers, $\theta_N(m)$, together with its assumptions, and ask how this distribution will transform when its abscissa is not cell size or mass, but rather the sedimentation constant, s. This transformation will be constructed by first deriving $\theta_m(m)$, then $\theta_m(s)$. We will also describe $\theta_N(s)$. Our convention for identifying each distribution is that the abscissal variable appears in the parentheses and the quantity measured appears as a subscript. Thus $\theta_m(s)$ would be the amount of bacterial mass within the interval s to $s + ds$. $\theta_m(s)$ and $\theta_N(s)$ directly correspond to the distribution expected to be observed from examination of an ideal "isokinetic" gradient, where each particle moves at a constant velocity throughout the run and the total distance traveled is directly proportional to its sedimentation constant, $s_{20,w}$. In later sections we will describe methods to calculate distributions that would be observed for actual experimental conditions with any type of gradient.

[3] The fact that age at division is less precisely regulated than is size at division strongly implies that the regulatory mechanism for cell division responds only indirectly to elapsed time since last division and more directly to the level of some biological function at some point in the cell cycle. We have shown previously (4) that the almost two-fold difference in precision between age at division and size at division is quantitatively in accord with a model in which the latter alone is regulated. If cell age alone were regulated, then cell size at division would necessarily have a larger coefficient of variation than the 20–25% found for age at division.

The Frictional Coefficient

The Svedberg equation relates s to hydrodynamic parameters as follows:

$$s = dr/dt/\omega^2 r = m(1 - \bar{v}\rho)/f = m(\rho_p - \rho)/\rho_p f,$$

where r is the position of the particle measured from the center of rotation, ω is the speed of angular rotation, t is time, $\bar{v}$ is the partial specific volume of the cells, and ρ and ρ_p are the density of the medium and particle, respectively. Of special importance here is f, the frictional coefficient, since it, like m, changes with size of the particle. That is, in most of what is to follow we presume that as the cell grows, m and f change but $(1 - \bar{v}\rho)$ or $(\rho_p - \rho)$ essentially does not. For prolate spheroids, Perrin (18) showed in 1936 that:

$$f = 6\pi\eta b\{(a/b)(1 - (b/a)^2)^{1/2}/\ln[(1 + (1 - (b/a)^2)^{1/2})(a/b)]\},$$

where η is the viscosity of the medium, a is the major semi-axis, and b is the minor semi-axis of a prolate ellipsoid. Although this expression is quite complicated, it happens that for ellipsoids with proportions in the range of many actual rod-shaped bacteria this expression can be approximated quite precisely by $f = 6\pi\eta(a/b)^{1/2}$. Fig. 3 shows that $f/6\pi\eta b$ is very nearly equal to $(a/b)^{1/2}$ over a range of proportions bracketing those of most enteric bacteria. Values of 2–6 for the axial ratio a/b are typical for *E. coli*. (Compare curve designated Perrin with that marked root square.)

Most enteric bacteria resemble hemispherically capped cylindrical rods more than they do ellipsoids. However, equations applicable to right cylinders are considerably more difficult to derive, and no one has attempted an equation for a cylinder with hemispherical ends. Fortunately, Bloomfield et al. (19) have derived an equation

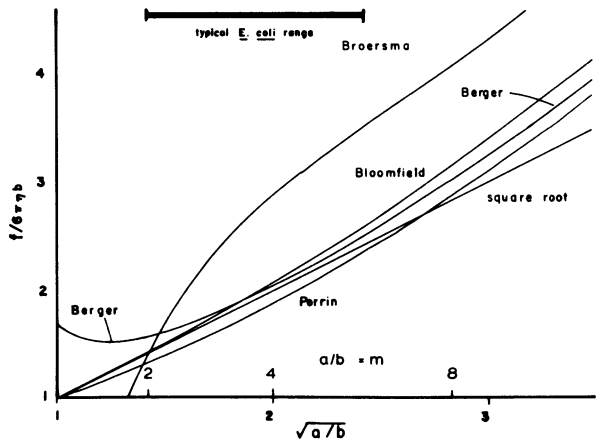

FIGURE 3 Theoretical dependency of the frictional coefficient on asymmetry. Shown are variously derived functions for f, where a and b are half-length and equatorial radius, respectively, as described in the text. It can be seen that within the range of proportions typical of enteric bacteria, i.e. 2–6, for a/b, all but the Broersma equation are closely approximated by the square root expression.

quite useful for short right cylinders. The method of calculation depends on treating the case as an assembly of many small spheres. Their equation can be written:

$$f = 6\pi\eta b[a/b/(\ln(2a/b) - \gamma/2)],$$

where $\gamma/2$ is given by:

$$\gamma/2 = 0.301 - 2b/\pi a.$$

The equation is given in this format because other attempts at solution of this problem by other techniques can be presented by using the same equation for f, but different expressions for $\gamma/2$. For example, Burger (20) derived an expression where $\gamma/2 = 0.11$, and Broersma (21) has expressions for rods moving sidewise and endwise which average to give $\gamma/2 = 0.825 - 2(1/\ln 2a/b - 0.43)^2 - 4(1/\ln 2a/b - 0.3)^2$ for rods in random orientation. These equations, too, are shown in Fig. 3 but are widely divergent from $f/6\pi\eta b = 1$ at $a/b = 1$. Both the square root rule and the Perrin expression for ellipsoids obey this necessary feature. In principle Broersma's is the most accurate expression for cylindrical rods more asymmetric than typical of enteric bacteria, but the Bloomfield et al. expression, while it should not work as accurately for longer rods, is the only treatment applicable to shorter rods in the range of interest here.

On the basis of the calculations of Fig. 3, we conclude that the value of f applicable to enteric bacteria will be slightly above Perrin's accurate expression for prolate ellipsoids. The square root function is a good approximation since it splits the difference with the Bloomfield et al. approximation, and therefore is probably more accurately applicable to enteric bacteria than the exact expression for rod or prolate-ellipsoid. Since the diameter of enteric bacteria during balanced growth is nearly constant (22), a is proportional to m, and f is proportional to $\sqrt{m}$. Consequently the sedimentation constants of cells of different sizes in a zonal centrifuge run will be proportional to $m/\sqrt{m} = \sqrt{m}$. The proportionality constant is $[(\rho_p - \rho)/\eta(\rho_p)^{1/2}](b/3^3\pi)^{1/2}$ or $[(\rho_p - \rho)/\eta(\rho_p)^{1/2}](b/6^2\pi)^{1/2}$ depending on whether the bacterium is modeled as an ellipsoid or a right cylinder.

Thus, on the assumption of this square root approximation, the s value of a cell about to divide, $\bar{s}_c$, is only $(\sqrt{2} - 1) = 41\%$ greater than either of the two cells that result from the division. This compression of the two-fold range of mass between 50% $\bar{c}$ and 100% $\bar{c}$ into a range of 70.7% $\bar{s}_c$–100% $\bar{s}_c$ means that velocity gradients must be of high resolution if they are to be of much use in the separation of cells in different parts of the cell cycle. The inventor of the gradient technique (Mitchison [23]) speaks disparagingly of this technique largely, we believe, because of this compression of range.

This is the appropriate point to return to the question of the suitable function to be chosen for f. For an hypothetical bacterium of the shape of an ellipsoid of revolution, the Perrin equation predicts that s for a cell about to divide will range from 52–31% larger than that of either of the two progeny as the value of a/b of the newborn cells ranged from 1 to 4 (b always constant). Over the same range of birth proportions the

prediction of the Bloomfield et al. equation is 36–32%. Consequently the square root function which yields 41% for all sizes again seems quite appropriate for structures of an intermediate shape. As will be seen below the square root rule greatly simplifies computation of distributions of bacteria in gradients.

Transformation to s*-Distributions*

At the beginning of this section, we asked how the mass distribution will transform when the variable m is replaced with s. To transform $\theta_N(m)$ into the distribution function of sedimentation constants appropriate to the square root approximation, we first note that $\theta_N(m)\mathrm{d}m$ was the fraction of the total number of cells in the mass range m to $m + \mathrm{d}m$. So if $\theta_m(m)\mathrm{d}m$ is the fraction of the combined mass of cells in the range m to $m + \mathrm{d}m$, then this distribution can be derived from the $\theta_N(m)$ simply by multiplying by m, and is given by:

$$\left.\begin{aligned} \theta_m(m) &= C'm/m^2 = C'/m; \bar{c}/2 \leq m \leq \bar{c} \\ \theta_m(m) &= 0; \qquad\qquad m < \bar{c}/2; \bar{c} < m \end{aligned}\right\},$$

the canonical form of the mass distribution by combined cell masses. To transform to the function $\theta_m(s)$ (where $\theta_m(s)\,\mathrm{d}s$ is the fraction of the cell mass in the range of sedimentation constants s to $s + \mathrm{d}s$) one substitutes ks^2 for m and $2ks\mathrm{d}s$ for $\mathrm{d}m$ where k is a proportionality constant, yielding:

$$\theta_m(s)\mathrm{d}s = (C''/ks)ks\,\mathrm{d}s = C''\mathrm{d}s$$

or

$$\left.\begin{aligned} \theta_m(s) &= C''; \qquad \bar{s}_c/\sqrt{2} \leq s \leq \bar{s}_c \\ \theta_m(s) &= 0\ ; \qquad s < \bar{s}_c/\sqrt{2}; \bar{s}_c < s \end{aligned}\right\},$$

the canonical form of the sedimentation constant by combined cell masses. Thus $\theta_m(s)$ for this idealized case of precise cell division where the cells grow to reach mass $\bar{c}$ and sedimentation constant, $\bar{s}_c$, is a rectangular, flat-topped distribution that we call the mesa distribution.

Another appropriate distribution, shown in Fig. 6, is that for cell numbers as a function of s, i.e.,

$$\left.\begin{aligned} \theta_N(s) &= C'''/s; \bar{s}_{\bar{c}}/\sqrt{2} \leq s \leq \bar{s}_c \\ \theta_N(s) &= 0; \qquad s < \bar{s}_c/\sqrt{2}; \bar{s}_c < s \end{aligned}\right\},$$

the canonical form of the sedimentation constant by cell numbers. This would be the appropriate form if analysis of isokinetic gradients were made with a particle counter.

For the distributions we have been considering, the normalization constants are different and have different dimensions. The mass distribution by cell numbers, $\theta_N(m)$, has:

$$C = \bar{c}\ (\text{mass units}).$$

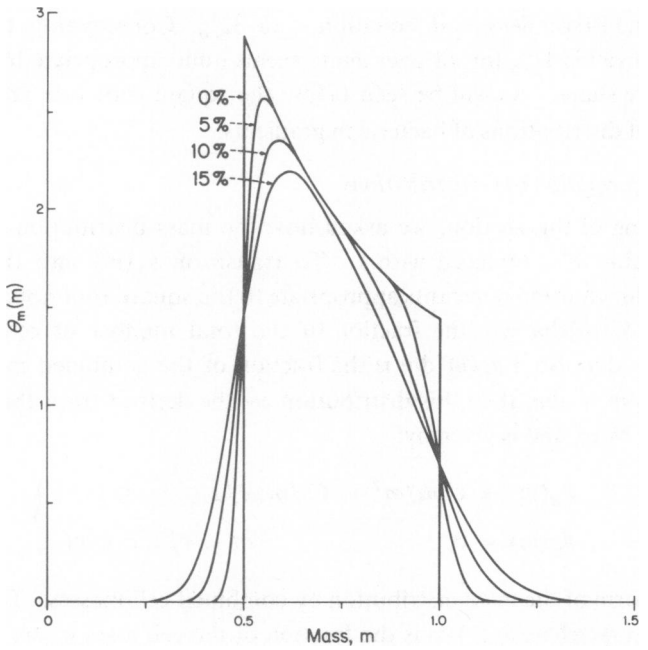

FIGURE 4 The inverse first power distribution: the mass distribution by combined cell masses. See legend to Fig. 2.

The mass distribution by combined cell masses $\theta_m(m)$ has:

$$C' = 1/\ln 2 = 1.4427.$$

The sedimentation constant distribution by combined cell masses $\theta_m(s)$ has

$$C'' = 1/(1 - 1/(\sqrt{2} - 1) = 3.4142 \text{ (per Svedberg)}.$$

The sedimentation constant distribution by cell numbers $\theta_N(s)$ has

$$C''' = 2/\ln 2 = 2.8854.$$

These canonical distributions are shown as the 0% cases in Fig. 2, 4, 5, and 6. These figures also give the corresponding distributions assuming a 5, 10, and 15% coefficient of variation in critical size (size at cell division).

To calculate the curves in Fig. 2, 4, 5, and 6 for the noncanonical cases, a computer algorithm was set up. This computer program superimposes many canonical distributions, one for each different critical cell size ranging from 50 to 150% of the mean critical size, in 100 steps. Then, each of these canonical distributions is subdivided into intervals corresponding to 1% of the average size at division and their contributions summed. This summation method agrees numerically quite well for the $\theta_N(m)$ distribution with that presented by Koch (24) using numerical methods to evaluate an integral expression in terms of mean, coefficient of variation, and skewness coefficient, γ_1.

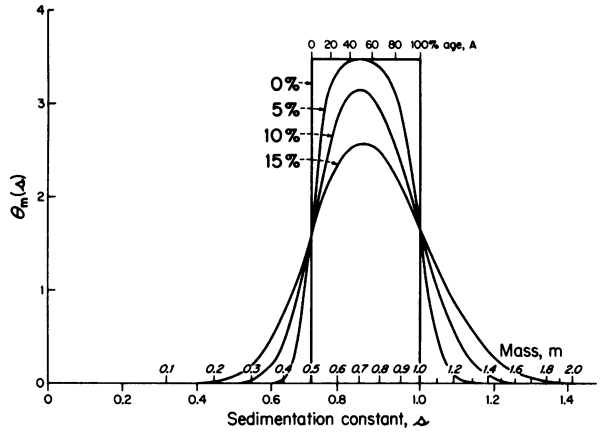

FIGURE 5 The mesa distribution: the sedimentation constant distribution by combined cell masses. Shown are curves for different coefficients of variation for mass at division as indicated. For the canonical form, marked 0%, various skewing factors exactly cancel out, thus producing a flat-topped, "mesa"-like curve. This basic form only applies when the square root law for f is valid.

It was assumed that the distribution of the critical sizes within the population in all of these cases follows normal Gaussian distributions. This assumption can be justified by the experimental finding from Marr's Laboratory (personal communication) that the size distribution of newly born cells eluted from membrane filters was normally distributed. However, our calculations show that the distributions presented here are relatively insensitive to the shape of assumed distributions of sizes at division.

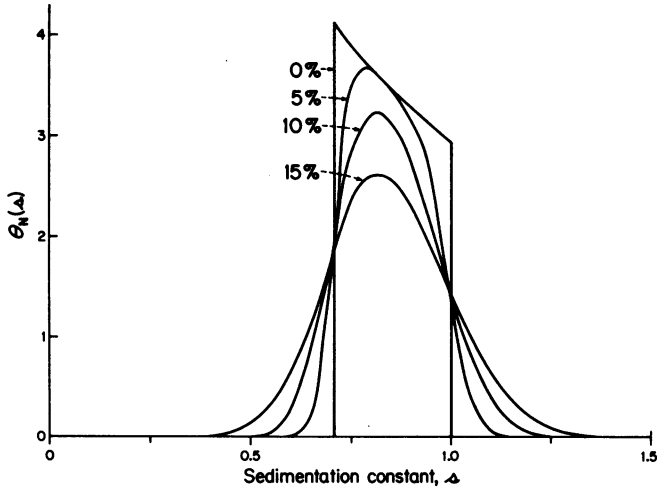

FIGURE 6 The sedimentation constant distribution by cell numbers. See legends to Figs. 2 and 5.

CHOICE OF GRADIENT

Only with specially constructed "isokinetic" gradients will particles sediment at constant velocity throughout the gradient. The necessary condition for this sedimentation behavior can be characterized as follows.

The Svedberg equation with the above approximation where m/f is replaced by a constant times $(2\rho_p b^2/9\eta)(a/b)^{1/2}$ can be rearranged as shown below to separate quantities that (*1*) vary across the population of particles, (*2*) vary along the time course of sedimentation, and (*3*) do not vary at all in a particular centrifugation run. Factor 3 has been evaluated on the assumption that the volume is $\frac{4}{3}\pi ab^2$. It is variation in the second factor that is solely responsible for variation in dr/dt during a single centrifugation.

$$\begin{matrix} & & & (1) & & (2) & & (3) \\ dr/dt & = & 2\omega^2 b^2[(a/b)^{1/2}(\rho_p - \rho)r/9\eta] & = & [(a/b)^{1/2}] & [(\rho_p - \rho)r/\eta] & [2\omega^2 b^2/9]. \end{matrix}$$

Noll (25, 26) describes nonlinear gradients that can be constructed to make the second factor very nearly constant. He shows that for certain choices of gradient materials and centrifuge heads and tube lengths that an exponential gradient can approach within 1% of the desired values for an isokinetic gradient. Nevertheless, the viscosity and density changes along the concentration gradient limit any particular exponential gradient to isokineticity for only a small range of particle densities, tube dimensions, etc. Moreover, isokinetic gradients are not necessarily the best solution for prokaryotic cell-cycle fractionation. Other gradients can have desirable features such as simplicity of construction, constant width of a band, etc. The merits of a number of gradient designs are discussed by Price (27, 28).

For cell separation, as distinct from ribosomes, proteins, etc., there is a further kind of restriction in the design of a density gradient because of the comparatively low density of the cells. The density of the bacteria we have studied is 1.13, whereas proteins typically range around 1.38 and ribosomes around 1.49. This means that the most dense part of the gradient should not exceed 1.10, thus the gradients must be much less steep and consequently less stable to convection. In part we have circumvented this difficulty by using gradients made of D_2O. Because equilibration of water across the plasma membrane is rapid the effective density of the cell increases as the cell sediments.

Henry and Scheie (29) reported the density of *E. coli* to be 1.086, and Kubitschek reported it to be 1.103–1.108 (30), well below our value of 1.13. In our experimental paper these discrepancies are discussed (Blumberg and Koch, in preparation). Here we need only note that extremely shallow gradients must be used for bacteria when nonpermeating solvents are employed. This in itself precludes all but linear density gradients, since a nonlinear gradient with the same concentrations at top and bottom would contain at least one region yet shallower than its linear counterpart.

MODELS OF ZONAL DISTRIBUTIONS

To aid in both the design of gradients and the interpretation of them we constructed a computer program to compute the factor in the second bracket of the equation above.[4] In combination with the program described above which calculates the mesa distribution function $\theta_m(s)$, the time course of the sedimentation of bacterial populations can be calculated.

Figs. 7 and 8 show the computed outcome of the centrifugation of a hypothetical population of bacteria in balanced growth whose critical size has a coefficient of variation of 10% through two types of linear gradient used in this laboratory in the past. We have chosen parameters to correspond to centrifugation in the HB-4 head of the Sorvall centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.). Fig. 7 is for a linear gradient 0–100% in D_2O and with 5% sucrose throughout while Fig. 8 is for a linear 5–15% Ficoll gradient. For convenience in comparing different theoretical and experimental curves, we define a separation index as the ratio of the distance between the half maximal heights to the distance from the meniscus to the point of maximal cell concentration. This is listed on the curves as a percentage. For an ideal isokinetic gradient, cells of a population in balanced growth with a 10% variation of size at division give a separation index of 36.4% which, of course, does not change as the sedimentation proceeds. Such a condition is closely approximated by the D_2O gradient of Fig. 7. The progressive compression seen in the Ficoll gradient of Fig. 8 arises because here the centrifugal force does not increase fast enough with the increasing distance from the axis of rotation to offset the effects of Ficoll in decreasing the bouyant force and increasing the viscosity. It is therefore the same phenomenon as that designated "gradient-induced zone narrowing" by Price (28). (For this reason, and because Ficoll tends to aggregate cells of our strain of *E. coli* as observed microscopically, we have discontinued use of this type of gradient.) On the other hand, the D_2O gradient is relatively free of this compression effect and is in fact virtually isokinetic. This is because the viscosity of pure D_2O is much less than that of 15% Ficoll and because $(1 - \bar{\nu}\rho)$ changes little as the result of D_2O exchange with cell water as the cells go down the gradient. The calculations presented in this case assume that the equilibration is instantaneous.

LINEAR MODELS OF GROWTH

In an earlier paper (5), the problem of cells increasing in mass and size at a constant rate throughout the cell cycle was considered. The canonical mass distribution of cell

[4]The computer must be given the volume, cross section, area, and distance from center of rotation to calculate the quantities in the first two brackets, and parameters describing how viscosity and density change with concentration of gradient material. The program can be easily altered to allow a variety of nonlinear gradients. The computer can display the rate against distance along the gradient. In its current configuration, it can chart also the course of a particle with a particular size and shape and also it can display distributions, at a fixed time, of a population of particles as a function of their size (or shape).

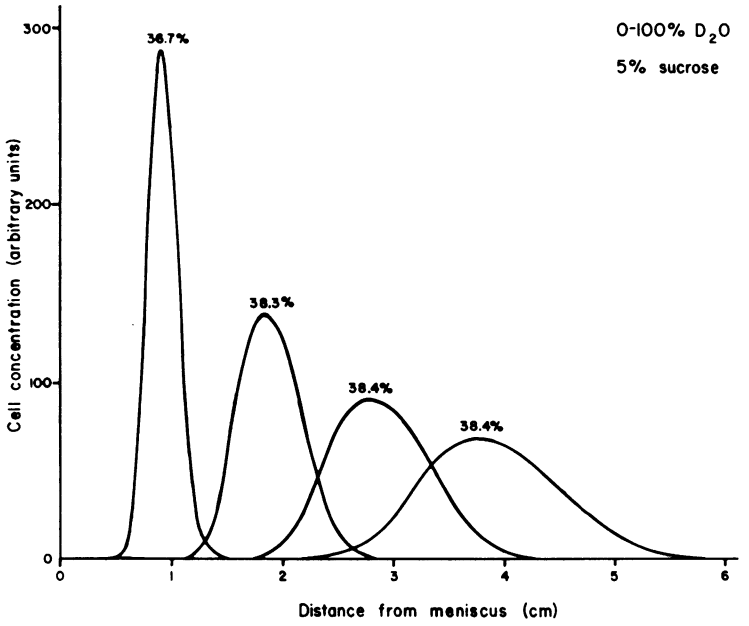

FIGURE 7 The theoretical course of centrifugation through a D_2O gradient. These curves were constructed from the computer output in which the sedimentation of an ideal population of cells with a coefficient of variation for mass at division of 10% is modeled. Depicted are the distributions of combined cell mass after four successive time increments of equal duration. This example and that of Fig. 8 were constructed to correspond to actual gradient runs made in our laboratory in which fractions were measured turbidometrically for bacterial content. Constants chosen were consistent with the loading of 40 ml gradients into 50 ml cellulose nitrate tubes which were spun in an HB4 Sorvall rotor at room temperature (25°). The linear gradients are 0–100% in D_2O and constant in M-9 and 5% sucrose. Radial distances are 6.48 cm to the meniscus and 12.97 cm to the gradient bottom. Constants assembled from the literature and the assumption of average density and content of cell solids given in Table I leads to $\rho_p - \rho = 0.0844 - 0.0002909D$ and $\eta = 1.045 + 0.0020522D$ where D is the percent of D_2O in any portion of the gradient. The percentage shown above each curve is the separation index described in the text.

numbers for the case of constant rate of elongation is markedly different from that for the exponential increase. In the former there is a two-fold increase in frequency for the birth size relative to the cell-division size, instead of fourfold as in Fig. 2. This major change in proportions causes essentially little change in the first and second moments of the distribution while dramatically changing the third and higher moments.

Inclusion of the effects of variability of individuals in the population in terms of cell mass at division were also made in the original analysis (5) and later by Kubitschek (16). The conclusion drawn from both these studies was that despite the two-fold difference in frequency of smallest cells seen in the canonical models for linear vs. exponential growth it would be extremely difficult to distinguish the two cases on the basis of the size distributions. This is because there is little difference in the coefficient

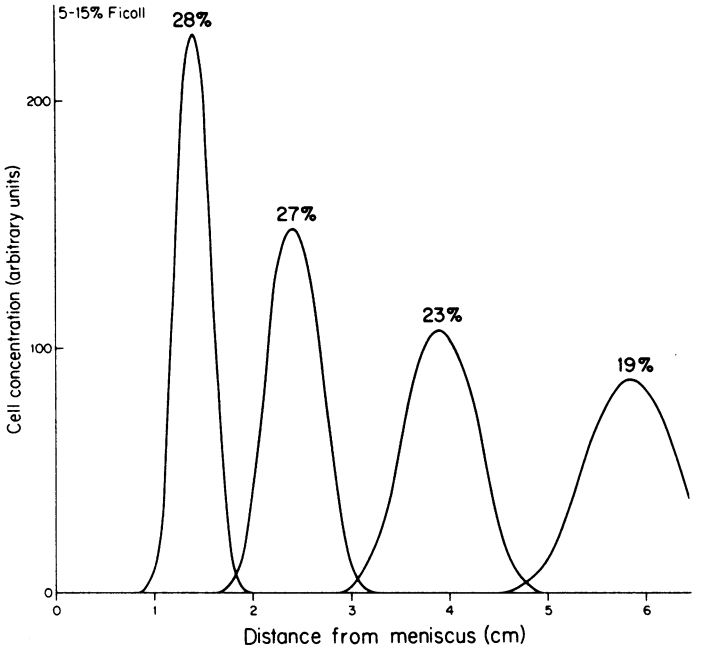

FIGURE 8 The theoretical course of centrifugation through a Ficoll gradient. This corresponds to a 5–15% linear Ficoll gradient made in M-9, other conditions as specified in Fig. 7. Note the gradual compression and slowing as the centrifugation proceeds. Each successive curve is for twice the length of centrifugation time as the previous curve.

of variation of sizes, and the difference in the skewness statistic becomes very small if there is appreciable variation in the size at division in balanced growth. For example, if there is 10% variation in cell size in division, the coefficient of variation of $\theta_N(m)$ for the linear case is 22.09% and for the exponential case is 22.71%. The skewness statistics are 0.38 and 0.57, respectively.

Although a linear growth model for macromolecular synthesis seems excluded as noted above, it seemed a possibility consistent with a number of experimental papers that the mass growth is exponential while volume growth linear. For this model, the cell is at maximum density at birth, shifts to a minimal density near the middle of the cell cycle because the linear rate of volume increase had raced ahead of mass increase and then returns to maximal density at the end of the cycle where exponential rate mass growth overtakes linear increases in cell size.

To calculate the density changes assume that the dry weight of the cell's constituents increases exponentially according to $w = (w_{\bar{c}}/2)2^A$, and that the volume changes linearly according to $v = (v_{\bar{c}}/2) + A(v_{\bar{c}}/2)$ as the age A, ranges from 0 to 1. Thus for this variable-density model, the dry weight concentration, w/v, must change proportionally to $2^A/(1 + A)$. The second difference from the model used in previous sections of this paper is that the axial ratio, a/b is not proportional to w but to v.

The function $2^A/(1 + A)$ is unity at $A = 0$ and $A = 1$; its maximum deviation from

TABLE I

SEDIMENTATION PROPERTIES OF CELLS BASED ON THE VARIABLE-DENSITY MODEL OF ECKER FOR CELLS IN BALANCED GROWTH

	Portion of Cell Cycle			
	Birth*	Point of Minimum Density	Cell* Division	Mean Over Cycle
A. General Properties				
Age	0	0.4427	1	0.4427
Dry Weight	0.5 $\bar{w}_c$	0.6796 $\bar{w}_c$	1 $\bar{w}_c$	0.6931 $\bar{w}_c$
Volume	0.5 $\bar{v}_c$	0.7213 $\bar{v}_c$	1 $\bar{v}_c$	0.7213 $\bar{v}_c$
Relative dry weight Concentration $2^A/(1+A)$	1 $\bar{w}_c/\bar{v}_c$	0.9421 $\bar{w}_c/\bar{v}_c$	1 $\bar{w}_c/\bar{v}_c$	0.9609 $\bar{w}_c/\bar{v}_c$
B. Applied to *E. coli* B/r growing in minimal medium				
Dry weight content	28.099%	26.4717%	28.099%	27%#
Cell density‖	1.1380	1.1143	1.1380	1.121

*Birth and cell division are points of maximum density.

#Assuming mean over cycle : 27 g dry mass per 100 g wet weight.

‖Assuming mean density of cellular dry solids measured in this laboratory of 1.46. This and the 27% absolute dry weight content corresponds to this average density.

unity is at $A = (1/\ln 2) - 1 = 0.4427$ where its value is 0.9421. The mean of the function is $2(\ln 2)^2 = 0.96091$. These values together with the assumptions that the organism is made up of 27% dry solids of density 1.46 suffice to compute the cell density. These two numbers are consistent with our own unpublished observations with *E. coli* B/r mentioned above and those of George Hegeman (personal communication), of the density and dry weight content of cells grown in minimal culture medium.

The theoretical values for the $2^A/(1 + A)$ function are shown in Table I for the parts of the cell cycle where it is a maximum or a minimum. In the bottom portion of the table these theoretical calculations are applied to the *E. coli* B/r case. It can be seen that the density is expected to vary 0.0237 g/ml through the cell cycle. Our experimental determination of the range of cell density (Blumberg and Koch, in preparation) is only 0.008 g/ml or 30 times less, so that this variable density model is experimentally excluded in the case of *E. coli* B/r.

DISCUSSION

Synchrony experiments are evidently one of the most powerful approaches to questions involving the cell cycle of bacteria. Of the many methods used to achieve

synchrony—amino-acid starvation, use of inhibitors, nutritional shifts, membrane elution fractionation, etc.—we feel that the method of choice is zonal gradient sizing of cells. When experiments are performed such that all critical events, e.g., incorporation of label or induction and biosynthesis of a gene product, occur during undisturbed balanced growth, and then fractionation is achieved rapidly in a way that does not depend upon the subsequent physiological behavior of a perturbed cell population, then it seems to the authors that artifacts are minimal and experimental results amenable to clear and direct interpretation. In addition, since we believe (4) that the determinants in the normal cell cycle which control key events are more directly related to cell size than to age, then an experimental method that generates size classes will be *a priori* more tightly coupled to the event under study than a method based upon age separation, such as the membrane elution technique.[3] This will be true whether the goal is to select a population of cells for subsequent synchronous growth or to separate cells at different stages in the cell cycle.

When the latter type of experiment is performed, it is usually necessary to determine the amount of cellular mass per fraction, and this is typically done either by counting cells electronically or by measuring cell mass turbidometrically. For either case it is now possible to calculate, with the aid of a computer, what the expected distribution should be for a given centrifuge run in which the cell population is sedimented and fractionated in an infinitely precise manner. We have developed this modeling approach primarily as a tool for assessing the degree of resolution attained in an actual run, through comparison of the experimentally obtained distribution with the ideal expectation for that run. These comparisons will be presented in future experimental papers. Thus the above derivations and computations were made in an attempt to take into account all of the variables that could be rigorously treated, so that by comparison with actual experiments we could quantitate the effects of those other variables so far not taken into consideration. We have taken into account to the first approximation the growth characteristics of the enteric bacteria and how these effect sedimentation parameters such as particle density and the frictional coefficient; we have also taken into account the design of the gradient. On the other hand, we have not taken into account any convective stirring or any process that prevents the organisms from separating into hydrodynamically independent entities concomitantly with cell division.

Of course, various species of microorganisms and various strains within a species differ widely in their natural tendency to produce mini-cells, filaments, and chains and to adhere to each other as aggregates. Most experimental studies have been carried out with well-behaved strains in these respects. These possibilities can be checked by microscopic examination of samples under analysis (although this should be done on a sample that has been subjected to pelleting in a centrifuge, if this is part of the procedure). Alternatively they can also be checked or at least monitored by analyzing the width of the size distribution measured in a particle counter. Recently Zimmerman et al. (30) have presented data with a modified Coulter counter which should have a greatly improved resolution. Measurements of these published figures show ratios in

the neighborhood of 53% of the width at half height to the modal value. This figure corresponds to very precise cell division (fairly small coefficient of variation of the size at division), and leaves no room for aggregates, etc. The theoretical mass distribution by cell numbers shown in Fig. 2 predicts a range of 54–65% in the ratio of the width at half maximal height to the modal value as the coefficient of variation of size at division is increased from 5 to 15%.

Although our interest has been to isolate enteric microorganisms in various stages of the cell cycle, we hope this approach will be found fruitful for application to other systems as well.

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Copies of the Wang 720 c programs mentioned in the text will be sent those requesting them.

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