GENERATION TIME STATISTICS OF *ESCHERICHIA COLI* USING SYNCHRONOUS CULTURE TECHNIQUES

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by

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ABSTRACT

The distribution of generation times and the correlation coefficient between the generation times of mother and daughter cells are determined for a B strain of *Escherichia coli* under various conditions of growth.

Synchronously dividing cultures of *E. coli* are produced by the Mitchison-Vincent density gradient centrifugation technique. Very precise data are yielded by cell number counts at frequent intervals with a Coulter electronic particle counter. Culture growth is followed for three and sometimes four generations.

Doubling times between 30 and 80 minutes are obtained at 37°C with mineral salts medium supplemented with suitable carbon sources, namely, glucose + methionine + histidine, glucose, sucrose, glycerol, or succinate. On glucose, doubling times between 40 and 90 minutes are obtained by varying the temperature between 37° and 26°C.

A mathematical description of synchronous growth is taken from the literature. The rate of increase of cell number concentration is expressed as the sum of a series of functions with properties related to the generation time distribution of the cells and the correlations between the generation times of related cells. Smoothing of the cell number data by digital filtering and subsequent numerical differentiation yields a series of peaks having little apparent skewness for all growth rates. This is confirmed

by fitting a sum of Gaussian functions to the peaks by a nonlinear least squares procedure. The parameters of the generation time distribution and the correlation coefficients between the generation times of parent and progeny cells are then extracted directly from the means and variances of the Gaussian functions.

The uncertainty associated with each data point is estimated to be within 1½% and the errors in the extracted parameters are determined by repeated simulation of the data analysis procedure using computer generated noisy data.

Under all growth conditions the generation time distribution is of Gaussian form with a coefficient of variation of 0.22 ± 0.02. The mother-daughter generation time correlation coefficient was significantly negative at doubling times between 40 and 64 minutes; a constant value of -0.47 ± 0.06 was consistent with the the observations. At doubling times of 30 and 80-90 minutes this coefficient tended to be closer to zero but with a higher uncertainty. In succinate medium at 37°C (doubling time 80 minutes) synchrony was noticeably weaker after the first generation compared with the results at higher growth rates. The growth data for this medium indicate a correlation coefficient very close to zero.

The implications of the results are discussed in terms of the predictions of a number of models for the control of cell division that have appeared in the literature.

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CHAPTER 1

INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

Of vital importance in biology is an understanding of the mechanism by which the basic unit of life, the cell, controls its own division. The growth and division of single cells is the common link in the growth and development of all living organisms. The regulation of this growth and division is important, for instance, to the cancer researcher investigating the differences between normal and malignant cells.

Information on the statistics of the process of cell division can provide some insight into the nature of the control mechanism. More specifically, we can study the variability in the time between successive divisions of cells of a particular species and the association between the interdivision times of one generation and those of subsequent generations. A number of theoretical models for the control of division have appeared over the years which make predictions concerning the form of the distribution of interdivision times and the correlations between the interdivision times of related cells. In addition, the development of adequate models for the kinetics of growth of cell populations requires knowledge of this distribution and the degree to which the interdivision times of related individuals are

associated.

In this thesis we undertake an experimental investigation into these statistical features of the division process.

In general, the growth of a single cell from its inception at the division of its parent through the steps required to enable it to divide in its turn, can be studied more easily in the simpler organisms, such as the bacteria, than in higher cells. The unicellular bacterium *Escherichia coli* is studied in this thesis. This organism is probably better understood than any other in terms of its molecular biology. Cell cycle studies on *E. coli* will therefore be easier than on organisms about which little is known concerning the fundamental molecular processes of the cell. It is recognized, of course, that differences will appear between the processes occurring in the relatively simple bacterial cells and those taking place in more complex organisms. However, research with microorganisms has frequently furnished valuable models for workers with higher organisms.

Before outlining the specific objectives of the work presented here it is worthwhile to consider the approaches of other workers to our problem, namely, that of determining the statistics of the process of bacterial cell division.

1.2 PREVIOUS APPROACHES

The distribution of generation times*

Measurements of growth and of the division cycles of individual bacteria and other microorganisms have generally been made by means of direct microscopic observation. Kelly & Rahn (1932) were the first to publish extensive measurements on the interdivision times of individual cells. These workers located seven or eight well separated cells on an agar block in the field of view of a microscope and measured the interdivision times of three generations of descendants of these cells.

A correct sample for the generation time distribution can be obtained as follows. Select a fixed number of generations and observe every individual in every line of descent from the ancestor for this number of generations. A biased sample will result if observations of generation time are made on all the descendants of a single organism and the experiment is broken off at an arbitrary moment. This sample will be biased in favour of short generation times since, near the end of the measurement period, those cells with longer generation times would not have had time to divide. The diagram of Fig.1.1 will make this clear. The horizontal lines represent the interdivision times of individuals in a family tree. The length of the line for each individual is proportional to its interdivision time. The individuals extant at time X are six third-generation and four fourth-generation organisms. The times a,....,f, *i.e.* two complete

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*The terms *lifespan*, *interdivision time* and *generation time* all appear in the literature for the period between successive divisions.*

generations from the ancestor A, are unbiased. The inclusion of g and h introduces bias to the sample of times a,..., h. The figures of Kelly & Rahn (1932) suggest that this scheme for unbiased sampling was not rigidly followed and as such their results may be biased.

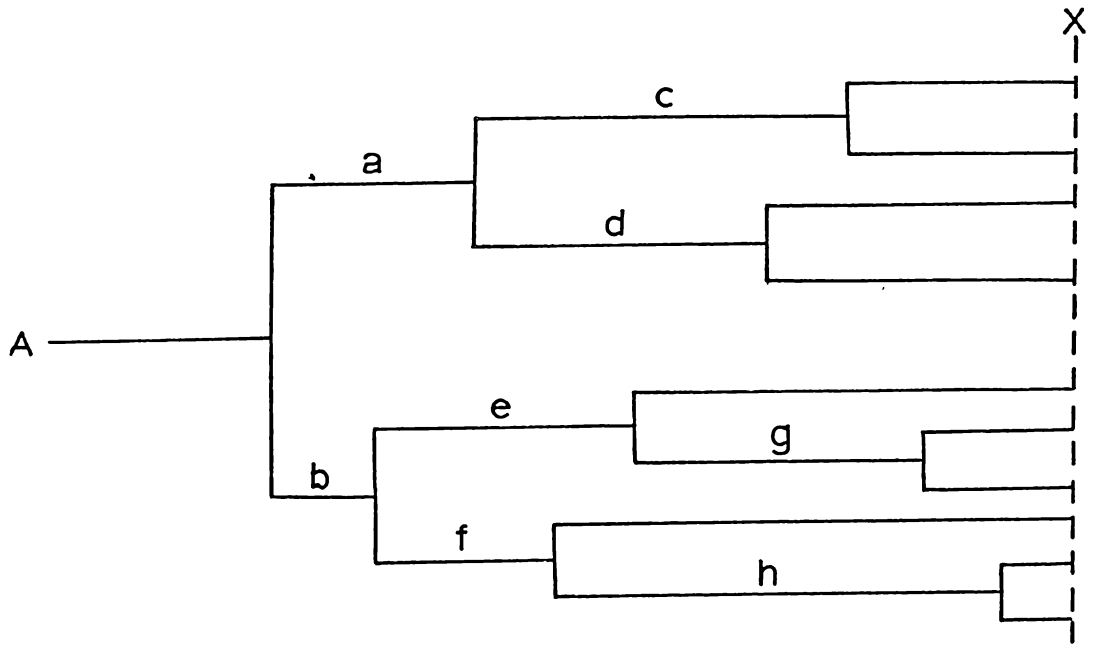

Fig. 1.1 A family tree of microorganisms stemming from an individual A, illustrating bias. From Powell (1955)

Another problem not discussed by Kelly & Rahn is the existence of a steady-state. Apart from the statistical problem that only a small number of bacteria can be observed at one time, microscopic measurement of generation times has also to contend with the difficulty of maintaining the cell population in a constant environment during growth. Although the number of cells in the population obviously is not

constant we apply the term steady-state to the condition where the population is in a state of balanced growth* and the growth rate is therefore constant. Steady growth is undoubtedly difficult to maintain when cells are limited to movement in only two directions on a microscope slide.

The most detailed and systematic data published on generation times are those of Powell & co-workers (Powell, 1955, 1956a, 1958; Powell & Errington, 1963). The culture chamber designed by these workers (Powell, 1956b) allows rather better control of the environment than the agar block method. This chamber contained a cellophane membrane on which the organisms were grown and permitted microscope observation under phase-contrast illumination while the aerated growth medium was continuously circulated under the cellophane. Metabolites could diffuse through the cellophane partition. The method is still hampered, of course, by the large errors of measurement in optical microscopy and by the necessity for a large number of observations in order to obtain statistical reliability.

All of Powell's data indicate that the generation time distribution is a uni-modal function. These measurements indicate also a tendency towards positive skewness in the distribution. In other words, the peak or modal value of the distribution is consistently found to be smaller than the mean value. The data for *Escherichia coli* and related bacteria yield a coefficient of variation** of around 20%.

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*This condition is discussed fully in Chapter 3.

**Denotes the ratio of the standard deviation of the distribution to its mean value.

Although the range of media used in these experiments was limited the measurements supported the hypothesis (Powell, 1958) that the coefficient of variation was higher the greater the chemical complexity of the medium.

Measurements of the generation times of *E. coli* were recorded also by Kubitschek (1962a). The cells were likewise grown on agar coated slides but instead of direct microscopic observation the colonies were photographed, usually at 10 second intervals, through a phase-contrast microscope. The developed film was then examined with a dissecting microscope. A histogram of generation times published by Kubitschek (1962a) is shown in Fig. 1.2. The frequency of divisions is plotted against the generation time, τ, in units of the mean doubling time for the cell population. The spread in the distribution is similar to the values obtained by other workers and a slight positive skewness is indicated. Kubitschek (1962a) has pointed out that the photographic method has the advantage of more accurate timing, although the optical resolution is poorer, when compared with direct observation by eye.

Schaechter *et al.* (1962) also used phase-contrast microscopy and photography to obtain data on the generation times of several bacterial species, including *E. coli*. Cells were grown on a slide having a thin layer of agar and the results on the spread of the distribution of cell generation times agree with those reported by Powell (1958), Kelly & Rahn (1932) and Kubitschek (1962a). However, no significant skewness was found in these distributions, with the exception of that for *Salmonella typhimurium*. Koch & Schaechter (1962) were moved to suggest that the skewness of the generation time

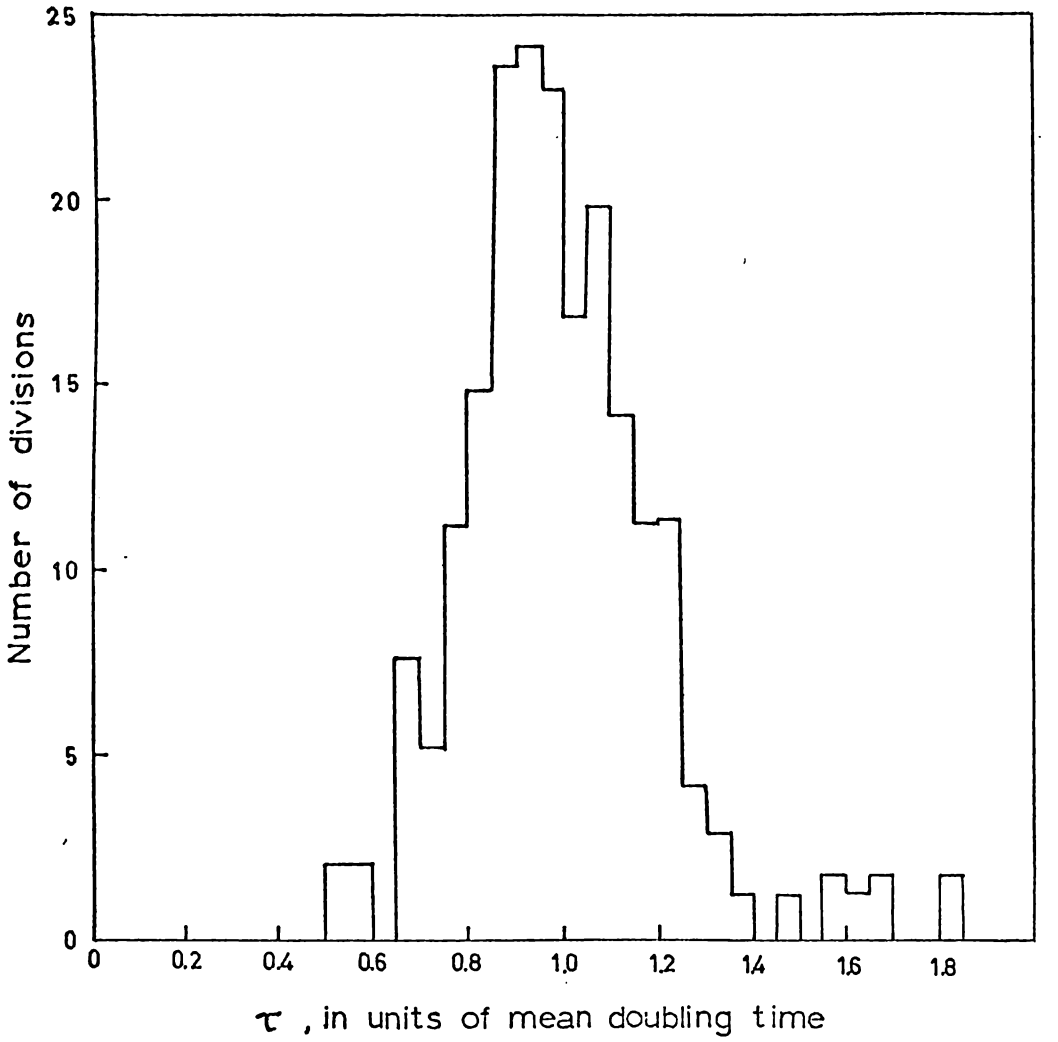

Fig. 1.2 The generation time distribution for *E. coli* B/r (Kubitschek, 1962a).

distribution has generally tended to decrease with improvement in experimental technique.

Kubitschek's data for *E. coli* led to a distribution of generation times more highly skewed toward longer generation times than that found in the studies of Powell & Errington (1963). These two studies coupled with that of Schaechter *et al.* in which no significant skewness was evident, indicate three different distributions of generation time for the same bacterial species. In all of these experiments the data appeared to be collected in a manner which avoided cut-off bias. In addition, all seemed to satisfy the requirement of balanced growth.

However, when the criterion for determining the instant of fission is considered we immediately see that Kubitschek's study differs from the other two. Powell's group and Schaechter's group considered the time of fission to correspond to the time of separation of the mother cell into two mechanically independent daughter cells. On the other hand, Kubitschek's criterion was cytoplasmic separation; the formation of a septum isolating the contents of a mother cell into the daughter cells which remained tightly attached to each other. Variability in the time interval between completion of a septum and the ensuing mechanical separation could lead to distortion of the distribution of times based on the latter process, as Kubitschek (1967) has pointed out. It was intimated earlier by Powell (1955) that different distributions could be expected for generation times based on the two different criteria.

A completely different approach was adopted by Shehata & Marr (1970). They produced a culture growing in division synchrony by selecting a sample of newborn cells according to the technique of Helmstetter & Cummings (1963).* Analysis of the subsequent growth of this synchronous culture allowed them to estimate the distribution of generation times. Harris (1959) showed that, if the generation times of mothers and their daughters are independent, then the growth rate of a synchronous culture is given by

$$N'(t) = N_o f(t) + 2\int_0^t N'(t-\theta) f(\theta) d\theta \qquad (1.1)$$

in which N(t) is the number of bacteria at any time, t, N_o is the number of newly formed bacteria collected at zero time, and f (t) is the distribution of generation times. The integral represents the part of the division rate contributed by cells formed by divisions after zero time. If generation times are not independent then this term is an approximation. Significant error is introduced through this approximation only for long generation times. Shehata & Marr carried out the computation of the generation time distribution for three strains of *E. coli*. The distributions were found to have coefficients of variation in the range 0.18 to 0.22 and all exhibited a small degree of negative skewness.

Correlations between generation times

The question of the relationship between the generation time of a parent cell and the generation times of its daughters has attracted the attention of many biologists.

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*See Chapter 3 for a discussion of methods of producing synchronous cultures.

Experiments by Hughes (1955) purported to show that the growth rate (or generation time) was an inherited characteristic from one generation to the next. Hughes isolated single cells from large and small colonies of *E. coli* and allowed them to grow for a fixed time under identical conditions. A high correlation was found between the size of the colonies so formed and the size of those from which the parent cells were isolated. In other words, fast and slow growing mothers had, on the average, fast or slow growing daughters respectively. This inference of positive correlations between the growth rate or generation time of mother and daughter cells, according to Powell (1958), does not necessarily follow from the results. Powell criticised these experiments on the grounds that Hughes had not taken sufficient account of the effect of the initial conditions on the development of colonies. The size of a colony after a given period of growth will be highly dependent on the age of the ancestor at the beginning of the growth period, and on any lag induced by the manipulations.

The extensive data on generation times of Powell (1955, 1956a, 1958) and Powell & Errington (1963) allow the correlation between mother and daughter cell generation times to be computed. A difficulty, discussed by Powell (1958) is immediately apparent. This is the effect of errors in the estimation of the times of fission of mother and daughter cells on the correlation coefficient. An error in the determination of the time of fission of the mother cell will affect the estimates of the interdivision times of the mother and daughter cells in opposite ways and will, therefore, introduce negative correlation between these times. We can

establish this result mathematically as follows. This brief analysis will also serve to illustrate the meaning, in mathematical terms, of the correlation coefficient.

Consider the schematic illustration of Fig. 1.3 of three successive divisions of a cell. The periods T_1 and T_2 represent the true generation times of a mother and a daughter cell respectively. If errors ε_1, ε_2, ε_3 are associated with the corresponding estimated times of division, then the measured generation times will be τ_1, τ_2, where

$$\tau_1 = T_1 - \varepsilon_2 - \varepsilon_1$$
$$\tau_2 = T_2 + \varepsilon_3 + \varepsilon_2$$

We can reasonably assume that the ε are all distributed alike and are independent of the T and of one another. Then,

$$\text{var}(\tau) = \text{var}(T) + 2\ \text{var}(\varepsilon) \qquad (1.2)$$

$$\text{var}\ (\tau_1 + \tau_2) = 2\ \text{var}(T) + 2\ \text{cov}(T_1, T_2) + 2\ \text{var}(\varepsilon) \qquad (1.3)$$

are the expressions for the variances of each of the quantities τ_1, τ_2 and their sum $\tau_1 + \tau_2$. We denote the covariance between a pair of variables, to be defined shortly, by 'cov'. To obtain equation (1.2) we have used the fact that the variance of a sum of independent random variables equals the sum of their variances*. In deriving equation (1.3) we cannot assume that T_1 and T_2 are independent so that

$$E\ (T_1\ T_2) \neq E\ (T_1)\ E\ (T_2)$$

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*See, for example, Fisz (1963).

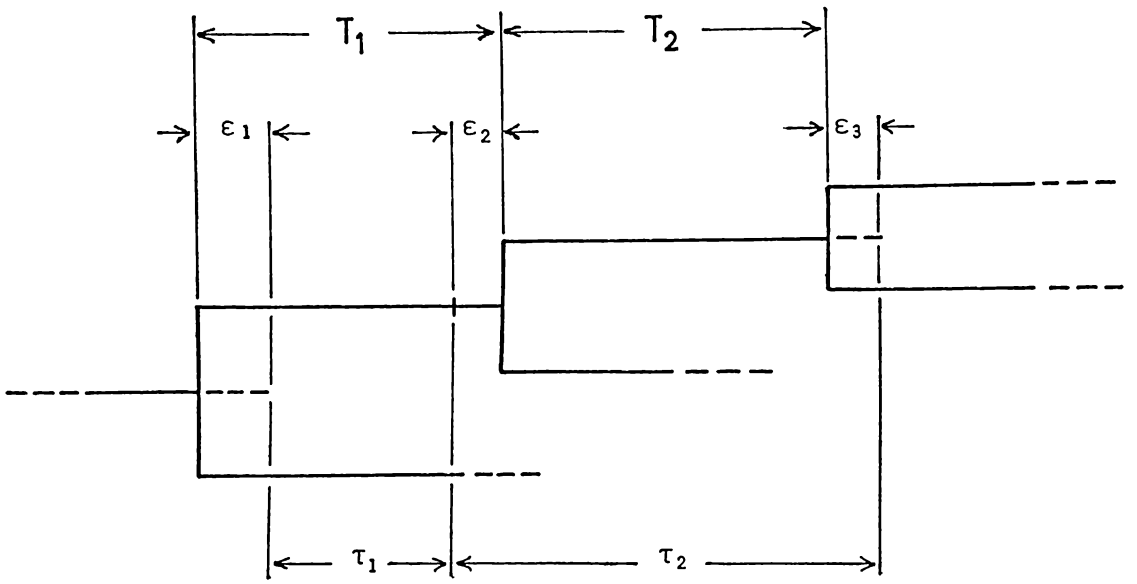

Fig. 1.3 Effect of errors, ε, on correlation between generation times. τ_1, τ_2: measured values; T_1, T_2: true values. From Powell (1958)

where E (T) is the expected or mean value of the random variable T. Hence, we introduce the covariance of T_1 and T_2, cov (T_1, T_2), into the expression for var $(\tau_1 + \tau_2)$. By definition,

$$\text{cov}\ (T_1, T_2) = E\left[\{T_1 - E(T_1)\}\{T_2 - E(T_2)\}\right]. \qquad (1.4)$$

Expanding the right-hand side yields

$$\text{cov}\ (T_1, T_2) = E\,(T_1 T_2) - E(T_1)\,E\,(T_2). \qquad (1.5)$$

Through the definition of the variance of a random variable we obtain equation (1.3). We can now define the correlation coefficient between the observed mother and daughter generation times τ_1 and τ_2. We call this coefficient $\rho(H)$ and it is given by

$$\rho(H) = \frac{\text{cov}\,(\tau_1, \tau_2)}{(\text{var}\,\tau_1)^{\frac{1}{2}}\,(\text{var}\,\tau_2)^{\frac{1}{2}}} \qquad (1.6)$$

where, of course, $\text{var}\,\tau_1 = \text{var}\,\tau_2 = \text{var}\,\tau$. It is easily shown (Fisz, 1963) that the correlation coefficient ρ satisfies

$$-1 \leqslant \rho \leqslant 1.$$

Through the definition of the variance we find that

$$\text{var}\,(\tau_1 + \tau_2) = 2\,\text{var}\,\tau + 2\,\text{cov}\,(\tau_1, \tau_2). \qquad (1.7)$$

Hence, (1.6) may be written

$$\rho(H) = \frac{\text{var}\,(\tau_1 + \tau_2)}{2\,\text{var}\,(\tau)} - 1. \qquad (1.8)$$

Substituting the expressions (1.2) and (1.3) we obtain

$$\rho(H) = \frac{\text{cov}\,(T_1, T_2) - \text{var}\,(\varepsilon)}{\text{var}\,(T) + 2\,\text{var}\,(\varepsilon)}. \qquad (1.9)$$

Thus $\rho(H)$, the observed mother-daughter generation time correlation coefficient, will be biased towards the value $-\frac{1}{2}$ by the common error (ε_2) which affects τ_1 and τ_2 in opposite ways. Because the magnitude of var ε was uncertain, it was neglected by Powell and co-workers in their computations of $\rho(H)$. Throughout their measurements $\rho(H)$ appeared to be variable and not significantly different from zero. Powell & Errington (1963) concluded from their data on *E. coli* and other organisms that no quantitative description could be offered of the manner in which generation times are inherited. These workers found likewise no discernible pattern in the

values they calculated for the correlation coefficient between grandmother and granddaughter generation times, $\rho(H_2)$. On the basis that $\rho(H_2)$ arises solely from the mutual correlation with the mother cell generation time it is expected that

$$\rho(H_2) = \{\rho(H)\}^2 \qquad (1.10)$$

Powell & Errington found that $\rho(H_2)$ was not everywhere positive nor systematically closer to zero than $\rho(H)$, as equation (1.10) would suggest.

Kubitschek's (1962a) results on *E. coli* indicated no significant correlation between the generation times of mother cells and their daughters except when daughter cells had unusually short generation times. In these cases the generation times of their mothers were significantly longer than the mean implying a negative correlation. However, Kubitschek found significant negative correlations extending over longer periods of two or more generations (Kubitschek, 1966).

Mother-daughter correlations in generation time observed for *E. coli* by Schaechter *et al.* (1962) were generally found to be more negative than those reported by Powell and by Kubitschek. However these values varied in different experiments with the same strain and Schaechter & co-workers were not able to establish definitely that they were significantly less than zero.

All the studies by the three groups we have mentioned indicate a strong positive correlation between the generation times of sister cells. Attention should be drawn, though, to the fact that this correlation can be biased towards the value $+\frac{1}{2}$ through any error in estimating the time of inception. Such an error will be common to both sisters. Exactly analogous reasoning to that used in deriving equation (1.9) will reveal this result (Powell, 1958). A positive correlation between the generation times of first cousins was also found by Powell (1958). These are cells with a grandmother as their most recent common ancestor. In the study of Powell & Errington (1963) similar positive correlations were found for other species of bacteria. In both of these reports the generation times of second cousins were found to be positively correlated.

The evidence suggests then, that the generation times of closely related bacterial cells are not independent.

Summarising, it is clear that the microculture techniques discussed here have not furnished satisfactory data to enable conclusive generalizations to be made regarding the form of the generation time distribution for bacteria and for *E. coli* in particular. The nature of the correlations between the generation times of related organisms is also not at all clear, especially that between mother and daughter cells.

1.3 OBJECTIVES OF THIS WORK

The resolution of the considerable amount of uncertainty regarding the form of the generation time distribution for *E. Coli* and the magnitude and sign of the correlation between the generation times of mother and daughter cells was the primary aim of the work presented in this thesis.

While most other approaches to the problem have used microculture techniques we have adopted an approach, essentially that used by Shehata & Marr (1970), whereby measurements are carried out on large samples in liquid culture undergoing synchronous growth. Equation (1.1), used by Shehata & Marr, is a special case of a more general development provided by Harvey (1972a). The derivation given by Harvey allows for dependence between parent and progeny generation times.

Synchronous culture techniques have long been an important tool for the biologist. However, to apply effectively the analysis of Harvey (1972a) we require synchronous growth data of the highest possible quality having the smallest reasonable interval between successive data points. Such data is sadly lacking in the biological literature. Harvey (1972b) applied his analysis to data of Helmstetter (1969) which seemed to satisfy, more than any other data available, the requirements of precision and close spacing of the data points. However, Helmstetter's membrane selection technique of synchronization has suffered adverse criticism as to its effect on the cell cycle. A more

favourable procedure is the density gradient centrifugation method used in this work. With the ability of the electronic particle counter to count cells rapidly and accurately very satisfactory data have been obtained using the centrifugation technique. The data reported in this thesis satisfy the requirements we have mentioned and enable the generation time distribution of *E. coli* to be characterized under various conditions of growth. We discuss and evaluate in Chapter 3 the standard techniques for producing synchronous cultures of bacteria.

Besides obtaining the shape and the parameters of the generation time distribution, application of Harvey's method yields correlation coefficients between parent and progeny cells provided the data has been sufficiently extended in time and is of adequate precision. Our method of extraction of these parameters is somewhat different from that of Harvey and enables better estimates to be obtained. This is particularly so when cultures are analyzed in which the synchrony is essentially washed out within about three generations.

We are therefore able to characterize, using these techniques, the generation time distribution and the mother-daughter generation time correlation coefficient for *E. coli* under a range of growth conditions. Growth rates were altered by varying the growth media as well as changing the temperature under which growth takes place.

Theoretical models for the control of cell division have appeared from time to time in the literature. These

have usually furnished predictions as to the form of the generation time distribution and the extent, if any, of association between the generation time of one cell and that of a related cell. By aiming in this thesis to obtain good experimental data so that reliable estimates of these statistical parameters may be extracted we are then able to comment on the validity or otherwise of many of these models. The following chapter is largely devoted to a discussion of these various models for cell division regulation. In each case we look at the rationale behind its proposal and the predictions that develop. A brief introduction covering some basic concepts of cell biology and the mechanics of the cell cycle is provided at the beginning of this chapter.

The experimental results are presented in Chapter 4. Details of the experimental techniques and the conditions under which these results were obtained are given in this chapter. Discussed also is the treatment accorded the raw cell number data so that a true picture of the progress of each synchronous culture is obtained. Cell volume distributions are examined in order to establish the efficacy of the synchronization procedure and to confirm the presence of synchronous growth.

Chapter 5 begins with the mathematical description of synchronous growth presented originally by Harvey (1972a). This analysis lays the foundation for the extraction of the statistical parameters of interest. Details are given of the extraction procedure we use and all the results are analyzed in this way.

The final chapter includes a discussion of the significance of the results and their implications. In particular, we are able to comment on the viability of a number of models that have been proposed for the control of cell division. Possibilities for future work are considered after some of the shortcomings of the experimental work presented in this thesis are assessed.

CHAPTER 2

MODELS FOR THE CONTROL OF CELL DIVISION

2.1 INTRODUCTION

For the benefit of readers who may not be familiar with some of the basic principles of cell biology and the processes occurring in the living cell between divisions we begin this chapter with a discussion of some of the important features. In the main, we limit our considerations to the bacteria but it is pertinent to mention similarities and differences between the simple cells and those of higher organisms. It is to be hoped that some of the models to be discussed will find application in understanding the more complex processes occurring in the latter.

During the course of this introduction we touch on a number of models which have been proposed in an effort to understand the dynamics of cell growth and the regulation of cell division. Those models which yield relatively simple predictions for the generation time distribution and correlations between the generation times of related cells, and which have received a large measure of attention in the literature, are discussed individually later in this chapter.

Living organisms, while appearing to be of great diversity, reveal a fundamental unity when considered at the cellular level. From an evolutionary viewpoint it is perhaps not surprising to find many common features among distinct

biological systems. All organisms share a common chemical composition. The main macromolecules of all living beings, lipids, polysaccharides, protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) are composed of the same small molecules. Metabolic processes are essentially the same in the cells of all organisms. These are the processes by which the universal constituents of living matter are synthesised from external chemical building blocks and by which the energy necessary for such synthesis is generated. At the structural level, each cell possesses a nucleus surrounded by cytoplasm. The nucleus is that region of a cell in which the genetic information is principally localised. The carrier of this information is DNA, segments of which constitute the genes specific to the organism. The DNA carries in coded form all the information required to determine the specific properties of the organism. This information is translated, through the intermediacy of RNA, into specific patterns of protein synthesis. The proteins serve as the catalysts or enzymes responsible for the various operations of the cell; it is the specific pattern of protein synthesis characteristic of an organism that determines its distinctive gross properties.

All living organisms can be classified into three large divisions or kingdoms. These are the plants, the animals and the protists. The protists comprise the algae, protozoa, fungi and bacteria, and are distinguished by their relatively simple biological organization compared to the plants and animals. Many are unicellular, this being generally true of the bacteria and protozoa.

The development of the electron microscope with its ability to study the fine details of cellular structure has led to the recognition that there are two different kinds of cells among organisms. Dougherty (1957) proposed the terms eukaryotic and prokaryotic to distinguish the two cellular types. The former, the more highly differentiated kind, is the unit of structure in plants, animals, protozoa, fungi, and most groups of algae. The less differentiated prokaryotic cell is the unit of structure in bacteria and blue-green algae.

This division into eukaryotes and prokaryotes is of great evolutionary significance. In the prokaryotes the universal functions of cells, *viz.*, biosynthesis, growth, respiration, photosynthesis, and movement, take place within a material structure very much simpler than that of the other cellular organisms. The question arises as to whether the prokaryotic cell represents a stage in the evolution of the more complex eukaryotic cell, or whether these two kinds of cells have completely different evolutionary origins.

The major differences between these two groups of cells lie in their nuclear structure and in the processes of DNA synthesis and replication.

The resting, or interphase, nucleus of a eukaryotic cell is enclosed by a nuclear membrane. The genetic material is carried within this envelope on a number of different structural subunits, the chromosomes. Each chromosome is a very long thread consisting of DNA linked to a special kind of basic protein, known as a histone. The replication of the

individual chromosomes occurs in the interphase period, the period between cell divisions. Howard & Pelc (1953) introduced a convenient nomenclature marking the various phases of the eukaryotic cell cycle. They called the period of DNA replication the S phase. Usually some time elapses between the formation of the cell at division and the beginning of DNA replication; that interval is called the G_1 phase. By the time the S phase is completed the cell is usually not ready to divide, and the interval between the end of S and the onset of cell division is termed G_2. The completion of G_2 is marked by the beginning of the period of cell division known as the M phase (for mitosis). The durations of the G_1, S, G_2 and M phases are different in different kinds of cells, but the variations between individual cells of the same kind are small. By far the greatest variation is found in the G_1 phase. When a cell cycle is long, most of the prolongation is in the G_1 phase; when a cell cycle is very short, there is no measurable G_1 phase at all. The durations of the S and G_2 phases tend to be relatively constant. A useful summary of the procedures used to determine the lengths of these various phases is given in Nachtwey & Cameron (1968).

We mention briefly at this point a model devised by Smith & Martin (1973) to explain the regulation of proliferation in mammalian cells. The demonstration of the extreme variability of the G_1 period in these cells and the fact that this variability accounts for most of the variation of the generation time suggested that the major events controlling cell proliferation occur in G_1. Smith & Martin proposed

that the variability in G_1 arises as a result of a probabilistic transition occurring within this phase. The model divides the cell cycle into two parts - the B phase (containing S, G_2, M and part of G_1) where the cells are proceeding towards division, and the A state (contained in G_1) where the cells are awaiting a random triggering event before proceeding. A cell may remain in the A state for any length of time subject to the condition that under steady-state conditions the probability per unit time (called the transition probability) of the cells leaving the A state and entering the B phase is a constant. In other words, cells leave the A state exponentially with respect to the time they have spent in that state in much the same way as a radioactive material will decay at a rate determined by its half-life. In a population of cells the shortest interdivision time is shown by a cell that leaves the A state immediately it enters. Interdivision times greater than this minimum (equal to the duration of the B phase, T_B) should be exponentially distributed in a population of cells with identical T_B values and identical transition probabilities. In practice, T_B is not invariant; however, provided the variability of T_B is relatively small, the exponential distribution of A state durations should be reflected in the distribution of generation times as an exponential decay for long generation times. Experimental data on mammalian cell generation times have generally indicated skewness toward long generation times and Smith & Martin (1973) show that their model appears to be consistent with such data. Shields (1976) has asserted that the theory can account satisfactorily for the distribution of generation times in a wide variety of cell

types including bacteria. Shilo *et al.* (1976) have applied the model to data for yeast cells. However, it has been pointed out by Nurse & Fantes (1977) and by Wheals (1977) that the data given by Shilo and co-workers could be fitted equally satisfactorily with a lognormal or a reciprocal normal distribution of generation times. Experimental data have generally been tested against the model by plotting on a logarithmic scale the percentage of undivided cells against age at division. This representation seems to be rather insensitive to the precise nature of the generation time distribution at least down to the 2% level (Wheals, 1977). This shortcoming could apply to much of the data that has been presented in the literature. The model, as proposed, can account for positive correlations between the generation times of sister cells (Minor & Smith, 1974) but would seem unable to predict correlations between mother and daughter cell generation times. Smith & Martin (1974) have compared their hypothesis, that the initiation of DNA synthesis is a random event, with other interpretations of the variability of generation times. Mention should also be made that much of the data on animal cells may be subject to some of the criticisms put forward in the introduction to this thesis. More particularly, it is very important that evidence for balanced growth is provided in studies of these cells. It is frequently quite difficult to maintain constant growth conditions over a period of many generations.

Cultivation of bacteria is considerably less problematical and we now turn our attention to these much simpler organisms. Prokaryotic cells possess a loosely defined

nuclear area rather than the definitive membrane-bound nucleus characteristic of eukaryotes. They have one chromosome which is probably a single DNA molecule without any associated histone. Most of our present information has been obtained with the rod-shaped bacterium *Escherichia coli**. The chromosome of *E. coli* consists of a single closed circle of DNA about 1200 μm in length when unfolded. The biochemical mechanism of its replication is still not understood and even less is known about the spatial and temporal organisation of such a long molecule in a cell which is only about 2 μm in length. Much is known, however, about the way its replication is integrated into the bacterial cell cycle. Replication begins on the chromosome at a fixed site (the 'origin') and proceeds in both directions from that point until the two replication forks reach a point (the 'terminus') which appears to be located on the circle approximately opposite to the origin. In the undisturbed cell cycle of *E. coli* strain B/r, growing at 37°C with generation times of 20 to 60 minutes, the time required to duplicate the chromosome is approximately 40 minutes. Under these conditions, cell division takes place about 20 minutes after the completion of each round of chromosome replication. We discuss in more detail later, in connection with the Cooper-Helmstetter model, this situation where the replication time may be twice as long as the cell cycle itself. In these fast growing cells a new round of replication is initiated at every doubling of the cell mass even though the previously initiated replication forks may have yet to reach the terminus. In

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**References to the original experiments can be found in a number of review articles on the bacterial cell cycle, for example, Donachie, Jones & Teather (1973).*

such a population each chromosome will therefore have several sets of replication forks. We can contrast this situation with that in eukaryotes where the replication fork moves much slower but has a much shorter distance to go since the chromosome is divided into many replicating units. In fact the eukaryotic chromosome may have many thousands of replication forks. While DNA synthesis is continuous in fast-growing *E. coli* it is almost always periodic during the cell cycle of eukaryotes. Helmstetter *et al.* (1968) have pointed out the similarity between the constant C + D of their model for the *E. coli* cycle and the constant $S + G_2$ in higher cells; C being the time for a complete round of replication and D the time between the end of a round and the succeeding division. It must be borne in mind, however, that a new C can start during D whereas a new S does not start during G_2, and that the constancy of $S + G_2$ is a tendency rather than a rule.

The growth of a typical batch culture of microorganisms can be distinguished by a number of phases, as shown in Fig 2.1, where we have plotted the cell number concentration in the culture against time. The four principal phases are the lag phase, the phase of exponential growth*, the stationary phase and the death phase. When a microbial population is inoculated into fresh medium growth does not usually begin immediately. A lag phase, of highly variable length, depending on the conditions, intervenes. In general, lags are caused when the inoculum is taken from an old culture,

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*Also referred to as the logarithmic phase.

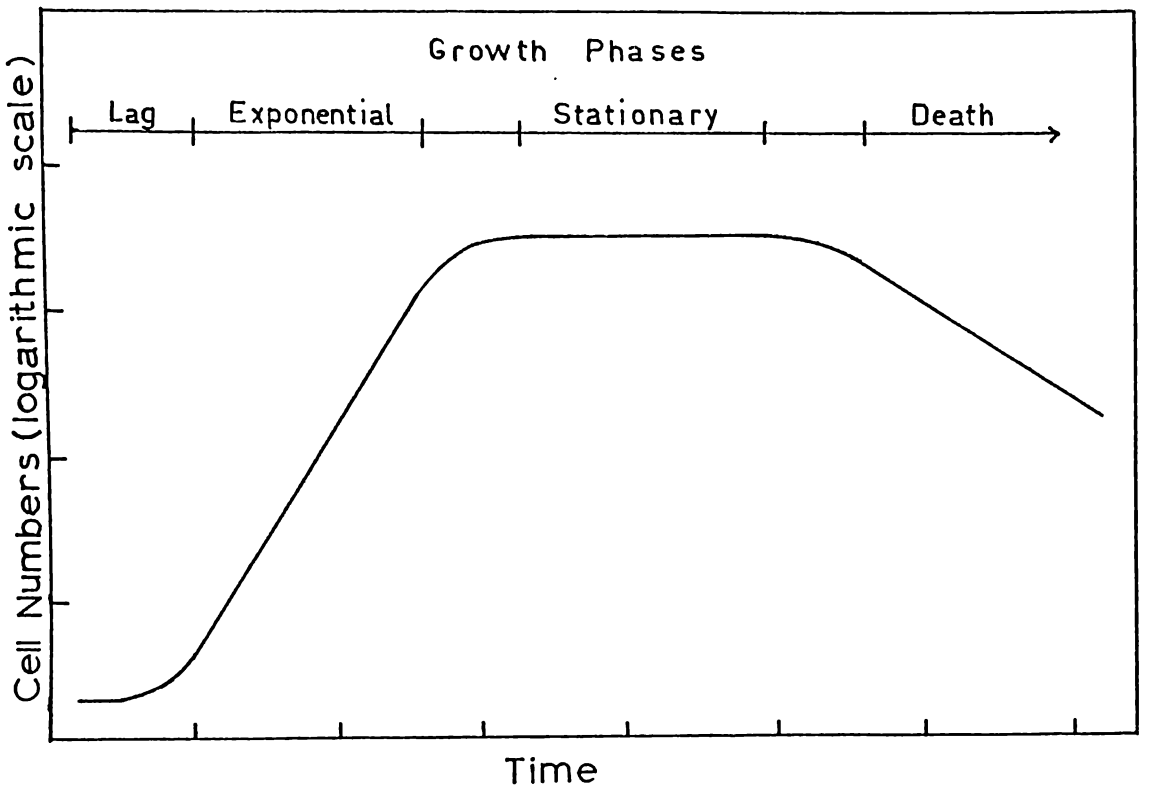

Fig. 2.1 Typical growth curve for a bacterial population in batch culture.

being in the stationary or death phase, or by a transfer from a chemically different medium. Cells in the stationary phase of growth are smaller with an altered chemical composition when compared with exponentially growing cells. Stationary phase cells inoculated into fresh medium must resynthesize essential cell constituents before growth can occur at the maximal rate characteristic of the exponential phase. Similarly, if transfers are made into media with different energy sources, particularly poorer sources, a lag generally occurs as the enzymes required for utilization of the new nutrient are synthesized. The exponential phase of growth begins when the growth rate reaches a constant value. The growth eventually starts to decline as a nutrient is exhausted or because some toxic metabolic product has accumulated. The stationary phase is the period during which

growth of a population ceases. This phase is followed by the death phase in which the live cell count declines. Cell mass may also decline if lysis of these cells occurs.

The exponential phase is a consequence of the fact that each cell in a population divides into two cells. For this phase we can write

$$\frac{dN}{dt} = \nu N \tag{2.1a}$$

$$\frac{dC}{dt} = \mu C \tag{2.1b}$$

where ν and μ are the specific rates of cell number increase and population mass increase, respectively. During steady or balanced growth μ and ν must have the same value, otherwise the size of the organisms would increase or decrease indefinitely. Under these conditions, we can represent the growth of a culture by

$$\frac{dx}{dt} = kx \tag{2.2}$$

where x denotes population numbers or population mass and k is the associated specific growth rate. The rate of exponential growth is usually expressed as the doubling time t_D of the population. By integrating equation (2.2) between appropriate limits we can obtain an expression for t_D in terms of k, thus:

$$\int_x^{2x} \frac{dx}{x} = k \int_0^{t_D} dt$$

i.e.

$$t_D = \frac{\ln 2}{k} \tag{2.3}$$

Although the growth of a population of cells results from growth of the individuals within that population, the quantitative relationships of population growth may be quite different from those of cell growth. The rate of increase in volume or mass of the individual cell can be any function of time which permits a doubling at the end of the inter-division period. The growth law for single cells has been the subject of much discussion in the literature. Experiments have failed to yield conclusive results as to the patterns of growth. In some of the theoretical models to be discussed in the following sections, assumptions are made concerning the precise form of single cell growth.

A variety of more complicated models for the control of cell division than those which we present have appeared in the literature. We have discussed a few of the more mathematically tractable proposals. We have not considered models in which age and size of a cell are considered as the fundamental determinants of cell growth and division (Bell & Anderson, 1967). Nor have we discussed the more recent proposal of Lebowitz & Rubinow (1974) in which the variables age and generation time are introduced to characterize the cell population.

2.2 MODELS BASED ON CELL AGE

2.2.1 Rahn's Hypothesis

Rahn (1932) proposed a model for the control of cell division in which he assumed that a cell divides as soon as some fixed number of events have all occurred within the

cell. Rahn supposed that these events corresponded to the duplication of essential entities, which may be genes, in the cell. The events were assumed to occur independently of one another and the time required for duplication would not be the same for each entity but would be subject to probabilistic laws. Under further assumptions about the rate of entity duplication we can derive an expression for the distribution of interdivision times given by this model as follows.

Let the integer-valued random variable $X(\tau)$ represent the number of entities which have replicated in a cell of age τ. The probability that x entities will have replicated in a cell of age τ is denoted $P_x(\tau)$. Here, $x = 0,1,2,\ldots,g$ where g is the number of entities to be replicated. We assume that the rate of entity replication is constant and equal to β and that the probability that a cell will have duplicated $x+1$ entities at age $\tau + \Delta\tau$, given that x entities were present at age τ, is given by

$$\beta (g - x) \Delta\tau + o(\Delta\tau)$$

In other words, the probability of the transition $(x) \rightarrow (x+1)$ in the interval τ to $\tau + \Delta\tau$ is proportional to the number of entities which have not yet replicated, $g - x$. $o(\Delta\tau)$ means that

$$\frac{o(\Delta\tau)}{\Delta\tau} \rightarrow 0 \text{ as } \Delta\tau \rightarrow 0.$$

In addition, the time interval is considered to be small enough so that the probability of two or more entities being

duplicated in τ to $\tau + \Delta\tau$ is at least $o(\Delta\tau)$. We can now write down the following balance equation for the probabilities,

$$P_x(\tau + \Delta\tau) = \{1 - \beta(g - x)\,\Delta\tau\}\,P_x(\tau) + \beta(g - x + 1)\,P_{x-1}(\tau) + o(\Delta\tau). \tag{2.4}$$

This is clear, since, apart from contributions of $o(\Delta\tau)$, $x\ (\neq 0)$ duplications at time $\tau + \Delta\tau$ can result from two mutually exclusive events:

either (1) $X(\tau) = x$ and no entities are replicated in τ to $\tau + \Delta\tau$,

or (2) $X(\tau) = x - 1$ and one entity is replicated in τ to $\tau + \Delta\tau$.

Transposing $P_x(\tau)$ from the right-hand side, dividing by $\Delta\tau$, and then taking the limit $\Delta\tau \to 0$, it follows that

$$\frac{dP_x(\tau)}{d\tau} = \lim_{\Delta\tau \to 0} \frac{P_x(\tau + \Delta\tau) - P_x(\tau)}{\Delta\tau}$$

$$= -\beta(g - x)\,P_x(\tau) + \beta(g - x + 1)\,P_{x-1}(\tau) \tag{2.5}$$

$$x = 1, 2, \ldots, g.$$

The equation for $x = 0$ is obtained more simply, since $x = 0$ at time $\tau + \Delta\tau$ only if $x = 0$ at time τ and no entities are duplicated in $\Delta\tau$.

Hence,

$$P_o(\tau + \Delta\tau) = (1 - \beta g\,\Delta\tau)\,P_o(\tau) + o(\Delta\tau). \tag{2.6}$$

In this case, we find

$$\frac{dP_o(\tau)}{d\tau} = -\beta g\, P_o(\tau). \tag{2.7}$$

The equations (2.5) and (2.7) are to be solved subject to the initial conditions

$$P_x(0) = \begin{cases} 1 & , x = 0 \\ 0 & , x = 1,2,\ldots,g \end{cases} \tag{2.8}$$

that is, no entities have replicated in a cell of age zero. The method of solution using Laplace transforms and the process of induction is given in Appendix A; we merely state here the result for the probability that all g entities have duplicated by age τ:

$$P_g(\tau) = (1 - e^{-\beta\tau})^g. \tag{2.9}$$

If we let $f(\tau)\,d\tau$ be the probability that a cell of age τ will divide in the interval τ to $\tau + d\tau$ then $f(\tau)$, the frequency function of interdivision times, is

$$f(\tau) = \frac{d}{d\tau} P_g(\tau)$$

i.e.,
$$f(\tau) = g\beta e^{-\beta\tau} (1 - e^{-\beta\tau})^{g-1}. \tag{2.10}$$

The distribution, equation (2.10), was first described by Yule (1925) and is widely known as Yule's distribution.

Powell (1955) has fitted this distribution to his data on generation times obtaining the parameter values in Table 2.1. Strictly, g should take only integer values

because of its identification with the number of genes in the organism. The value of β giving the best fit under this constraint could then be determined.

TABLE 2.1 Experimentally Determined Parameters for Yule's Distribution (Rahn's Hypothesis)

Organism	g	β, min^{-1}
Bacterium aerogenes	59.9	0.222
Bact. coli anaerogenes	27.2	0.191
Streptococcus faecalis	59.2	0.187
Proteus vulgaris	29.6	0.146

2.2.2 Kendall's Hypothesis

Kendall (1948) proposed a similar model for the control of division in that fission was assumed to take place as soon as a fixed number of events g have occurred in the cell. He supposed, however, that these events must occur step by step. Each step may or may not be a process of duplication; other synthetic processes could be deemed to be important. Assuming also that the expected rate of occurrence of the next event is the constant β we can easily derive a system of differential equations for the probability $P_x(\tau)$ that x events ($x = 0,1,2,\ldots,g$) have occurred within a cell by age τ. By similar reasoning to that above for Rahn's model we see that for $x \geq 1$, exactly x events can occur in the interval $(0, \tau + \Delta\tau)$ in three mutually exclusive ways:

(1) x events during $(0, \tau)$ and no events during $(\tau, \tau + \Delta\tau)$;

(2) $x - 1$ events during $(0, \tau)$ and one event during $(\tau, \tau + \Delta\tau)$;

(3) $(x - k)$ events during $(0, \tau)$ and $k \geqslant 2$ events during $(\tau, \tau + \Delta\tau)$.

The probabilities of these events are, respectively,

$$P_x(\tau)\ \{1 - \beta\,\Delta\tau - o(\Delta\tau)\},$$

$$P_{x-1}(\tau)\ \{\beta\,\Delta\tau + o(\Delta\tau)\}, \quad \text{and}$$

a probability of smaller order of magnitude than $\Delta\tau$. This means that

$$P_x(\tau + \Delta\tau) = P_x(\tau)\,(1 - \beta\Delta\tau) + P_{x-1}(\tau)\,\beta\Delta\tau + o(\Delta\tau) \tag{2.11}$$

which gives, in the limit as $\Delta\tau \to 0$,

$$\frac{dP_x(\tau)}{d\tau} = -\beta P_x(\tau) + \beta P_{x-1}(\tau) \qquad x = 1,2,\ldots,g\,. \tag{2.12}$$

For $x = 0$ the second and third contingencies mentioned above do not arise and therefore (2.11) is to be replaced by the simpler equation

$$P_o(\tau + \Delta\tau) = P_o(\tau)\ (1 - \beta\Delta\tau) + o(\Delta\tau) \tag{2.13}$$

which leads to

$$\frac{dP_o(\tau)}{d\tau} = -\beta P_o(\tau)\,. \tag{2.14}$$

The initial conditions are

$$P_x(0) = \begin{cases} 1 & x = 0 \\ 0 & x = 1,2,\ldots,g\,. \end{cases} \tag{2.15}$$

The solution of the well-known system of differential equations (2.12) and (2.14) is easily found* to be the Poisson distribution

$$P_x(\tau) = \frac{e^{-\beta\tau}(\beta\tau)^x}{x!} . \qquad (2.16)$$

Hence, the expected rate at which the g-th event occurs at age τ is $\beta P_{g-1}(\tau)$. The probability that a cell of age τ will divide in the interval τ to $\tau + d\tau$ is just

$$f(\tau)\, d\tau = \beta P_{g-1}(\tau)\, d\tau .$$

The frequency function of generation times predicted by the model is therefore

$$f(\tau) = \frac{\beta^g e^{-\beta\tau} \tau^{g-1}}{(g-1)!} . \qquad (2.17)$$

This is the frequency function for a two-parameter gamma distribution (Keeping, 1962) and is the well-known Pearson Type III frequency function**. In a situation where the parameter g can take non-integer values the factorial in equation (2.17) will be replaced by the gamma function $\Gamma(g)$.

One advantage of the Pearson Type III distribution is that the parameters g and β are simply related to the mean generation time $\bar{\tau}$ and the variance in the generation times

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*See Appendix A.

**See, for example, Elderton & Johnson (1969).

σ_τ^2 by the expressions

$$g = \frac{\bar{\tau}^2}{\sigma_\tau^2} \qquad (2.18a)$$

$$\beta = \frac{\bar{\tau}}{\sigma_\tau^2} \qquad (2.18b)$$

These parameters are therefore easily extracted from experimental data and this is undoubtedly one of the reasons that more work has been done with the Pearson Type III than with Rahn's distribution.

Data collected by Powell (1955, 1958) to which he fitted the Pearson Type III distribution yielded numerical values for the parameters given in Table 2.2. The last four sets of parameters were extracted from the same data as were the parameters given in Table 2.1 for Yule's distribution. Powell (1955) remarked that satisfactory fits to his data can generally be achieved with the Pearson Type III distribution and that the Yule distribution usually gives a less satisfactory fit. In Fig. 2.2 we have shown Kubitschek's (1962a) result of fitting these two distributions to his data for *E. coli* B/r. The parameters giving the best fits are:

	Yule	Pearson Type III
g	400	30
β, min^{-1}	0.16	0.033

Both frequency functions give an acceptable fit to the data.

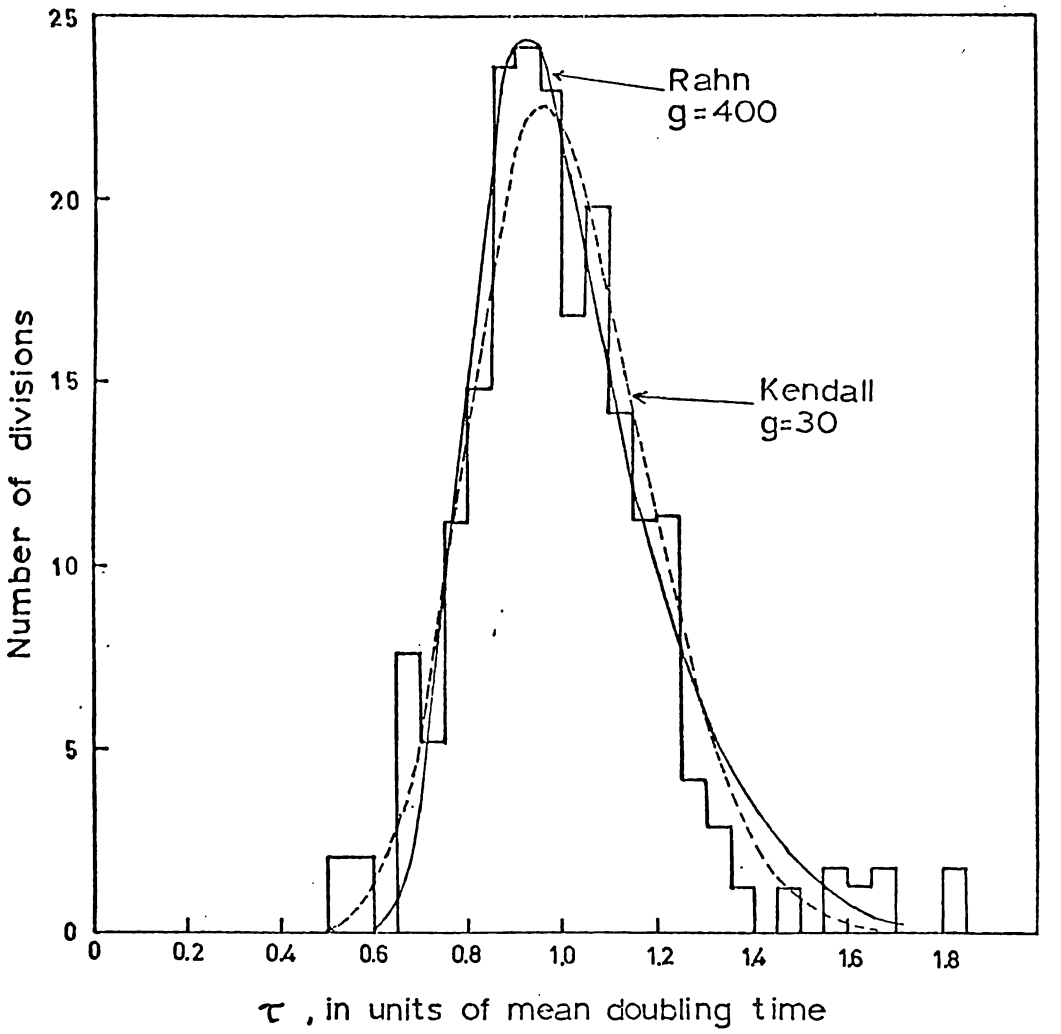

Fig. 2.2 The distributions predicted by Rahn's and by Kendall's hypotheses fitted to the generation time distribution for *E. coli* B/r. From Kubitschek (1962a).

TABLE 2.2 Experimentally Determined Parameters for Pearson Type III Distribution (Kendall's Hypothesis)

Organism	$\bar{\tau}$, min	σ_τ, min	g	β, min^{-1}
Bacillus mycoides	28.7	14.2	4.07	0.142
Streptococcus faecalis	26.1	3.5	56.5	2.16
Pseudomonas aeruginosa	38.1	5.4	50.6	1.33
Bacillus megaterium	22.3	7.8	8.26	0.370
Bacterium aerogenes	-	-	13.4	0.637
Bact. coli anaerogenes	-	-	9.53	0.465
Streptococcus faecalis	-	-	13.4	0.538
Proteus vulgaris	-	-	9.84	0.361

Kendall (1952) later considered a more general case where the g sequential events take place at different rates $\beta_1, \beta_2, \ldots, \beta_g$. In analogous fashion to the derivations of equations (2.12) and (2.14) the following system of differential equations clearly applies:

$$\frac{dP_o(\tau)}{d\tau} = -\beta_1 P_o(\tau) \tag{2.19a}$$

$$\frac{dP_x(\tau)}{d\tau} = -\beta_{x+1} P_x + \beta_x P_{x-1}(\tau) \tag{2.19b}$$

The solution of this system of equations is given in Appendix A for the special case where $\beta_1 = \beta$, $\beta_2 = 2\beta$, ..., $\beta_g = g\beta$. The predicted distribution of generation times is again found to be Yule's distribution

$$f(\tau) = (1 - e^{-\beta\tau})^{g-1} g\beta e^{-\beta\tau} \tag{2.20}$$

Thus we find that two different mathematical models based on quite different physiological models predict identical generation time distributions. Measurements of f (τ) cannot, therefore, distinguish the two. Marr *et al.* (1969) have pointed out that modifications could possibly be made to Rahn's or Kendall's hypotheses in many ways in order to fit the experimental data as closely as one wishes. It is worth emphasising here that, while measurements of generation time distributions may be of use as criteria for discarding models or for generating new ideas, agreement between a model and the experimental data is not sufficient to validate the model.

The models we have discussed so far have a significant drawback in that they do not allow for any correlation between the generation times of sister cells or parent and progeny cells. The generation time in these models is essentially probabilistic and each cell is governed by the same probability density function for determining its generation time. We have discussed in the introduction to this thesis the interdependence of the generation times of closely related cells generally shown by experimental results.

The fact that these models, based essentially on cell age as a determinant of division, cannot predict correlations, particularly between the generation times of sister cells, was one of the reasons that led Koch & Schaechter (1962) to consider a different model for the control of cell division.

2.3 MODELS BASED ON CELL SIZE

2.3.1 Koch & Schaechter's Hypothesis

Several observations concerned with cellular growth and division prompted Koch & Schaechter (1962) to propose a model in which division is controlled by cell size rather than cell age. We have already mentioned the unsatisfactory nature of models based on cell age in which interdivision times are completely independent. Schaechter *et al.* (1962) determined the generation time correlation coefficients between sister cells and between mother and daughter cells for a number of bacterial species. Some of their results are presented in Table 2.3. Shown also are their results for the coefficient of variation for the distribution of generation times and the distribution of lengths of cells at division in these experiments. The diameters of the cells were not measured but considerable data are available indicating that this dimension remains approximately constant during the development of individual cells (Adolph & Bayne-Jones, 1932; Deering, 1958; MacLean & Munson, 1961). More recent measurements by Marr *et al.* (1966) have substantiated the assumption that increase in volume during growth of *E. coli* consists of an increase only in length. Schaechter *et al.* (1962) showed in addition that the apparent refractive index of the cells remained constant during their development and thus considered cell elongation to represent a reasonable measure of the increase in cell mass. The smaller spread in the distribution of cell mass at division compared with that for cell age at division, as is clearly shown in Table 2.3, implies that the latter is less precisely regulated than is

TABLE 2.3 Data from Schaechter *et al.* (1962) on the variability in cell generation times and division lengths and the correlations between generation times of sisters and mothers and daughters

Organism	Experiment	Coefficient of Variation		Generation Time Correlation Coefficients	
		Division Length	Generation Time	Sisters	Mother-Daughter
Escherichia coli B/r	A-1	0.085	0.176	0.183	-0.539
	A-3	0.089	0.137	0.795	-0.195
Salmonella typhimurium	B-4	0.099	0.169	0.567	-0.142
	B-5	0.107	0.152	0.616	-0.401

size at division. Indeed, it may be inferred that, whatever the mechanism of regulation of cell division, this control is more closely coupled to the level of some biological function in the cell cycle than to the time elapsed since the last division. Koch & Schaechter (1962) reported that the mean size of bacteria at division changes in a regular fashion when cells are transferred to a medium in which growth rates and bacterial size are different, so emphasising the dependence of cell division on cell size.

Koch & Schaechter's (1962) proposed model was based on the following postulates:

First Postulate: The rate of growth in size of an individual cell is a deterministic function of its size. As a subpostulate Koch & Schaechter assumed that the growth of individual cells of a culture in exponential growth is also exponential, with the same growth rate constant as that of the culture. That is,

$$\frac{dy}{dt} = ky \qquad (2.21)$$

where k is the growth rate constant of the culture and y is the amount of any cell constituent or some combination of constituents in a cell. The particular case where y represents the total protoplasmic mass, m, was considered.

Second Postulate: The size (mass) of a cell at division is under genetic and environmental control. In other words, division follows when, on the average, a cell has reached a critical size dependent on physiological and biochemical parameters.

Third Postulate: The size at division may vary slightly about the mean value due to random causes. Random variations of cell size at division may be due to

(i) the critical mass varying slightly from individual to individual; or

(ii) the possibility that appearance of visible evidence of division in individual cells may be premature or delayed.

It is immaterial to the discussion which of these sources of variation holds. As a subpostulate, Koch & Schaechter assume that this variation is small, Gaussian, and uncorrelated with events in previous cell cycles or in sister cells.

Fourth Postulate: Cell division results in an equal or nearly equal division of cytoplasmic mass between the daughter cells.

On the basis of these assumptions Koch & Schaechter (1962) showed that the observed coefficients of variation in the age of cells at division (approximately 20%) found in the experiments of Schaechter *et al.* (1962) could be explained entirely on the basis of the observed coefficients of variation in the critical mass (about 10%) in the same experiments. No significant skewness was found in the generation time distributions obtained in these experiments which is in accord with the assumptions of the Koch & Schaechter hypothesis. A number of workers however have emphasised the positively skew nature of the generation time distribution indicated by their experiments. Koch & Schaechter (1962) suggested three possible reasons, one or

more of which may explain the deviation from symmetry in the generation time distribution. First, the distribution of sizes at division may not be normal. Appealing to the central limit theorem they show that the distribution of generation times tends to be more normal than the distribution of division sizes. The latter would have to be strongly skewed in the negative direction in order to explain the skewness of the generation time distribution observed in some instances. This seems unlikely although it is experimentally difficult to exclude. A second explanation is that fluctuations in environmental conditions are more often harmful than beneficial with the result that life lengths are predominantly increased. In this connection, Koch & Schaechter (1962) observed that the skewness of the distribution has decreased as experimental techniques have advanced. The third explanation is that unequal division produces the asymmetry in the generation time distribution. Koch & Schaechter show that such imprecise division will lead to positive skewness in the distribution and they suggest that, on this basis, their experiments (Schaechter *et al.*, 1962) indicate that the division process in the cases considered is quite even.

Koch & Schaechter (1962) proceed to show that their model predicts a sister-sister generation time correlation coefficient of + 0.5, in the case where division of the mother cell is precisely even. Uneven division tends to decrease the sister-sister correlations. It will be evident that this positive correlation may result mainly from the fact that sister cells have a common mother whose critical mass

may be smaller or larger than the average, so giving both daughters a handicap or a head start, respectively.

The correlation coefficient between the generation times of mothers and daughters is found, similarly, to be -0.5 in the case of even division. Unequal division renders this coefficient more positive, tending toward zero for highly asymmetrical division. The need for a negative mother-daughter correlation is self-evident in this model. If a cell divides prematurely in one generation an immediate recovery occurs in the next generation with a fluctuation toward longer than average generation time.

Powell's (1964) detailed account of the consequences of the Koch & Schaechter hypothesis suggests, through suitable mathematical simplifications, how these consequences can best be compared with experiments. The reader is referred to the original paper for the mathematical analysis. It is of interest here, however, to mention the so-called 'real' and 'artificial' distributions of generation times introduced by Powell in this publication and elaborated upon later (Powell, 1969). If the generation times are independent of one another the frequency function of generation times $f(\tau)$ can be generated by selecting a group of fissions in any way and recording the generation times of all the organisms resulting immediately from those fissions. This procedure furnishes a biased sample of generation times in cases where the generation time of an organism depends on that of its mother. This is clear because our apparently random selection of fissions provides generation times which

are influenced by the generation times of the mothers. A sample of new organisms collected from a culture in exponential growth contains more cells with short-lived parents than cells with long-lived parents. The resulting distribution was called by Powell (1964) the 'population' distribution, $P(\tau)$. We can realise a sample of generation times which is essentially unbiased with respect to the parent generation times by recording the generation times of all the nth generation progeny of selected organisms, provided n is taken large enough for these generation times to be uninfluenced by the original selection. This distribution, $f(\tau)$, is 'artificial' in the sense that it is not directly represented in the growing culture. $P(\tau)$ and $f(\tau)$ are, of course, identical when there is negligible correlation between the generation times of related organisms. Otherwise, the difference between the two distributions is not great, especially if the coefficient of variation of the generation times is small (Painter, 1975). Some extreme examples considered by Powell (1956a) show that an error of less than 3% is incurred if the growth rate is calculated from the generation times disregarding the mother-daughter correlation. It should be mentioned that sister-sister correlations in generation time have no effect on the growth rate. This may be seen (Powell, 1956a) as follows. Consider a group of fissions occurring during some short interval Δt, represented in Fig. 2.3. The pair of sisters resulting from each fission have generation times which may be correlated. The branches of the tree to the right of $t + \Delta t$, representing part of the subsequent development of the daughters, are

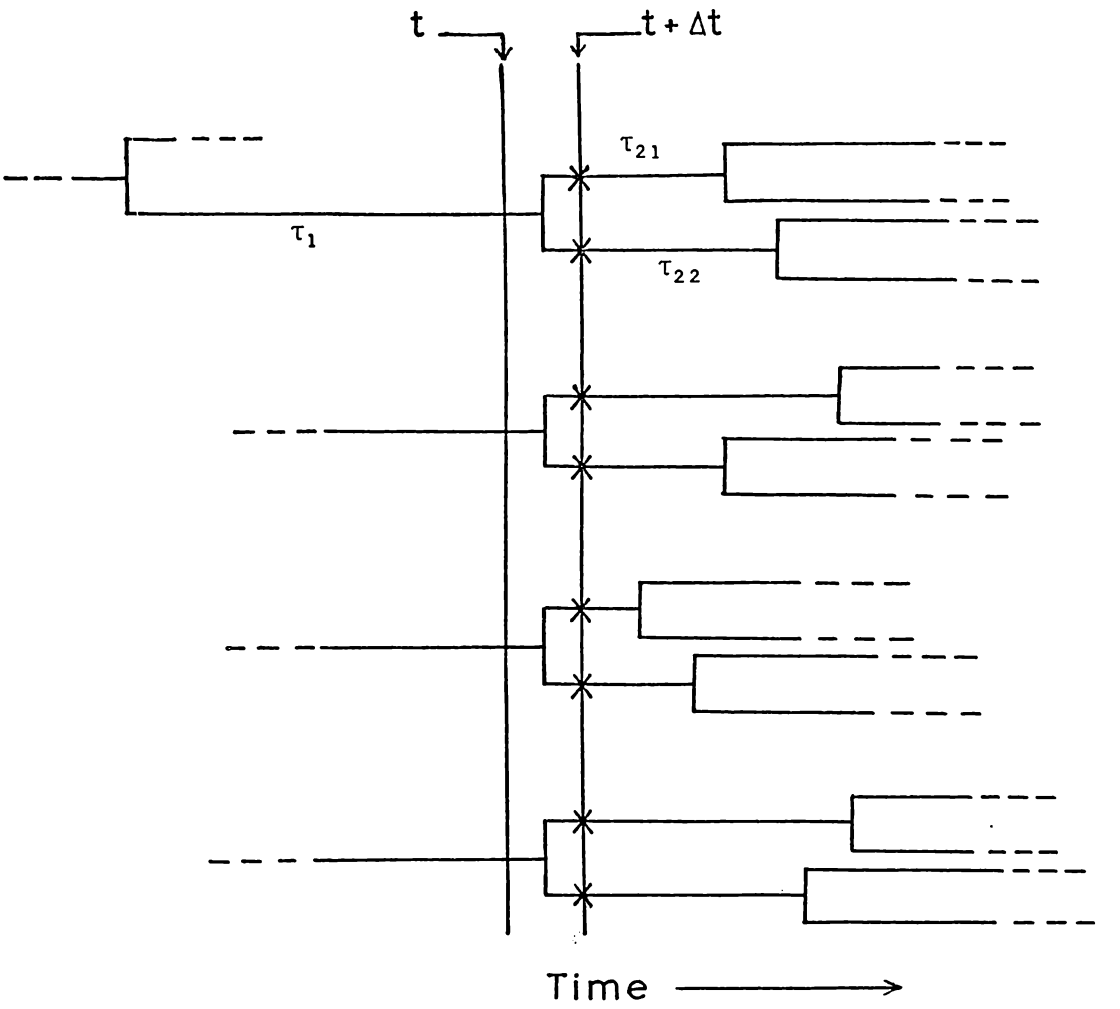

Fig. 2.3 Schematic illustration of the independence of growth rate and sister-sister correlation in generation times. A typical triad of a mother and two daughter cells is represented by τ_1, τ_{21}, τ_{22}. From Powell (1956a).

severed at the points X and reattached after a random shuffling. We can form the pairs such that there is no correlation between the members, although their contribution to the growth rate remains the same. This holds for all intervals Δt. For steady growth then, the growth rate is independent of correlations between sisters.

The fourth postulate of the Koch & Schaechter hypothesis was shown to be substantially correct for a strain of *E. coli* studied by Marr *et al.* (1966). Their measurements indicated that the distribution of p, where

$$p = \frac{\text{volume of daughter cell at division}}{\text{volume of parent cell at division}},$$

was not significantly different from Gaussian with a very small coefficient of variation (4%). They were led to conclude that cells of *E. Coli* divide equally with a very small random error in the division of cytoplasm between the two daughters.

Support for a deterministic process underlying the growth of microorganisms was presented by Koch (1966a). He showed, using data of Kubitschek (1962a) and Hoffman & Frank (1965), that the standard deviation of the total time elapsed between one cell division and the cell division of all the nth generation descendants did not increase significantly with increasing n. n, here, took values up to five in the case of Kubitschek's experiments and nine in the case of the Hoffman and Frank experiments. The implication is that this fluctuation in elapsed time depends only on the chance fluctuations that determine the initial size of the parent cell and the random fluctuations that terminate the last division, quite apart from the number of intervening divisions with their associated fluctuations. These observations strongly support the deterministic growth of cell constituents and the precise equipartition of cell constituents at division.

Koch & Schaechter (1962) assume exponential volume growth for the individual cells. Their measurements (Schaechter *et al.*, 1962) are in accord with this notion although they admit that it is difficult, at the single cell level, to distinguish between an exponential growth law and certain other functions, particularly the linear function. Much interest has centred on the linear versus exponential models for the growth of individual cells. Owing to the small difference in volume predicted by these two models experimental verification is difficult and conflicting reports appear in the literature. Measurements of individual living bacteria using light microscopy, for example those of Schaechter *et al.* (1962), do not appear to have the required resolution to distinguish between various growth patterns. An alternative method was developed by Collins & Richmond (1962) who derived an equation enabling the determination of volume growth rates of individual cells from the distribution of cell volumes in steady-state cultures. The use of the equation requires knowledge of the volume distributions of the total cell population as well as the subpopulations of dividing and newborn cells. Anderson & Bell (1971) have studied the inherent accuracy of this equation through an analysis of error propagation within it. Reproducible cell volume distributions are provided by electronic counter-pulse height analyzer systems. This method has been applied by Marr and his collaborators (Harvey *et al.*, 1967; Marr *et al.*, 1969) and indicates a growth curve of sigmoid shape for *E. coli* cells. The procedure, of course, relies on volume distribution spectra of suitable accuracy being available. Kubitschek (1971a) and Koch (1966b) have pointed

out the technical difficulties that may lead to distorted distributions of cell volumes* and so invalidate the conclusions.

Kubitschek has advocated linear cell growth in several publications. His measurements of mean cell volumes in a synchronous culture with a Coulter counter-multichannel analyzer system provide evidence for constant rates of cell volume growth over nearly all of the generation cycle in three different strains of *E. coli* and at different rates of steady growth (Kubitschek, 1968a). It was pointed out (Kubitschek, 1971a) that this method is not subject to the difficulty mentioned in the previous paragraph because the degree of distortion in the volume distributions is expected to be the same for cells of all volumes.

Harvey *et al.* (1967) have shown that deviations from exponential growth of the individual cells can lead to skewness in the distribution of generation times. Their argument is based on Powell's (1964) assertion that, under the Koch & Schaechter hypothesis, the generation time distribution, $f(\tau)$, will be symmetrical regardless of the symmetry of the distribution of volumes of dividing cells.

Koch & Schaechter's hypothesis is a definite advance on all previous ideas. However, it should be mentioned that it does not account for the correlations between the generation times of cousins and second cousins. Powell & Errington (1963) found positive association between the

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A brief discussion of these distortions is given in Chapter 4, Section 2.

generation times of first cousins and of second cousins so that the properties of a given organism affect the individual generation times of its progeny up to at least the third generation.

Marr *et al.* (1969) carried out a detailed analysis of synchronous growth in *E. coli* using a general formulation of the Collins-Richmond equation applicable to synchronous cultures (Painter & Marr, 1968). Their conclusion from this study was that the assumption of Koch & Schaechter, that division occurs at a critical size which is independent of the size at birth, was incorrect. These workers suggested that cell division was more likely to be regulated by size only indirectly.

2.3.2 Kubitschek's Model

Detailed evidence has been presented by Kubitschek (1970) for linear cell growth during the normal cycle for cultures in a steady-state of growth on compounds of low molecular weight. Linear growth suggests that the accumulation of most or all growth factors occurs at a correspondingly constant rate. This would be the case if uptake were limited by the presence of a constant number of active sites for binding or accumulation of growth factors. Uptake experiments were performed by Kubitschek (1968b) with several labelled compounds in steady-state cultures of *E. coli* and the rates of accumulation remained constant during most of the cycle. These results do not necessarily contradict those of Abbo & Pardee (1960) and Cummings (1965) who found that protein and RNA increased essentially

exponentially during the growth of synchronized bacterial cultures. Kubitschek (1968b) has pointed out that these workers measured macromolecular incorporation only, whereas total uptake must also include the additional contribution from any pools of precursor substances. Mitchison & Wilbur (1962) postulated the existence of soluble pools of low molecular weight compounds. The sum of the material in the pool and that in the macromolecular fraction might increase linearly, even though macromolecular mass increases exponentially.

The model suggested by Kubitschek (1971b), incorporating these observations, was based on the following assumptions:

(1) During most of the growth cycle, each cell contains a constant number of active sites for transport of growth factors from the medium into the cell.

(2) The rate of increase of cell mass increases with the number of sites.

(3) New sites are activated near or at the end of the growth cycle. On the average, the number of sites is doubled at this time.

(4) The number of sites activated at the end of the cycle increases with the surface area synthesized during the cycle.

This model is consistent with the evidence presented by Kubitschek (1971b) that cell generation <u>rates</u> follow a truncated normal distribution. Newly formed cells would inherit about half the number of sites in the parental cell.

The actual number of sites for each cell will fluctuate because of unequal division and because of variations in the final cell size and number of sites produced. If the number of these sites is large these fluctuations will lead to a distribution of sites that is approximately normal. This distribution must be truncated, since the number of sites cannot be negative, nor can they increase without bound upon the limited surface of the cell. Under the assumptions of the model such a distribution of active sites could lead to a truncated normal distribution for generation rates.

The model predicts also, that the generation times of sister cells are positively correlated. If division into daughter cells of nearly equal sizes occurs, then both of the daughters of a very large cell are likely to inherit more active sites than either daughter of a much smaller cell.

This simple model is designed to provide agreement with available evidence for linear cell growth and normal distributions of generation rates and relates these two phenomena to a common mechanism. A number of factors, such as the relationship of DNA synthesis to the production and activation of new sites and to cell division, are neglected, however.

2.3.3 Mass Distribution Model

Eakman, Fredrickson & Tsuchiya (1966) developed a statistical model for a microbial cell population in which the physiological state of the individual organism is assumed to depend only on its mass. These workers derived population

balance equations for the dynamics of microbial populations in batch and continuous cultures.

The reader is referred to the paper of Eakman *et al.* (1966) for the derivation of the general equations of the model. We restrict ourselves in the following to showing how, under simplifying assumptions akin to those of Koch & Schaechter discussed earlier, the model readily yields predictions for the generation time distribution and correlations between generation times of related organisms. Indeed, the development presented here could equally as well have been introduced through the hypothesis of Koch & Schaechter. This derivation, however, is more appropriately given as a special case of the generalized considerations of Eakman *et al.* (1966).

We consider a culture in steady, exponential growth in which the mass of each cell increases with the same growth rate constant that characterizes the culture as a whole. That is the rate of mass increase of a single cell is

$$\frac{dm}{d\tau} = km \equiv r(m) \qquad (2.22)$$

The growth rate constant is k and the rate of mass increase with age, τ, is a function only of mass, m. We are assuming that environmental conditions on the culture are constant with time. In the original development of Eakman *et al.* (1966) specific hypotheses about the uptake and release of materials were made. This allowed the introduction of structure into the model through geometrical considerations

pertaining to rodlike and spherical cells.

Denoting the initial mass of a cell at age $\tau = 0$ as m_o, we can express the growth rate of a single cell as an implicit function of its age and initial mass

$$\frac{dm}{d\tau} = r\{\dot{m}(m_o, \tau)\} \tag{2.23}$$

where $m = m_o e^{k\tau}$.

We define:

$p(m,m')\,dm$ = probability that a daughter cell formed from a mother cell of mass m' has a mass between m and $m + dm$.

$h(m)\,dm$ = probability that a cell will divide in the mass range m to $m + dm$.

By probabilistic arguments, which are given in Appendix B, an expression can be obtained for the distribution of generation times, $f(\tau)$, in terms of the distribution of division mass $h(m)$:

$$f(\tau) = \int_o^\infty h(m') \int_o^{m'} h\{m(m_o, \tau)\}\, r\{m(m_o, \tau)\} \times p(m_o, m')\, dm_o\, dm' \tag{2.24}$$

There is little conclusive evidence, as Eakman *et al.* (1966) point out, to support the assumption that the distribution of division mass deviates significantly from the Gaussian. Since cell mass cannot be negative, this distribution cannot be strictly Gaussian. It is assumed, therefore, that the distribution of division mass around a mean division mass m_c

is of a Gaussian type:

$$h(m) = \frac{2e^{-\left(\frac{m - m_c}{\varepsilon}\right)^2}}{\varepsilon\sqrt{\pi}\left\{1 + \operatorname{erf}\left(\frac{m_c}{\varepsilon}\right)\right\}} . \qquad (2.25)$$

The factor $1 + \operatorname{erf}\left(\frac{m_c}{\varepsilon}\right)$ is a normalizing factor* so that

$$\int_0^\infty h(m)\, dm = 1 . \qquad (2.26)$$

The standard deviation of this distribution is $\varepsilon/\sqrt{2}$. An analytic expression for $f(\tau)$ can be found if we assume that the two daughter cells are always of equal initial mass, that is,

$$p(m, m') = \delta(m - \tfrac{1}{2} m'), \qquad (2.27)$$

where δ is Dirac's delta function. This function satisfies the conditions (Dirac, 1958)

$$\int_{-\infty}^{\infty} \delta(x)\, dx = 1$$

$$\delta(x) = 0 \quad \text{for } x \neq 0$$

and has the following important property

$$\int_{-\infty}^{\infty} g(x)\, \delta(x - a)\, dx = g(a), \qquad (2.28)$$

where a is any real number and g (x) is any continuous function of x. This assumption is reasonable, for *E. coli*

The properties of the error function, erf (x), may be found in, for example, Abramowitz & Stegun (1965).

at least, in the light of experimental evidence mentioned in our earlier discussion of the Koch & Schaechter hypothesis. Under these assumptions, and using the property (2.28), equation (2.24) becomes

$$f(\tau) = \int_0^\infty h(m')\, h\{m(\tfrac{m'}{2}, \tau)\}\, r\{m(\tfrac{m'}{2}, \tau)\}\, dm' . \qquad (2.29)$$

Eakman *et al.* (1966) performed this integration numerically so allowing them to introduce more complicated expressions for r (m) than that used here. Substituting for r (m) in equation (2.29) using (2.22) and (2.23) we obtain

$$f(\tau) = \tfrac{1}{2} k e^{k\tau} \int_0^\infty m' h(m') h(\tfrac{1}{2} m' e^{k\tau})\, dm' . \qquad (2.30)$$

This expression has also been obtained by Powell (1964) and is equation (3) of that paper. Following Powell, the substitution

$$\eta = \sqrt{2}\, m_o\, e^{k\tau/2}$$

yields

$$f(\tau) = k \int_0^\infty \eta\, h\left[\eta \exp\{-\tfrac{k}{2}(\tau - \tfrac{\ln 2}{k})\}\right] h\left[\eta \exp\{\tfrac{k}{2}(\tau - \tfrac{\ln 2}{k})\}\right] d\eta \qquad (2.31)$$

which shows that f (τ) is symmetrical about $\tau = (\ln 2)/k$ whatever the form or degree of dispersion of h (m'). The arithmetic mean of τ is therefore $(\ln 2)/k$. Powell (1964) considered also the more general case when fission does not necessarily result in daughters of equal size, obtaining the result that

$$\bar{\tau} > \frac{\ln 2}{k}$$

where $\bar{\tau}$ denotes the arithmetic mean of τ.

Straightforward integration of (2.30) using the expression (2.25) yields

$$f(\tau) = \frac{k\varepsilon^2 B^2}{4} e^{-2 m_c^2/\varepsilon^2} q(k\tau) \times \left\{ 1 + \sqrt{\pi}\frac{m_c}{\varepsilon}\{1+q(k\tau)\}^{\frac{1}{2}} \frac{1+\operatorname{erf}\left[\frac{m_c}{\varepsilon}\{1+q(k\tau)\}^{\frac{1}{2}}\right]}{e^{-m_c^2\{1+q(k\tau)\}/\varepsilon^2}} \right\} \tag{2.32}$$

where $q(k\tau) = \dfrac{4}{4e^{-k\tau} + e^{k\tau}}$

and $B = \dfrac{2}{\varepsilon\sqrt{\pi}} \{1 + \operatorname{erf}(\frac{m_c}{\varepsilon})\}^{-1}$.

Typically, the coefficient of variation of the division mass distribution is about 10%. That is,

$$\frac{\varepsilon/\sqrt{2}}{m_c} \simeq 10\%$$

or, $$\frac{m_c}{\varepsilon} \sim 5\text{-}10.$$

Observing also that $\operatorname{erf}(\frac{m_c}{\varepsilon}) \simeq 1$ and $q(k\tau) > {}^4/_5$ since $k\tau$ is a positive quantity, we find, after expanding (2.32), that the first term is much smaller than the second and can be neglected. We obtain

$$f(\tau) \simeq \frac{k}{2\sqrt{\pi}} \cdot \frac{m_c}{\varepsilon} q(k\tau) \{1+q(k\tau)\}^{\frac{1}{2}} e^{\frac{m_c^2}{\varepsilon^2}\{q(k\tau)-1\}} . \tag{2.33}$$

A test of the fit of this distribution against real data will permit some justification of the assumptions of the model.

Eakman *et al.* (1966) derive expressions enabling the calculation of the correlation coefficient between generation times of sister cells. Expressions for the mother-daughter generation time correlation coefficient have not been obtained. Heuristic arguments, echoing those of the Koch & Schaechter hypothesis, have been given by Tsuchiya *et al.* (1966) to indicate the correlations that can arise. We can consider two extreme cases:

(1) If the function p (m, m') is such that there is a large probability that the daughter cells resulting from a fission will be of nearly equal size, whereas the distribution h (m) is such that the size of a dividing cell is rather variable, then the generation times of sister cells will be positively correlated. The daughters of a cell which was smaller than the average at fission are both likely to have generation times longer than the average, whereas the daughters of a cell which was larger than the average at fission are both likely to have generation times shorter than the average.

The coefficient of correlation of the generation times of a mother cell and either of its daughters will be negative, by a similar argument.

(2) If the function p (m, m') is such that there is a large probability that the daughter cells resulting from a fission will have quite different sizes, whereas the function h (m) is such that most fissions occur when cell sizes are very close to a certain value, then the generation times of sister cells will be negatively correlated. One daughter resulting from a fission is likely to have a generation time longer than the average while the other is likely to have a generation time shorter than the average. The generation time of a daughter will be more or less independent of the generation time of its mother, giving a near zero correlation coefficient.

2.4 A MODEL BASED ON CONTROL BY DNA REPLICATION

Hypothesis of Cooper & Helmstetter

Experiments by Clark & Maaløe (1967) and by Helmstetter & co-workers (Helmstetter, 1967; Helmstetter & Cooper, 1968) established the location of the DNA replication cycle with respect to the division cycle in *E. coli* strain B/r. The experiments were based on the idea that the start and finish of a round of DNA replication would appear as an abrupt increase and decrease, respectively, in the rate of DNA synthesis during the division cycle. These workers were therefore able to determine the time for a round of chromosome replication (C), the time between the end of a round of replication and the following division (D), and the sum (C + D) over a wide range of growth rates at constant temperature (37°C). The values for these parameters were fairly constant and equal to about 41, 22 and 63 minutes, respectively, in cells growing with generation times between 22 and 53 minutes. At generation times greater than 63 minutes the chromosome appears to be synthesized during the first two-thirds of the division cycle, so that $C = {}^{2}/_{3}\,\tau$, $D = {}^{1}/_{3}\,\tau$ and $C + D = \tau$.

Cooper & Helmstetter (1968) then proposed a model for the division cycle of *E. coli* B/r based on their observations for rapidly growing cultures (growth rates greater than one doubling per hour). They assumed that for growth at a given temperature and for growth rates greater than one doubling per hour:

(1) the time C required for replication to proceed from one end of the chromosome to the other is constant; and

(2) the time D between termination of the round of replication and cell division is constant.

Thus, division occurs C + D units of time after initiation of replication. In cells growing slower than C minutes per doubling there is a 'gap' or period devoid of DNA synthesis, and in cells growing faster than C minutes per doubling there is a period with multiply-forked chromosomes. The pattern of chromosome replication during the division cycle is illustrated in Fig. 2.4 for a range of growth rates.

Marr, Painter & Nilson (1969) have generalized this model and thereby predicted the growth of synchronous cultures. The model, as originally proposed by Cooper & Helmstetter (1968) does not account for any distribution in the times C and D, nor does it specify the control of initiation of rounds of replication. The time between initiation of successive rounds of replication determines the generation time so that the mechanism of control of initiation is crucial to the model. Marr *et al.* (1969) assumed that interinitiation times were independent and that initiation was controlled by timing from the preceding initiation. These workers derived an expression for the generation time distribution f (τ) in terms of the frequency function of interinitiation times and the frequency function of times from initiation to division (C + D). With their assumed forms for these latter two distributions they obtained good agreement with the synchronous growth data of Cummings (1965)

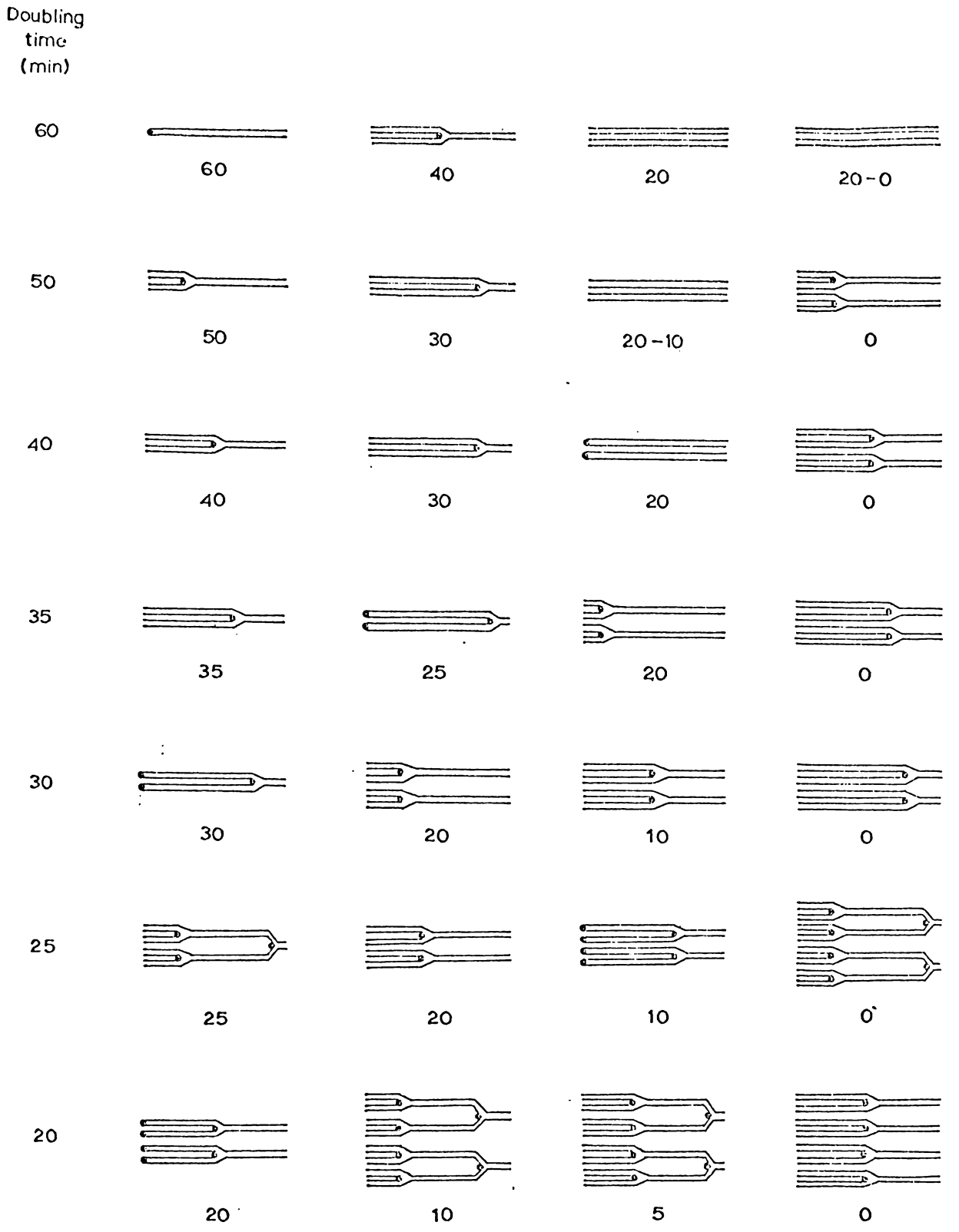

Fig. 2.4 Cooper-Helmstetter model for the pattern of chromosome replication in *E. coli* B/r. The construction is based on the approximate values C = 20, D = 40 minutes. The black dot indicates a replication point, and the numbers indicate the time in minutes prior to cell division at which the chromosome configuration is present in the cell. We have assumed, for simplicity, that chromosomes are linear and that replication is unidirectional. From Cooper & Helmstetter (1968).

for *E. coli* B/r. It is noteworthy that the associated distribution of generation times exhibited very little skewness.

It is evident that correlations between the generation times of parent and progeny cells could be accounted for through the fact that events which govern a particular division may extend over more than one generation.

More recent work (Pierucci, 1972) has established that C is approximately twice D for *E. coli* B/r at various temperatures of growth and in various growth media. It appears that a common mechanism controls chromosome replication and the progression of the cell towards division after completion of a round of replication. In slow growing cultures (generation times greater than 60 minutes at 37°C), the results indicate that chromosome replication extends throughout the first two-thirds of the division cycle (*i.e.*, $C + D = \tau$). These results, however, are not consistent with the proposal of Kubitschek & Freedman (1971), who used a different experimental approach, that $C + D$ is constant and equal to 72 minutes in exponentially growing cultures with generation times as long as 120 minutes.

As we have said the nature of the initiation mechanism is critical in this model and a satisfactory proposal is not available. This mechanism for initiation of DNA replication is at present almost completely unknown. We mention several proposals which have been put forward to explain the timing of initiation. Helmstetter *et al.* (1968)

suggested that initiation is governed by a positive mechanism whereby initiation occurs when an initiator protein accumulates to a certain level. Donachie (1968) has shown that initiation always occurs at a certain volume per chromosome origin. This is compatible with an accumulation of initiator. A negative control mechanism was postulated by Pritchard, Barth & Collins (1969) in which initiation occurs when the concentration of a periodically produced inhibitor of initiation has dropped below a certain level. It is likely that cell size could indirectly govern the initiation of new rounds of replication. In the model of Pritchard *et al.*, given a periodic synthesis of an inhibitor, it is conceivable that the increase of cell volume would lead (by dilution below some threshold) to periodic DNA synthesis and division.

CHAPTER 3

TECHNIQUES OF SYNCHRONIZATION

3.1 INTRODUCTION

The importance of phased growth techniques in the study of the time course of biochemical and structural events in living organisms is undisputed. In microbiological systems, analytical procedures are not generally sensitive enough to apply to the contents of a single cell and as such there is a clear advantage in having large numbers of cells at an identical stage of their life cycle. This is the object of synchronization. Investigation of the control processes in single cells through magnification by the number of cells in the synchronous culture, a factor of, say, 10^8, is then much more feasible.

If it can be shown that all cells in such a population are biochemically identical our objective is achieved. This identity, however, is difficult to prove and we must resort to some easily discernible event to support the claim that the individual cells are in the same metabolic state of their life cycle. Such an event is cell division, the only phase in the life cycle of the individual cell which can readily be observed. The periodic divisions in a synchronously dividing culture provide a natural time coordinate to which other changes, for example, the cycles of DNA replication, can be related.

The development of methods for synchronizing divisions

in cell cultures seemed to open the way for elucidation of the problem of the control of cell division by carrying out a few well-planned experiments. This phased growth approach, however, is beset with at least one major difficulty, and that is, that the operation which is effective in synchronizing cell divisions may itself be the source of non-steady-state conditions. It is well-known that environmental factors can influence the composition of microbial cells as well as the regulation of macromolecular synthesis. By observing cultures in which the cells are undergoing their 'normal' cycles of growth and division we can thereby minimise the effect of external factors on the underlying regulatory mechanisms. A culture of such normal cells can be considered to be a culture undergoing 'balanced' growth. This state was originally defined by Campbell (1957) as that in which every extensive property of the system increases by the same factor in the same time interval. This definition however designates all synchronous cultures as unbalanced because of the periodic variations in their properties, although individual cells of the culture may be normal at all times. This point is discussed by Anderson, Peterson & Tobey (1967) and a better criterion for the normality of cultures is their proposal that balanced states be defined in terms of the properties of individual cells. For a single cell, an unbalanced state is one in which it has a composition not shown by any cell in the course of the normal life cycle. The balance of a synchronous culture will depend on whether or not some cells are in states not attained by any cell in the original random culture. It is important, then, that the synchronizing agents do not

introduce transient distortions in the biochemical processes of the life cycle. This is not to say that methods which clearly upset the normal pattern of events in the cell should be abandoned. Such experiments may be of value but an awareness and appreciation of the perturbations to the cell cycle is necessary for a valid interpretation. In the studies in this thesis on the statistics of the process of cell division we wish to focus our attention on cultures which, as closely as possible, mimic the patterns of growth in normal cultures.

Numerous methods are available for synchronizing cell cultures with respect to the cellular division cycle. An exhaustive review of these is not attempted here and the reader is referred to the review articles appearing in the literature, for example, Helmstetter (1969). All the standard techniques were considered, however, in the course of determining a suitable procedure which satisfied the requirements we have discussed in the previous paragraph regarding balanced growth, and which produced results that we could analyse with confidence. The salient features of these techniques and our reasons for rejecting or adopting them are discussed in the subsequent paragraphs.

Methods for obtaining synchrony are commonly classified under two broad categories: induction methods and selection methods. In the former, cultures are treated with physical or chemical agents so that all of the individuals of a population are forced to enter the same stage with respect to the cell division cycle. This 'bunching' phenomenon, to use the terminology of Burns (1962), is achieved by treatments

which probably disrupt the metabolic pattern of events in all cells. Nutritional changes, temperature shifts, and light changes have commonly been used to induce such synchronization. For example, a method involving repeated changes of temperature at intervals of a generation time has been successful in synchronizing bacterial growth. Lark & Maaløe (1954) synchronized a culture of the bacterium *Salmonella typhimurium* by alternating 28-minute periods at 25°C with 8-minute periods at 37°C. In these experiments all of the cells divided during the brief period at 37°C. The selection methods, on the other hand, involve isolation of cells in the same stage of their life cycle from an exponentially growing culture. The terminology suggested by Abbo & Pardee (1960) points up the essential difference between the two categories. These workers used the term synchronized to refer to cultures produced by induction procedures where the treatment is fitted or entrained to the generation time of the cells. Cultures provided by the selection techniques were described as synchronous. These are, ideally, free of the metabolic distortions introduced by induction-type methods. A primary objective in the work presented here was to devise or adapt a technique which involves a minimum of disturbance to the cells. As such the induction methods have not been considered further. Although they have advantages in specialised applications we wish to avoid as far as possible any disruption of the normal pattern of events in the cell cycle.

We confine our attention, then, to the selection techniques which involve the isolation of cells of a

particular age or size class. The three selection techniques which we discuss in some detail have been extensively employed by various workers in microbiology. The first is a procedure for isolating newborn cells and the other two are able to fractionate cells according to their sizes.

Our initial attempts at synchronizing a bacterial culture were carried out, using the filtration method of synchronization, with cultures of the light-emitting bacterium *Photobacterium fischeri** . This organism was studied at the outset because it presented the attractive possibility of obtaining information on cellular control processes related to the light emission without the necessity for directly affecting the environment of the cells or the cells themselves. In other words, light emission from synchronous cultures could be monitored while the cells progress through their division cycles without interruption. The assumption is made, of course, that the correlation of the light emission with the normal cell cycle has been uninfluenced by the synchronizing technique. A number of workers have investigated the relationships between light production and growth in asynchronous cultures of this organism, for example, Kempner & Hanson (1968). Nealson, Platt & Hastings (1970) studied the luminescent system at the cellular control level using standard biochemical blocking procedures. The utility of synchronous cultures for yielding information at this level is clear, provided disturbance to the cells is kept to a minimum. Unfortunately these early attempts using the filtration method of size

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* *American Type Culture Collection, ATCC 7744.*

selection failed to yield observable synchrony in cultures of *P. fischeri*. Probable reasons for this failure are discussed in section 3.4. As we shall see, the methods of selection synchrony generally require considerable trial and error before satisfactory results are forthcoming. *Escherichia coli*, an organism which has been subjected to most synchronization procedures, was then adopted for this work. Details of our attempts at synchronizing cell division in *E. coli* cultures are presented in section 3.4, on filtration synchrony, and the subsequent section dealing with size selection by gradient-centrifugation.

The following section (3.2) deals with the usual methods of detecting synchrony. It has been pointed out earlier that the electronic particle counter has a number of attributes which we require in this work, but it is of interest to compare this method of enumeration with other procedures.

3.2 METHODS OF DETECTION

Reliable methods of cell counting are clearly necessary to confirm the presence of some degree of synchrony of cell division in cell populations. The sharpness of the first step in the growth curve for a synchronous culture is, of course, dependent on the extent of variability in generation times of the cells. It is quite conceivable that, for a species possessing a generation time distribution which is fairly broad, this step will be well-rounded. Imprecise counting methods will destroy any chance of confidently discerning such a step from the normal pattern of exponential

growth in an asynchronous culture. Fig 3.1 illustrates the transition in step shapes as the underlying generation time distribution broadens. A perfect step results when no dispersion is present in this distribution so that all cells divide at precisely the same instant of time. The straight line on the semi-logarithmic graph indicates the exponential growth observed in an asynchronously dividing population of cells. The difference between this line and the curve for a synchronous culture having a generation time distribution

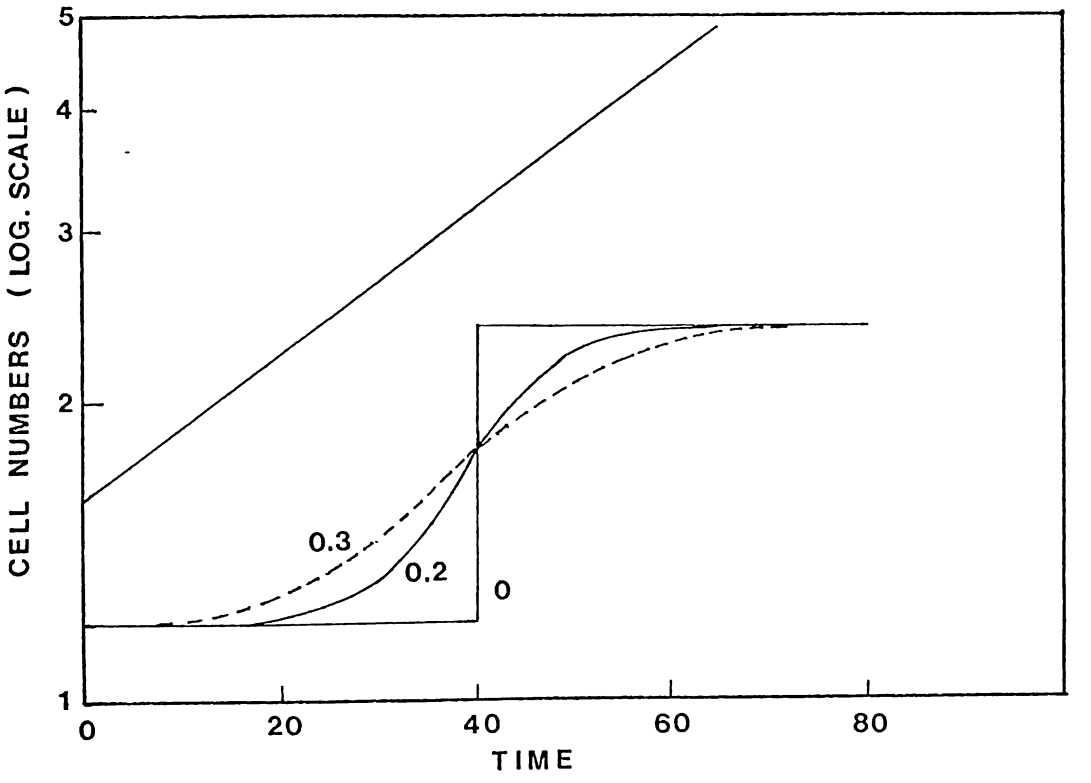

Fig. 3.1 Illustration of the effect of dispersion in generation time distributions on synchronous growth. The figures near the curves denote the coefficient of variation of the underlying generation time distribution. The straight line represents the growth of an asynchronous culture with a doubling time of 40 units. Scales are in arbitrary units.

with a coefficient of variation of 30% is not very marked. The generation time distributions were assumed in the diagram to be normal in shape but this is not important to the argument. Clearly then, while absolute accuracy is not an important consideration in the detection of synchrony, relative statistical precision is.

The usual methods of enumeration are:

(a) microscope counting chamber
(b) viable cell count
(c) nephelometry
(d) electronic particle counter.

A discussion of each of these methods follows. It is worth mention here that the standard microbiological method of measurement of growth in asynchronous cultures cannot be used to detect synchrony. This is the optical technique of determining the turbidity or optical density of a culture. This property is more closely related to cell mass than cell numbers. In fact, the per cent transmitted light through a cell suspension, which is a measure of the turbidity, is linearly related, within certain limits, to the cell mass density.

(a) Microscope Counting Chamber

Microscopic observation of a cell suspension provides a direct method of determining the number of cells in a population. With the aid of special counting chambers, such as the Petroff-Hauser or Helber chambers, the individual cells in an accurately determined, very small volume can be

counted. This procedure is tedious and high accuracy is hard to achieve. Its principal limitation in bacterial enumeration is that relatively high concentrations of cells, of the order of 10^7 cells/ml, must be present in the suspension. Otherwise statistically significant counts cannot be achieved. High magnification is required for seeing bacteria and even then some of the smallest cells may be missed. Especially in the case of bacteria, dead cells cannot usually be distinguished from living cells under the microscope so that this method provides a total cell count.

(b) Viable Cell Count

If we are interested in counting only live cells the viable count method must be used, by which we determine the number of cells in the population capable of dividing and forming colonies. By preparing appropriate dilutions of a bacterial population and then plating these out on suitable media, the viable count can be determined by counting the number of colonies that develop after incubation. Although this method is very sensitive, since, in principle, even one viable cell will be detected, there are a number of disadvantages. The most important of these, for our purposes, are:

(1) two or more adjacent cells may give rise to a single colony, so that we cannot be absolutely sure that a single colony arose only from a single cell;

(2) a practical upper limit to the number of colonies per plate is about 300 so that to reduce the sampling error replicate plates must be prepared; and

(3) high dilutions, of the order of 10^5-fold, may be necessary to achieve the appropriate colony number.

Dilution errors then become important.

While the method is important in certain microbiological applications, such as in the food industry, it is very time-consuming and usually subject to much error.

(c) Nephelometry

This is a light scattering technique whereby estimates of cell numbers can be obtained by measuring the percentage of light transmitted at a certain angle by the cell suspension. This method, described by Stárka & Koza (1959) is very quick but has not yet received much attention. The results of Abbo & Pardee (1960) indicate more scatter in the nephelometric determinations than in the microscopic estimations of cell numbers.

(d) Electronic Particle Counter

Bacteria and other cells may be counted very rapidly using electronic counters based on a principle developed by Coulter (1956). For readers unacquainted with this instrument a brief excursus on the Coulter principle and methodology will be useful.

The particle detector consists of a small aperture with two electrodes positioned on either side of it all immersed in an electrolyte (Fig. 3.2). The aperture diameter is usually 30 μm for counting bacteria. An electric current is passed between the electrodes and a suspension of particles in the electrolyte is forced to flow through the aperture. Each non-conducting particle produces a change in the electrical conductivity of the electrolyte within the

aperture, by displacing its own volume of electrolyte. This produces a voltage pulse of short duration which is amplified and displayed on an oscilloscope screen. The amplitude of the pulse is proportional to the volume of the particle detected, over a wide range*.

The appearance of the pulse display on the screen permits, for a homogeneous suspension, an immediate impression of average particle size and size distribution. Aperture blockages and other forms of interference can be monitored

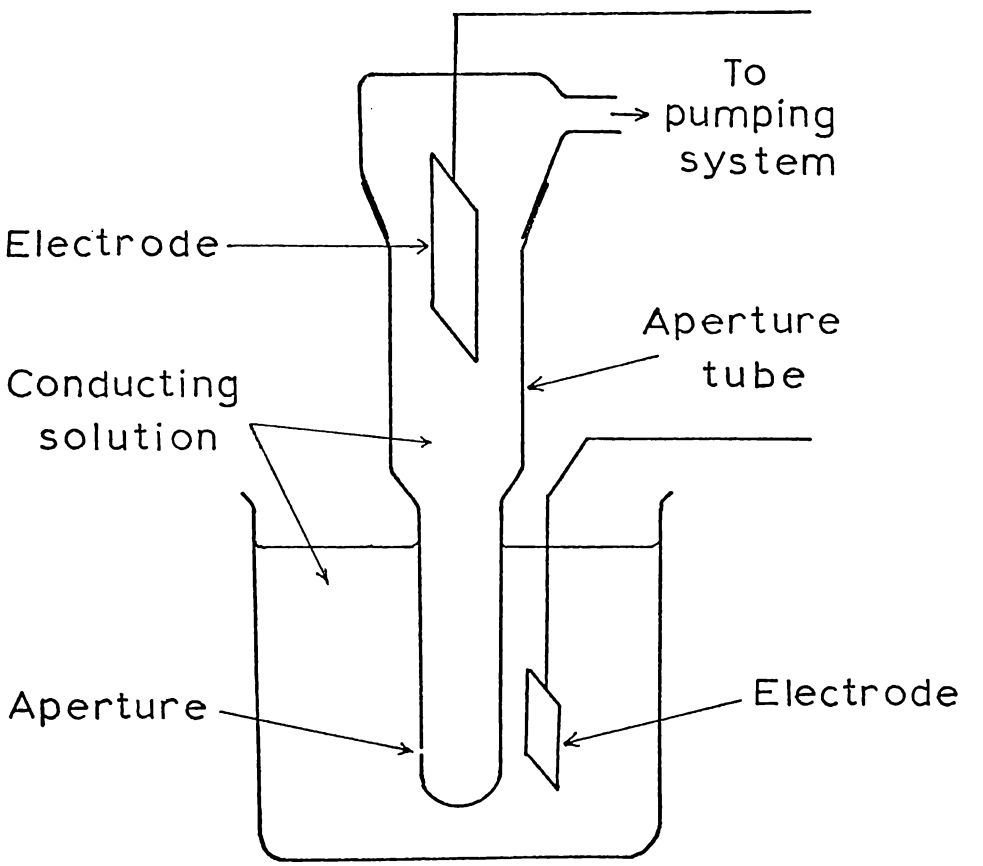

Fig. 3.2 Geometry of the aperture-electrode system of a Coulter electronic particle counter.

The limits of this proportionality are discussed in Chapter 4.

since these phenomena will generally produce irregularities in the pattern on the screen.

The aperture current control alters the current passing between the electrodes and through the aperture. Use of this control in conjunction with the attenuation control allows optimisation of the signal-to-noise ratio. The ability to detect smaller particles is enhanced by increasing aperture current until local heating within the aperture causes an increase in noise.

A threshold control allows the instrument to count only those particles having amplitudes exceeding the chosen threshold level. This enables the operator to obtain total cell counts by setting the threshold level so that the smallest particles are counted but the electronic noise is not. Some instruments have, in addition, an upper threshold control permitting particles within defined size ranges to be counted.

Counting is started and halted automatically as a defined volume of suspension passes through the aperture. For a volume of 0.1 ml and a 30 μm aperture a typical count cycle takes about 30 seconds. At high count rates one or more cells may pass through the aperture at the same time, resulting in only one pulse. We discuss in Chapter 4 the corrections that can be made for these coincident effects.

This method of enumeration cannot distinguish two cells stuck together from one large cell, nor can it distinguish between cells and other inanimate particles, such as dust. The suspending electrolyte should clearly be very clean to

reduce this background particle level to manageable proportions and to reduce the incidence of aperture blockages.

The main advantages of this method over others lies in its speed and accuracy. Higher statistical precision is possible since total counts of 10^4 or higher can be maintained. When adequate determinations of coincidence and background levels have been made accurate counts are possible, albeit of living as well as dead cells. If necessary, agitation of the sample suspension may be employed to break up cell clumps.

3.3 AGE SELECTION

A technique devised by Helmstetter & Cummings (1963, 1964) permitted the selection of newborn cells from a growing population. This membrane selection technique, as it has come to be called, makes use of the fact that a bacterium will bind irreversibly to a membrane filter and divide while so attached. In the experiments reported by these workers, cells of *E. coli* B/r were bound to a membrane filter, of pore size smaller than the cells, by passing an exponentially growing culture through the filter for a short period. A filter of 0.22 μm pore size was used and this was then inverted prior to elution with growth medium. The cells in a sample of the eluent collected during an interval of time which was short compared with the generation time of the cells were observed to grow synchronously. Presumably, the cells eluted are those that, on fission of the parent cell, become free of the attachment to the membrane. Fig. 3.3 will make this idea clear. If, as may happen occasionally, both daughters remain fixed to the membrane this would only affect

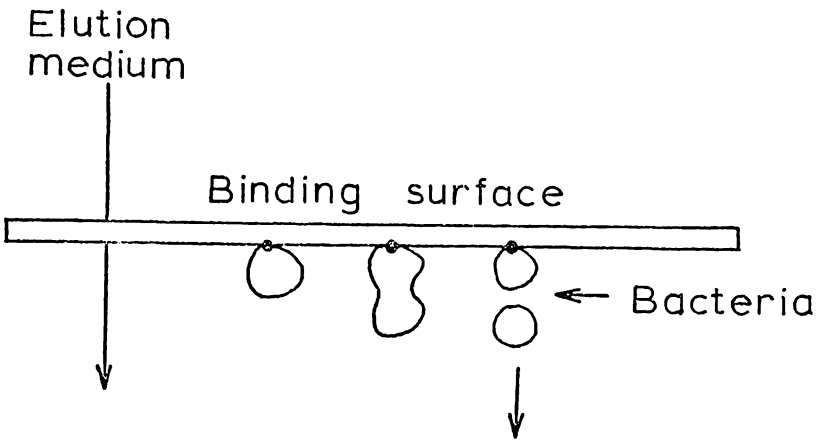

Fig. 3.3 Illustration of the principle of the membrane selection technique. Three successive stages in the division cycle of a bacterium bound to a membrane filter are indicated. If the attachment remains fixed, then only the daughter cell indicated by the arrow would be eluted from the surface as growth medium is passed through.

the concentration of newborn cells in the eluent.

Although considerable success has been achieved with *E. coli* B/r and, more recently, with *E. coli* K12 strains (Cummings, 1970; Shehata & Marr, 1970), the method does not appear to work at all well with most other bacterial strains (Rowbury, 1972). In addition, evidence exists that the technique perturbs the growth of the cell population (Helmstetter, 1967). Kubitschek (1970) has mentioned that pore size appears to be critical with this method and that if cells are attached to the membrane by one end then this area will be subjected to an altered environment. These attached ends are probably recessed within pores while the

daughter cell which is eluted suffers no such attachment and consequently would be 'shifted-up' to more favourable growth conditions. Kubitschek (1968a) has observed that growth and division were depressed temporarily in synchronous cultures prepared using this technique of membrane attachment. Further evidence for an environmental effect on the pattern of events in cells which have been adsorbed on membranes was reported by Kubitschek & Freedman (1971). They examined DNA synthesis in non-synchronized slowly growing cultures of *E. coli* B/r by chemical means and obtained results incompatible with those of Helmstetter & co-workers who employed the membrane filter procedure.

In the light of these criticisms it was felt that an alternative method for obtaining cell division synchrony was desirable, and consequently the membrane selection technique was not further pursued in this thesis.

3.4 SIZE SELECTION BY FILTRATION

Maruyama & Yanagita (1956) were able to produce a synchronous culture by isolating cells of similar size from an exponentially growing culture. They passed a concentrated suspension of *E. coli* through a stack of filter papers and eluted the filter pile under suction with fresh medium. The elution medium lacked a carbon source so as to prevent growth during the period of filtration. The smallest cells apparently travelled through the pile more easily than large cells and these cells subsequently grew synchronously.

Various modifications to the original procedure have

been reported. We mention two of these because of their application to *E. coli*.

To minimise possible disturbance to the cells during the procedure, Abbo & Pardee (1960) filtered an exponentially growing culture of *E. coli* B without concentrating the cells in advance. The bacteria were eluted with culture medium and particular attention was paid to minimising temperature variations during the procedure. These workers used a paper pile consisting of several grades of Whatman filter papers and filtration was stopped when the filtrate became very slightly turbid. Abbo & Pardee obtained a relatively high degree of synchrony through four or five generations.

Filtration through a single membrane filter was used by Anderson & Pettijohn (1960) to isolate the smallest cells of an exponentially growing cell population. Synchronous cultures of *E. coli* strains K12 (λ) and B were obtained after a rapid filtration through a single sheet of grade RA Millipore paper, pore size 1.2 μm. The whole operation was performed at constant temperature. The filtrate contained 1 to 2 per cent of the total cell population. Both these results and those of Maruyama & Yanagita indicated rapid deterioration of synchrony in the second generation. Abbo & Pardee suggested that close attention to avoidance of undue disturbance to the cells is necessary for good synchrony.

Different investigators using the filtration technique have obtained conflicting results regarding the sequence of biochemical events during the division cycle. For instance,

Abbo & Pardee (1960) found a continuous increase in DNA content during the division cycle of *E. coli*, whereas Maruyama (1956) found a step-wise increase. It would appear that this seemingly gentle procedure can significantly perturb the metabolic pattern of events during the cell cycle.

Although reproducibility seems to be a problem with the filtration method, its simplicity and speed are attractive. Attempts were therefore made to synchronize cultures of *Photobacterium fischeri* by the method of Abbo & Pardee. As used by these workers, the paper pile consisted of the following Whatman filter papers, from bottom to top: one No.42, twenty No.1, one No.42, and two No.1. The *P. fischeri* culture growing exponentially in complex medium* at 22°C (doubling time of 90 minutes) was poured directly onto the paper pile and filtered under suction. Additional fresh medium was used to elute the bacteria and a turbid filtrate appeared after about 20-25 minutes. The bacteria in the filtrate were recovered by centrifugation and resuspended in fresh medium. These operations appeared to arrest the growth of this organism and further work was undertaken with *E. coli*.

The membrane filter method of Anderson & Pettijohn (1960) was tried with a B strain of *E. coli*.** Cultures of this organism were grown in a modified M9-salts glucose medium, the details of which are provided in Chapter 4 of

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We used the minimal medium of Eberhard (1972) supplemented with 8 g/l nutrient broth.

***Kindly supplied by P.L. Bergquist, Department of Cell Biology, University of Auckland, N.Z.*

this thesis. Filtration through Millipore* membrane filters of pore size 0.8 μm yielded very few cells in the filtrate and efforts were directed at obtaining results using Nuclepore** membrane filters having 1.0 μm pore size. Besides being much thinner than the cellulosic membranes of the Millipore type, Nuclepore membranes have very uniform, cylindrical pores, compared to the highly irregular pore structure of the former variety. Filtration was performed at 37°C, the culture having grown to a density of about 1.1×10^8 cells/ml at this temperature. Approximately 80 ml of this culture was passed through the filter in two minutes and the filtrate was immediately transferred to an orbital incubator set at 37°C. Samples taken at five minute intervals were diluted 100-fold with 0.1 M HCl and counted with a Coulter counter. The raw counts, not corrected for background level or coincidence, are presented in Fig. 3.4. A very faint periodicity about a straight line of best fit to the points is discernible. The slope of this line indicates a doubling time for the culture of about 40 minutes corresponding to the doubling time observed in the parent culture. Although the uncertainty associated with each point is small and is expected to be less than 2%***, it is clear that no significant phasing of division has occurred in the filtrate. This is perhaps explained by the fact that some 2% of the population were able to pass through the filter.

Millipore Filter Corp., Bedford, Massachusetts.

**Nuclepore & Filtration Products, Pleasanton, California.*

***Full details of the error estimation and sampling methods are presented in the following chapter.*

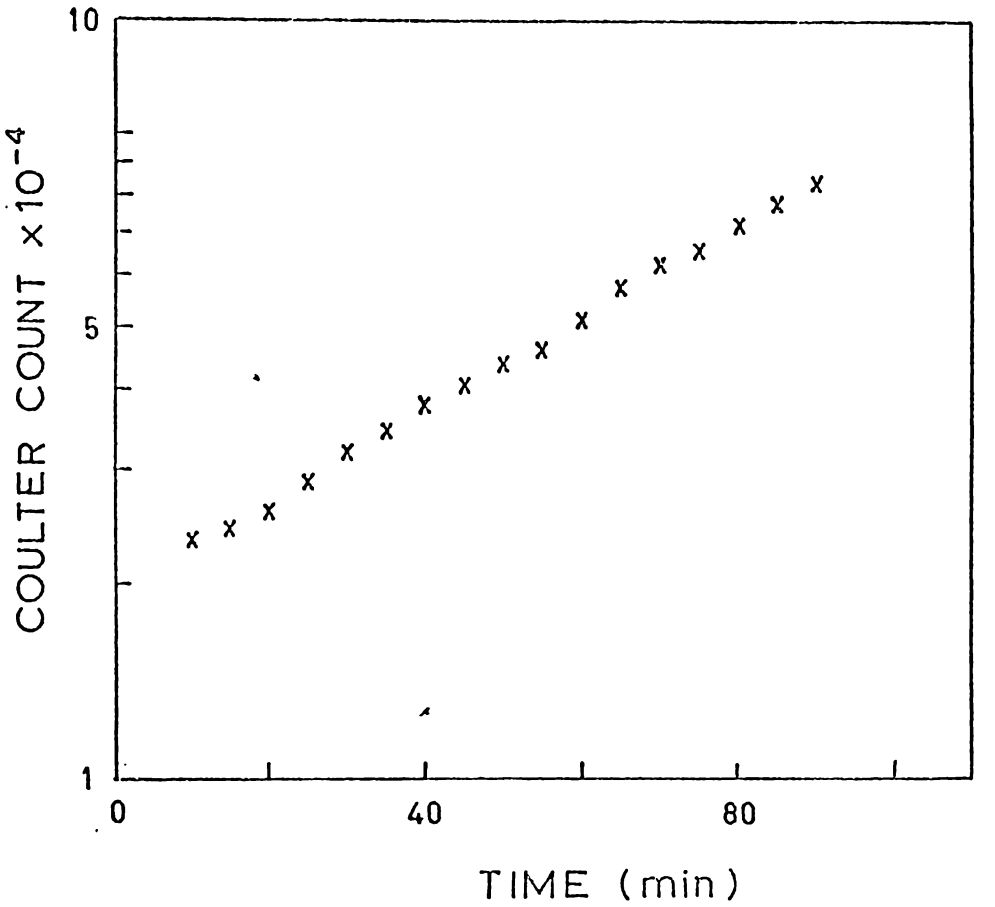

Fig. 3.4 Growth of a culture of *E. coli* B after filtration through a membrane filter (1.0 μm pore size).

Anderson & Pettijohn (1960), using 1.2 μm pore size membranes, were able to recover less than 2% of the total, even though their cells would be expected to be of similar size to those used here. These authors also used a glucose mineral salts medium and a B strain of *E. coli* growing at 37°C. Helmstetter (1969) has pointed out that filtration procedures require much trial and error before useful results can be obtained and, in fact, results have been difficult to reproduce in different laboratories. It is conceivable, in the cases of *E. coli* and *P. fischeri*, that a significant percentage of organisms of all ages could pass through the filter since these cells are rod-shaped and generally grow only by length extension.

Although only limited trials were carried out using this technique the difficulties experienced by other workers and the doubts about the efficacy of the procedure for rod-shaped organisms justified a different approach.

3.5 SIZE SELECTION BY CENTRIFUGATION

A very successful method of separating cells by size, which has since been used with a variety of organisms, was developed by Mitchison & Vincent (1965). These workers used their technique to produce synchronous cultures of *E. coli*, a fission yeast and a budding yeast. Their method essentially involves layering a concentrated suspension from an exponentially growing culture on top of a linear gradient of sucrose and centrifuging until a band of cells forms one-half to two-thirds of the distance down the tube. A synchronous

culture is obtained by picking off a sample of the small cells in the upper region of this band and inoculating these into growth medium. These cells, being, presumably, the youngest, should exhibit periodic increases in cell numbers at intervals of a generation time. The gradient serves to stabilize the liquid column against convection and facilitates layering of the suspension on top of the liquid.

Other materials besides sucrose have been commonly used to form gradients. Cells which are osmotically sensitive to sucrose may often be effectively separated in gradients of Ficoll or Dextran (Burdett & Murray, 1974). Solutions of Ficoll, a synthetic polymer of sucrose, are notable for their very low osmolarity. To avoid introducing complications by a change of carbon source, Bostock *et al.* (1966) used glucose gradients instead of sucrose with cultures of the fission yeast *Schizosaccharomyces pombe*.

Only a small fraction, of the order of 1-5% of the cells layered, is removed from the gradient and the remaining cells can be mixed and used to start an asynchronous control culture. Indications of any perturbations to the normal growth pattern of the cells, introduced by the experimental operations, may be revealed in this control culture. Normal exponential growth should be exhibited by the control over the duration of the experiment.

Because of its applicability to a wide variety of cells and because it appears to perturb growth less than any other technique, efforts were concentrated on developing this technique to the point where useful and reproducible results

could be obtained. The considerations used in developing a reliable procedure and some of the results of our early efforts are outlined in the following paragraphs. Complete details of the operations finally adopted are presented in Chapter 4, Section 2.

A number of theoretical considerations must be recognized to determine reasonable bounds on factors such as the steepness of the gradient and the concentration limits of the gradient material. The separation process featured here is of the 'rate' type and should be distinguished from the equilibrium type*. In this rate-zonal centrifugation process, separation depends primarily upon the differing sedimentation rates of the particles within the starting zone. The density range of the gradient medium should not encompass the density of any component within the zone (Sykes, 1971). Cells of the same type at different stages of their life cycle do not exhibit large differences in density and if the density of the gradient is close to that of the cells separation will be achieved almost entirely on the basis of cell size with maximal resolution. Unfortunately, materials such as sucrose, glycerol and Ficoll exhibit high viscosities at the densities usually required and this reduces the resolution attainable.

The concentration of cells in the starting zone is perhaps the most important single parameter determining the success of the separation procedure. The phenomenon of

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*See, for example, Mel & Ross (1975).

streaming occurs if the cell concentration in the band loaded onto the gradients exceeds a certain level called the streaming limit. Above this limit, which tends to vary inversely as the average volume of the cells, large numbers of filaments form below the cell band. Streaming does not appear to be a cell-aggregation phenomenon (Miller, 1973) nor is it caused by convection (Miller & Phillips, 1969). To achieve useful cell separations the cell load must be kept below this limit so that, if optimum resolution is sought, the final yield of cells will essentially be determined by the physical size of the gradient.

To provide a suitably high yield of cells so that numerous microscope counts could be carried out, large 275 ml sucrose gradients were prepared for our early attempts to synchronize *E. coli* B. These were formed with a gradient mixing device of the type described in the following chapter (see Fig. 4.2). The sucrose concentration in the gradient, which was prepared in 290 ml polycarbonate centrifuge bottles*, ranged from 10% at the top to 40% at the bottom. In a typical experiment cultures of *E. coli* B, growing in glucose mineral salts medium at 37°C, were pelleted at a density of about 4×10^8 cells/ml. The pellet was resuspended in 8 ml of growth medium and layered carefully on top of the sucrose gradient. Approximately 5×10^{10} cells were loaded on the gradient which was then spun in a Sorvall RC2-B centrifuge* using a GSA angle rotor. This centrifugation operation was carried out at room temperature (21°C). Through a trial and error procedure, good results were obtained at a

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*Ivan Sorvall Inc., Norwalk, Connecticut.

centrifugal force of 1500 g applied for 10 minutes. A broad band of cells then appeared roughly two-thirds of the distance down the gradient. Using a syringe, 10 ml were removed from the upper region of this band and inoculated into 10 ml of the parent growth medium. The latter had been filtered through a Millipore HA membrane (pore size 0.45 μm) and then prewarmed to 37°C. Aeration of the final culture was maintained in an orbital incubator. Cell numbers were determined microscopically with a Helber counting chamber* under phase optics. Samples (1 ml) were removed at intervals and added to 0.2 ml of 20% formaldehyde, so killing the cells. At least 300 cells were counted in each sample and the results are plotted in Fig. 3.5a. A step-like curve can be fitted to the points reasonably well and exhibits the expected doublings in cell numbers. The results of a similar experiment are shown in Fig. 3.5b, in which the scatter in the data points is a little less and the periodic increases in cell numbers are clearer. An uncertainty of 10% was assigned to each point in these curves and this is indicated by the error bars. This error was estimated from the dispersion observed in a series of counts of replicate samples from an *E. coli* culture at a density of approximately 2×10^8 cells/ml. About 300 cells were counted for each sample.

These results are encouraging and indicate that refinements in various ways to the whole operation would prove worthwhile. The zero on the time scale in the experiments of Fig. 3.5 was taken as the time of inoculation

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*Hawksley, Sussex

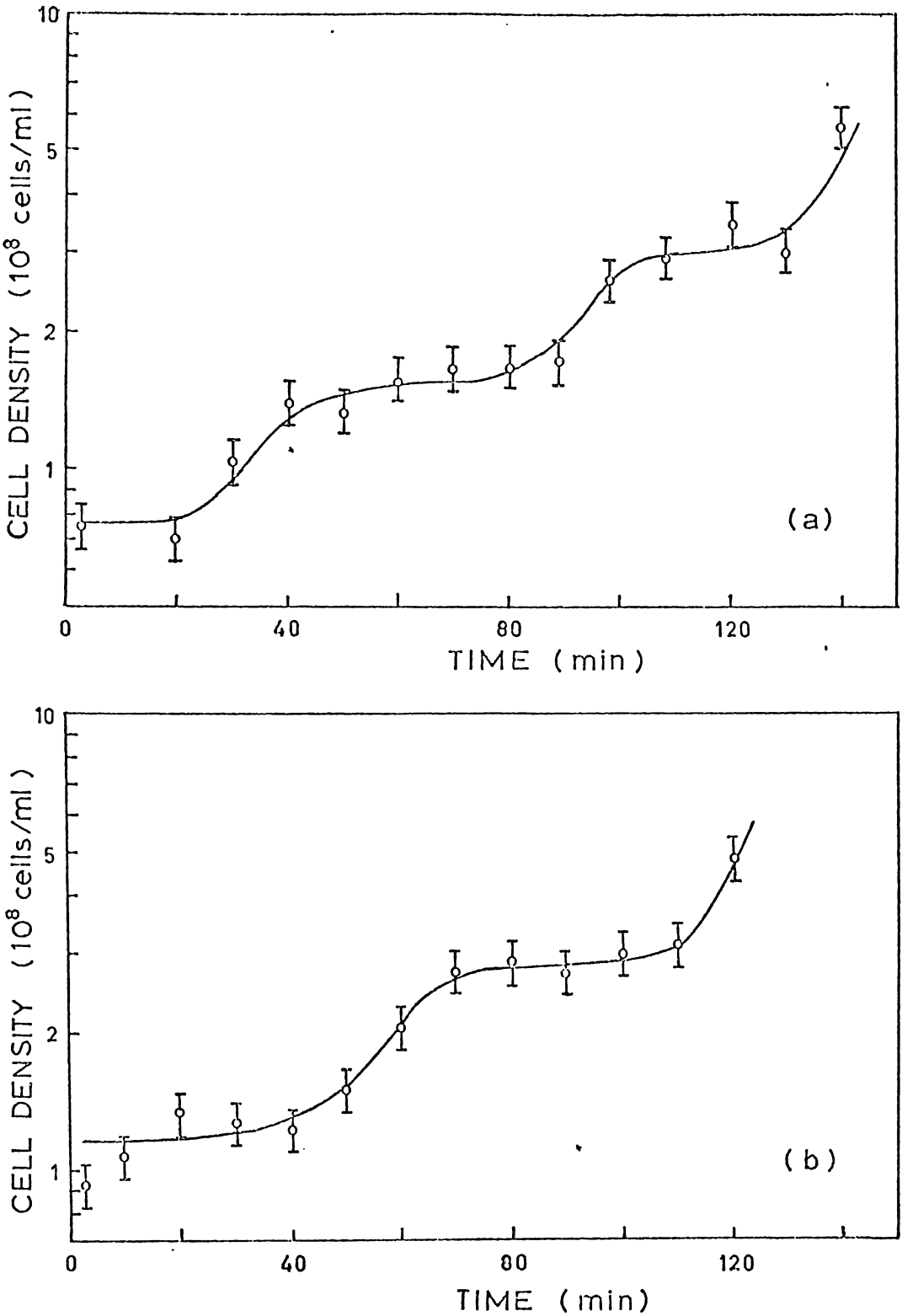

Fig. 3.5 Cell concentration curves from microscope counts of two synchronous cultures of *E.coli* B produced by the sucrose gradient centrifugation technique. Error bars (10%) represent standard deviations on the sample counts.

into growth medium of the cells taken from the gradient. The mean generation time of the cells indicated by the curves appears to be 50-60 minutes which is rather longer than the 40 minutes exhibited by exponentially growing cultures of this strain under the conditions mentioned. It is probable that growth has slowed down somewhat at the high densities (greater than 10^8 cells/ml) dealt with. With the use of a Coulter particle counter much lower densities can be accommodated and hence considerably smaller gradients may be employed. Swinging bucket rotors are preferable, also, to the angle type, since the effects of cells impinging on the walls in the latter may be detrimental to the quality of cell separation. After a few trials with gradients of 15 ml volume, somewhat larger gradients were deemed easier to prepare and handle. Glass centrifuge tubes of 45 ml capacity were tested with linear gradients of 5-20% sucrose and then 5-30%, the latter proving more satisfactory. The speed and duration of centrifuging depends on the gradient used, the temperature and the conditions under which the cells were grown. Slow growing cells of *E. coli*, being smaller than fast growing cells, will require a correspondingly longer duration of centrifugation for a constant centrifugal force. Cells will sediment faster as the temperature is raised, other parameters being held constant. The density range covered by a 5-30% gradient of sucrose (made up in water) is indicated in Table 3.1. The density of *E. coli* cells is close to the upper limit of this range so sedimentation should occur primarily on the basis of size.

TABLE 3.1 Density of Sucrose Solutions

Concentration*	Relative Density (20°C)
5%	1.0178
15%	1.0592
30%	1.1270

Koch & Blumberg (1976) have reported a value 1.13 for this density, although Kubitschek's (1974) estimate is slightly lower at 1.108 for *E. coli* B/r cells grown in glucose minimal medium. Other gradient materials have been used, and in all cases some trial and error was necessary to determine appropriate values for the duration and speed of centrifugation for the concentration ranges tested, bearing in mind the desirability of minimising the period which the cells must spend in the gradient. The values of these parameters are detailed in the following chapter together with the relevant experimental results.

All concentrations are weight %, i.e. g solute per 100 g solution.

CHAPTER 4

EXPERIMENTAL METHODOLOGY AND RESULTS

4.1 INTRODUCTION

This chapter falls into two main sections. The first deals with the details of the experimental procedure adopted in which the gradient-centrifugation technique is used to obtain synchronous cell cultures. Culture conditions and the cell counting and sizing techniques are discussed. One set of experiments was designed to provide data covering a range of growth rates at constant temperature. This aim was achieved by employing a variety of carbon sources for growth of the bacterial cells. Another set of data covered a similar range of growth rates obtained at various temperatures of growth using a common energy source.

In the second part we present the results obtained under these various conditions. Detailed analysis of these results is deferred until Chapter 5. Cell volume distributions recorded during the growth of one of the synchronous cultures are presented. These greatly enhance the credibility of the cell number data. The efficacy of the centrifugation technique in isolating the smallest cells of an exponentially growing culture is also illustrated by appropriate cell volume spectra.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture

The organism used throughout this work was a B strain of *Escherichia coli*[*]. Cultures were maintained on slopes of nutrient agar[**] at 4°C. These were subcultured for the purposes of all the experiments described here in a modified M9-salts solution (Gudas & Pardee, 1974) together with an energy source. The composition of the M9-salts solution is given in Table 4.1. After preparation in glass-distilled water the pH of the solution was checked and found to be close to the desired value 7.0. Sterilization was accomplished by autoclaving at a pressure of 15 lbs/in^2 for 15 minutes. $MgSO_4.7H_2O$ solution was autoclaved separately and added aseptically later. Solutions of the various carbon sources

TABLE 4.1 Composition of M9-salts Solution

	grams/litre
Na_2HPO_4	7.0
KH_2PO_4	3.0
NaCl	0.5
NH_4Cl	1.0
$MgSO_4.7H_2O$	0.2

were similarly sterilized separately. Glucose solutions were routinely sterilized at 10 lbs/in^2 pressure for 20 minutes. The mineral salts solution was supplemented, for a

[*] *See footnote, page 82.*

[**] *Difco Laboratories, Detroit, Michigan.*

particular experiment, with one of the carbon sources at the following concentrations: glucose 0.4%, sucrose 0.5%, glycerol 0.5%, or sodium succinate 0.4%. To obtain faster growing cells the amino acids methionine and histidine, at concentrations of 50 µg/ml, were added to the glucose-salts medium. Solutions of these compounds were sterilized by filtration through 0.22 µm pore size Millipore* membrane filters. All solutions sterilized by autoclaving were prefiltered through 0.22 µm pore size membranes.

Cultures (50 ml) were usually incubated overnight at the appropriate temperature with shaking. Slowly growing cultures with doubling times greater than 60 minutes generally took somewhat longer (2-3 days) to adapt to the new medium. For each synchronous culture experiment, parental cultures (100 ml) were grown in an orbital incubator** (130 revolutions/minute), usually for about ten generations, to final concentrations of between 5×10^7 and 2×10^8 cells/ml. Culture temperature could be maintained within $\pm 0.2^{\circ}C$ during growth in this incubator. Erlenmeyer flasks with a volume at least five times that of the culture were used during all experiments.

4.2.2 Synchrony

The gradient-centrifugation technique outlined in Section 3.5 was used to produce synchronously dividing cultures.

Millipore Filter Corp., Bedford, Massachusetts.

**A. Gallenkamp & Co., London.*

The same basic procedure was followed in all experiments. The first step involved pelleting the parent culture in a Sorvall RC2-B centrifuge*. Five minutes at 7000 g was usually satisfactory. This pellet was resuspended in 0.5-1.5 ml of the supernatant which we call the conditioned growth medium. The suspension was then layered carefully by means of a syringe upon a linear density gradient and centrifuged in a swinging bucket rotor**. In general, the band of cells moved about one-half of the distance down the gradient and, usually, a small fraction of the cells pelleted at the bottom of the tube. Plate I illustrates the formation of a band of cells in a sucrose gradient after centrifugation. A sample of the smallest cells was withdrawn, using a syringe, from the upper region of the turbid band of cells and was inoculated into the conditioned medium (usually 15-20 ml). This medium was previously filtered through a grade HA, 0.45 μm pore size, Millipore membrane filter. To retrieve the smallest cells, a syringe fitted with a bent hypodermic needle was used, since in a density gradient the liquid that is drawn into the syringe comes from above the point of the needle (see Fig. 4.1). In all experiments, the conditioned medium was used for the subsequent growth of the synchronous culture. This reduces the possibility of disturbance to the cells.

Linear density gradients were established in 40 ml volumes in 110 x 23 mm glass centrifuge tubes, by the method

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*Ivan Sorvall Inc., Norwalk, Connecticut.

**Super Minor Centrifuge, Measuring & Scientific Equipment Ltd., Crawley, Sussex.

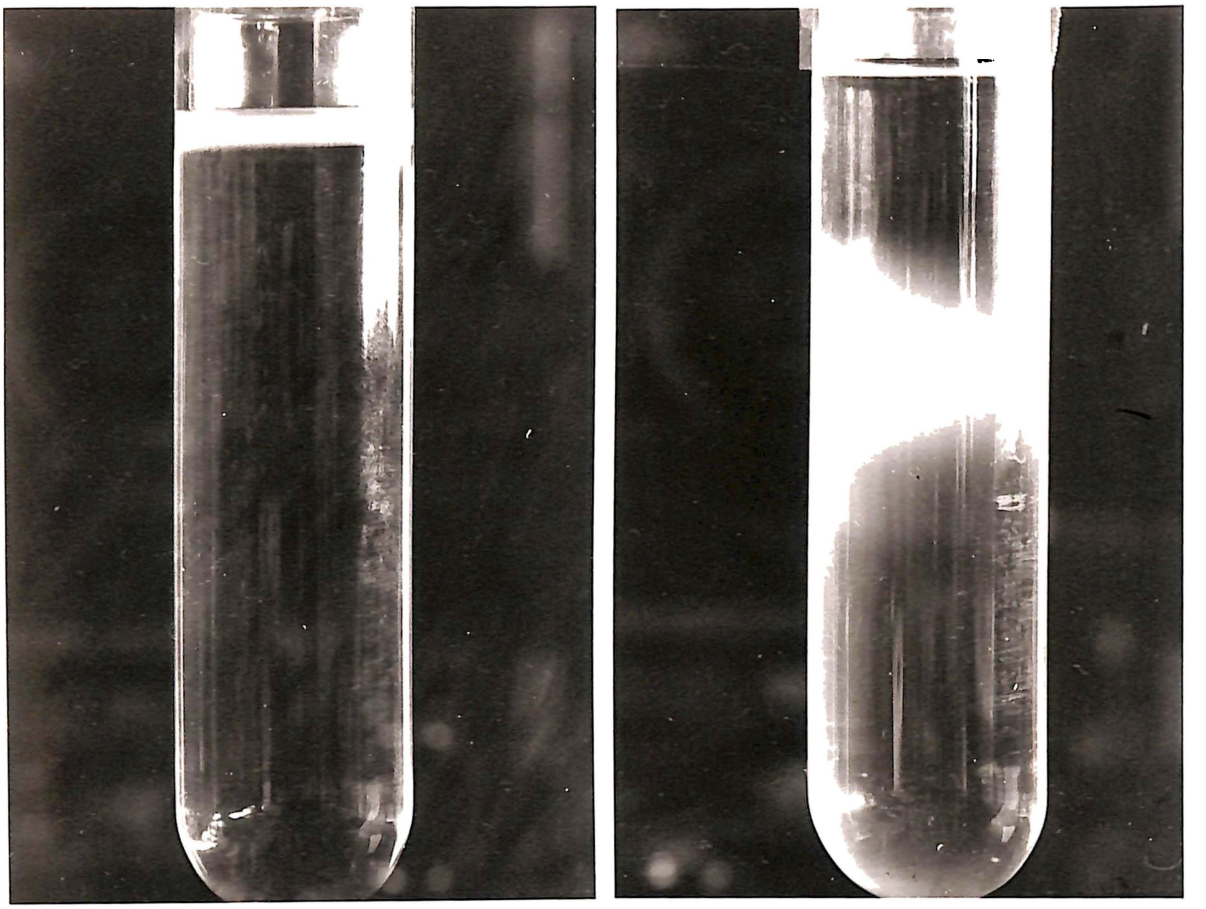

PLATE I

Formation of a band of cells in a 5 – 30 % sucrose gradient after layering a cell suspension on the gradient (tube on left) and centrifuging.

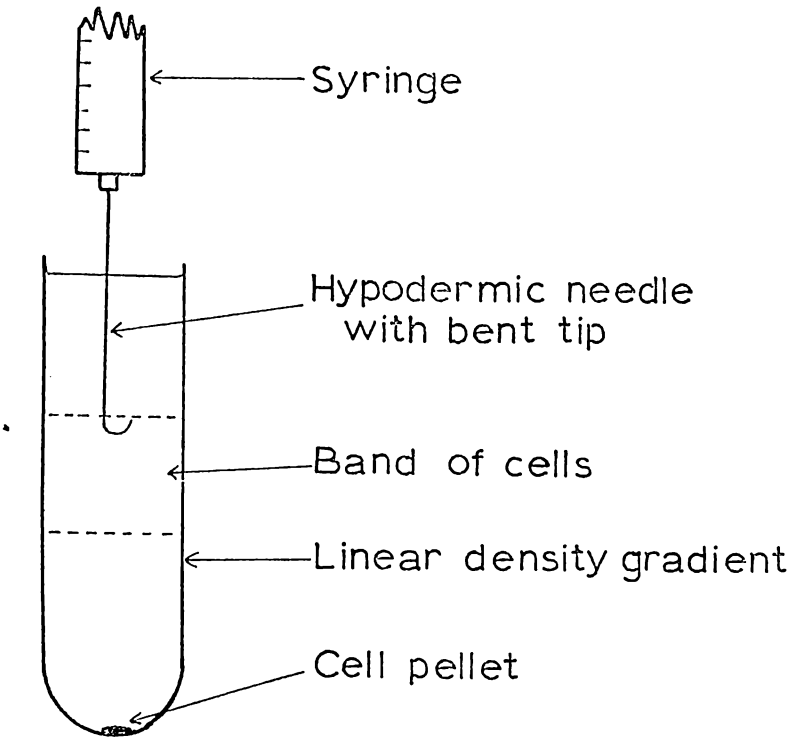

Fig. 4.1 Method of recovery of the smallest cells from a band in a density gradient using a syringe with a bent needle.

of Britten & Roberts (1960). The design of a simple gradient mixer is illustrated in Fig. 4.2. With both taps fully closed, chamber 2 is loaded with a suitable volume (that is, approximately 50% of the total gradient volume) of the highest density solution and chamber 1 is loaded with a slight excess of the lowest density solution. The weight of liquid in each chamber should be the same. The stirrer is started and the taps opened to give a flow rate of about 1-2 ml/min or less. Initially, the highest density solution emerges and is followed by the successively lighter mixture which floats on the underlying liquid in the tube with little mixing.

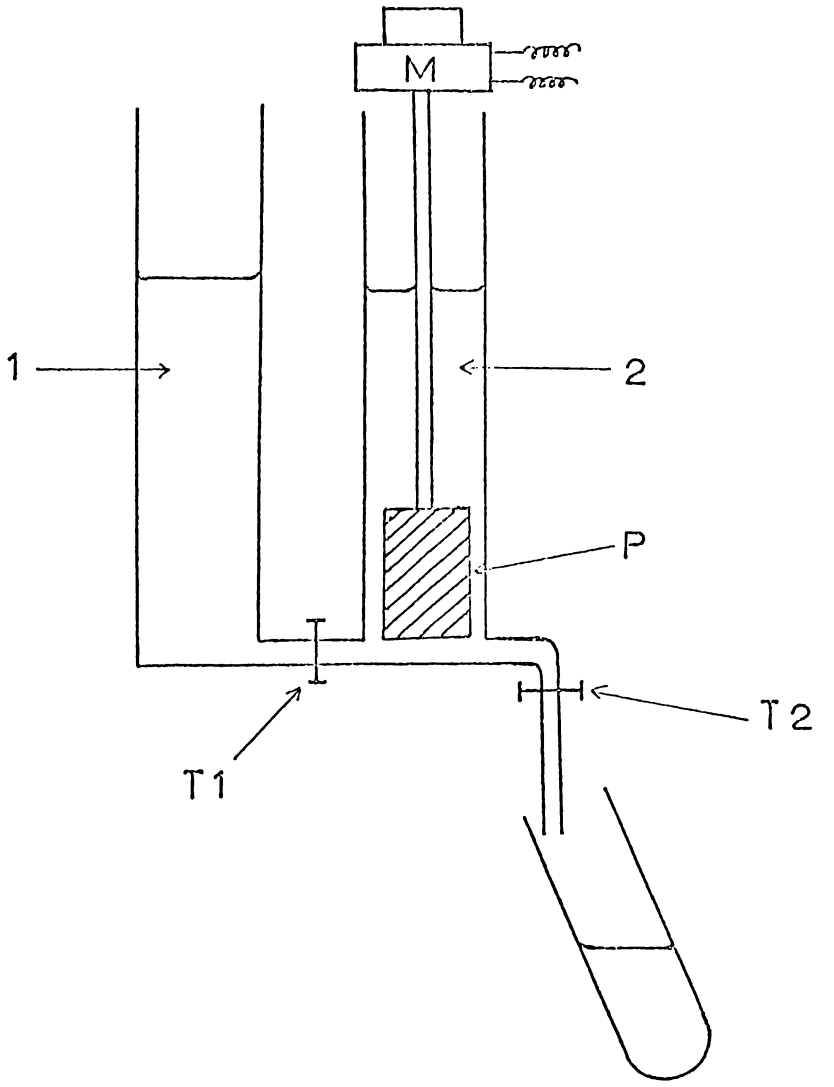

Fig. 4.2 A density gradient mixer designed to produce linear gradients. The two chambers (1 and 2) are of equal cross-sectional area. The motor (M) drives the stainless steel impeller (P) in the mixing chamber. Taps T1 and T2 are opened to allow the two solutions, of concentrations equal to the limits of the gradient, to be mixed and flow into the centrifuge tube.

A check on the linearity of a sucrose gradient formed by this procedure was carried out using a refractometer. Small samples (about 0.1 ml) were removed at intervals down the gradient and their refractive indices measured at 20°C. The sucrose concentration corresponding to each value of refractive index was read off a calibration curve (Fig. 4.3). The results of two such checks are shown in Fig. 4.4 for nominal 5-30% gradients, with and without overnight storage in a refrigerator. The expected errors in the data points are quite small, as indicated in the figures. An uncertainty of ± 0.5% in sucrose concentration has been attributed to each point, resulting mainly from the ± 2 mm uncertainty in depth determination. The graphs indicate reasonably good linearity, although some tapering-off occurs at the top and bottom of the tube corresponding to Fig. 4.4b. Schumaker (1967) has reported that this phenomenon may occur with gradient-forming devices.

In a typical experiment about 8×10^9 cells were layered upon the gradient with a syringe and, after centrifuging, about 0.5 ml, representing 1 to 3% of the cells layered, was removed from the upper region of the turbid band. The duration and speed of centrifugation depended, as we have emphasized earlier, on the type of gradient used, the temperature of the gradient during the operation, and the size of the cells. Gradients were formed from sucrose, glucose, glycerol or Ficoll*. The conditions of centrifugation and the gradient concentrations specific to the various

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*Ficoll Type 70 (average molecular weight 70,000) was obtained from Sigma Chemical Co., St. Louis, Missouri.

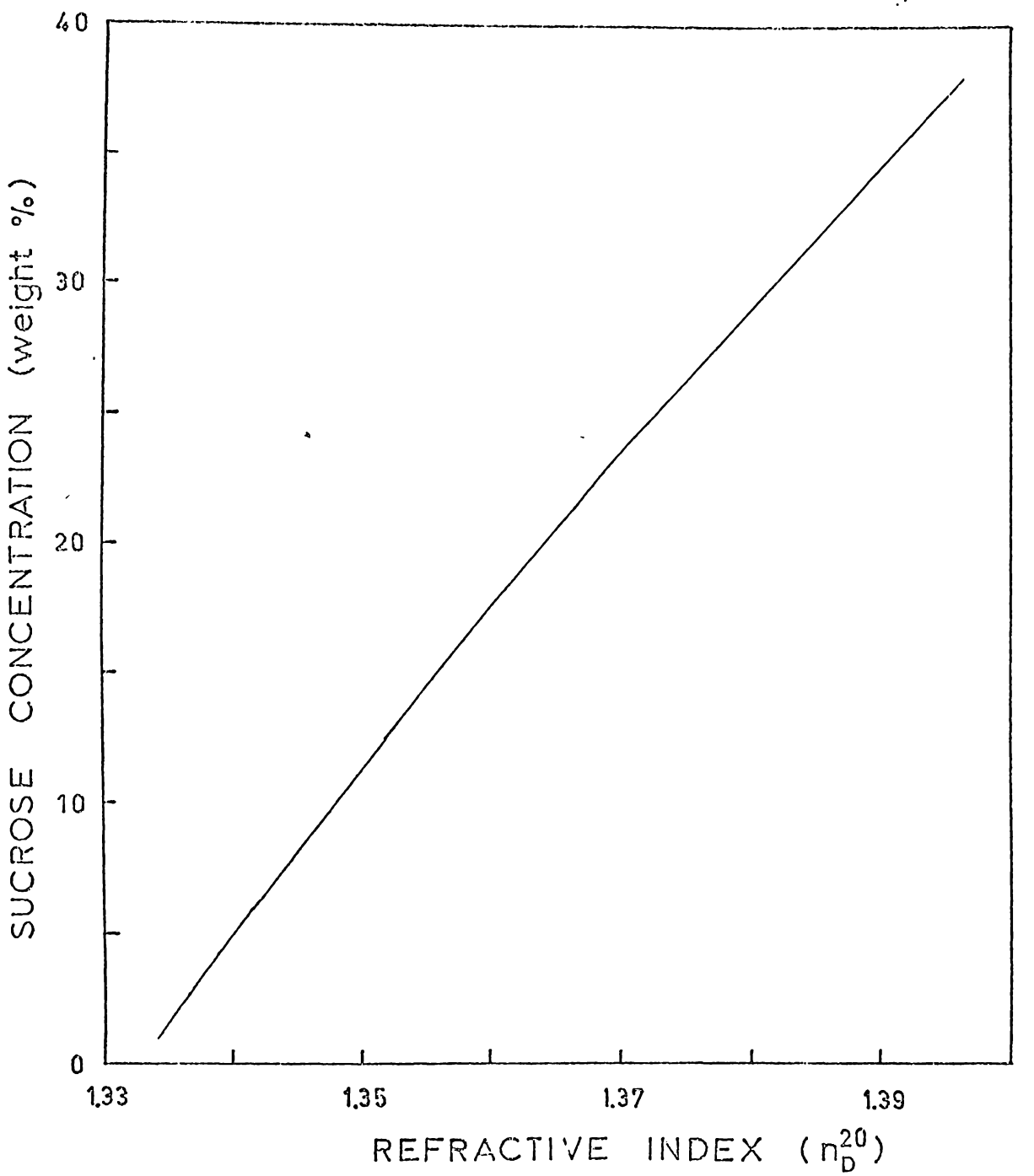

Fig. 4.3 Variation of refractive index at 20°C (n_D^{20}) with sucrose concentration (weight per cent).

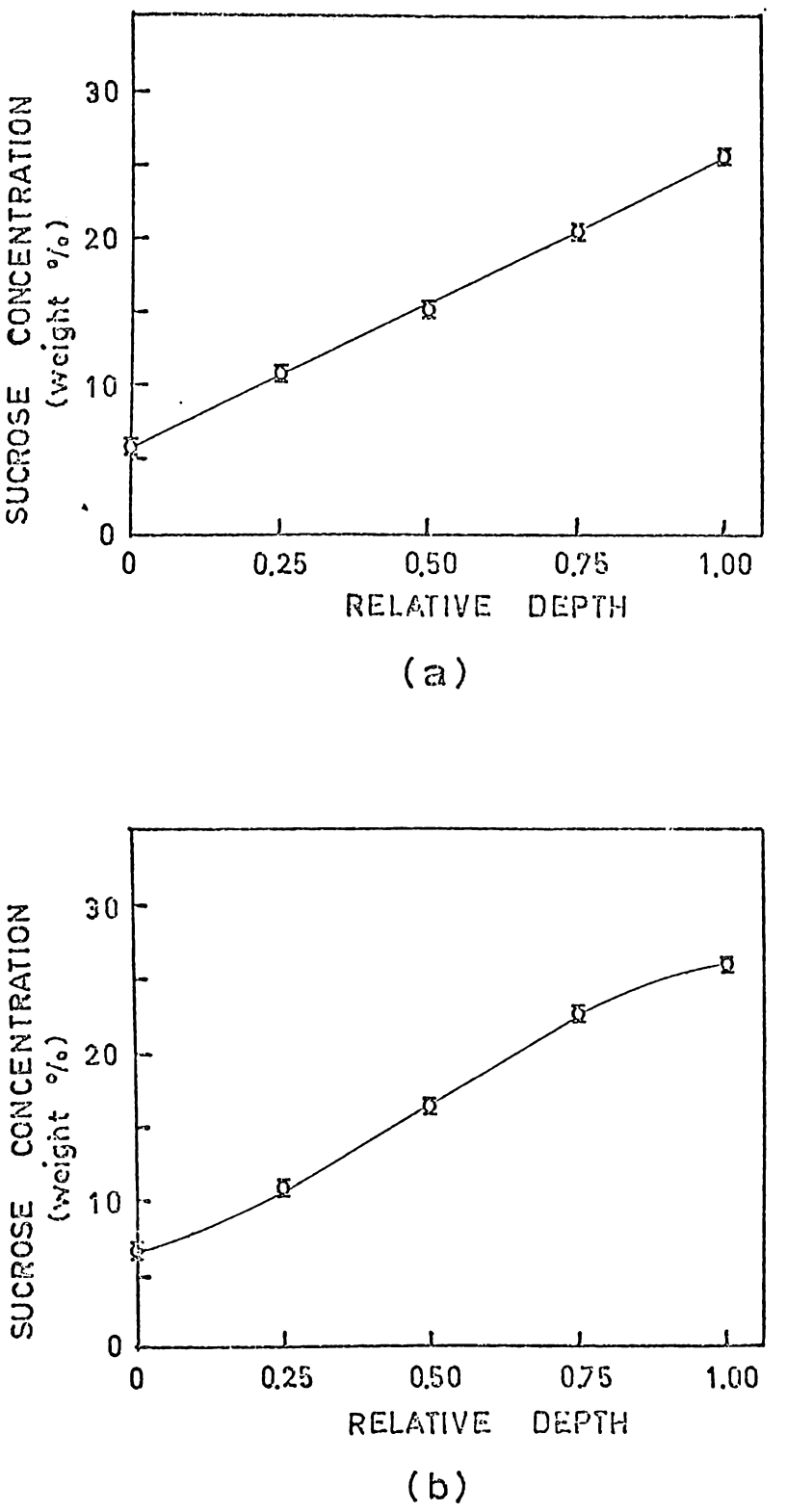

Fig. 4.4 Concentration linearity of sucrose gradients (5-30%).
(a) Without overnight storage.
(b) After overnight storage at 4°C.

experiments are detailed in Section 4.3 of this chapter.

4.2.3 Asynchronous Control Culture

By removing all the cells remaining in the band in the gradient, mixing, and inoculating a small fraction of these into the conditioned medium, an asynchronous culture could be generated, and this acted as a 'control'. Distortion of the normal pattern of exponential growth in this control culture would point to the possibility that the experimental manipulations had disturbed the cells in some way.

4.2.4 Cell Counting and Sizing

Cell counts were determined with a Coulter Model ZBI electronic particle counter* fitted with a commercial 30 μm diameter aperture. The principle of operation of this instrument has been discussed in the previous chapter. A useful description of the underlying theory of the electric sensing zone technique and its applications is provided in a review by Kubitschek (1969a). The rapidity with which cell concentrations can be determined with these instruments and their accuracy is well known. While complete volume distributions of particles can also be furnished very quickly, the accuracy of such distributions is not so certain.

Kubitschek's (1960, 1969a) simplified theory leads to a direct proportionality between particle volume and the amplitude of the pulse produced as the particle traverses the Coulter aperture, provided the particle cross-sectional area is not more than about 10% of the aperture cross-

Coulter Electronics Ltd., Harpenden, England.

sectional area. This holds for particles of any shape. A more rigorous analysis shows that particle shape does have a bearing on pulse amplitudes. In a comprehensive review by Allen & Marshall (1972) a number of these more refined treatments are discussed. Gregg & Steidley (1965) have shown that there can be as much as a 50% difference in the resistance change of particles of the same volume in going from a spherical shape to a long rod. This could clearly give rise to distortion of the size distribution of bacterial cells which grow by elongation at constant diameter, as do *E. coli*. Another possible source of distortion has been pointed out by Kubitschek (1962b, 1964) based on the fact that transit times for particles passing through the orifice will vary from a minimum for those particles moving along the aperture axis to much longer times for flow near the sides of the aperture. If the frequency response of the associated amplifying and pulse-shaping circuitry is not wide enough the sizes of those particles which go near the axis may be underestimated relative to the others. A suitably long aperture could clearly allow proper integration of all pulses. Unfortunately, the small diameter commercial apertures are often relatively short. Some apertures, in fact, may be so short that there is no finite region of uniform response (parallel equipotential planes) within the aperture (Kubitschek, 1969b). In these cases, even for amplifiers of the highest fidelity, the peak signal produced by a particle will depend upon its particular trajectory. Harvey (1968) has evaluated the performance of several instruments and shown that significant distortion occurs in the size distribution of latex spheres when compared with the distributions determined by electron microscopy.

The main feature of the distribution produced by these instruments is an exaggeration of the frequency of particles of larger volume, leading to an increased dispersion and skewness in the distribution. While the commercial instruments may be suited to rapid counting, they may require some modification to the aperture or to the electronics to effect accurate size distributions. A cursory study of the model ZBI counter indicated that, while the frequency response of the electronics in this instrument seemed adequate, pulses produced by the particles at the aperture appeared to be essentially differentiated before reaching the output and it was considered that particles of the same volume entering the aperture on different trajectories could produce output pulses of different amplitude (Rackham, 1977). Grover *et al.* (1969) also reached the conclusion that apparent particle volume depends on the particle trajectory through the aperture because of variations in electric-field strength near the aperture.

Volume distributions have been measured in this work by linking to the Coulter counter a 100-channel pulse height analyzer constructed in this laboratory (Rackham, 1977). This instrument sorts the output pulses from the Coulter counter into channels according to their size and keeps a running tally in its memory of the number of pulses in each size interval. While the memory registers are accumulating, their contents can be displayed on an oscilloscope screen as a graph of the number of particles of a given volume versus particle volume. At the end of a run this display can be plotted out on a servo-recorder to provide a permanent record.

A volume distribution obtained with this system is shown in Fig. 4.5, where polyvinyltoluene latex spheres* of 2.02 μm nominal diameter were suspended in 0.1 M HCl electrolyte and sized. The small peak at larger volumes is presumably caused by the presence of doublets. Aggregation of these spheres into doublets, triplets, etc. is known to occur in hydrochloric acid (Kubitschek, 1969a). The major peak exhibits significant skewness toward larger volumes, at variance with the symmetrical nature of the size distributions for these spheres obtained by electron microscopy. About 2×10^4 particles were sized at a count rate of approximately 800 particles per second in obtaining this distribution. At this count rate coincidence effects, discussed later in this chapter, are likely to be minimal (about 1%). In other words, the presence of two or more separate particles within the aperture sensing volume simultaneously, expected to produce a pulse roughly proportional to the sum of the volumes of the particles, may be neglected.

While the main causes of the distortion to size distributions in these instruments remains doubtful it must be borne in mind that such distortions exist when evaluating volume spectra for cell suspensions. Qualitative conclusions should be feasible, however, from observations of the shifts or trends in volume spectra. It is likely that the extent of distortion is similar in, say, distributions of newborn cells and distributions of extant cells, and then limited quantitative measurements could be made in a relative sense.

Dow Chemical Co., Midland, Michigan.

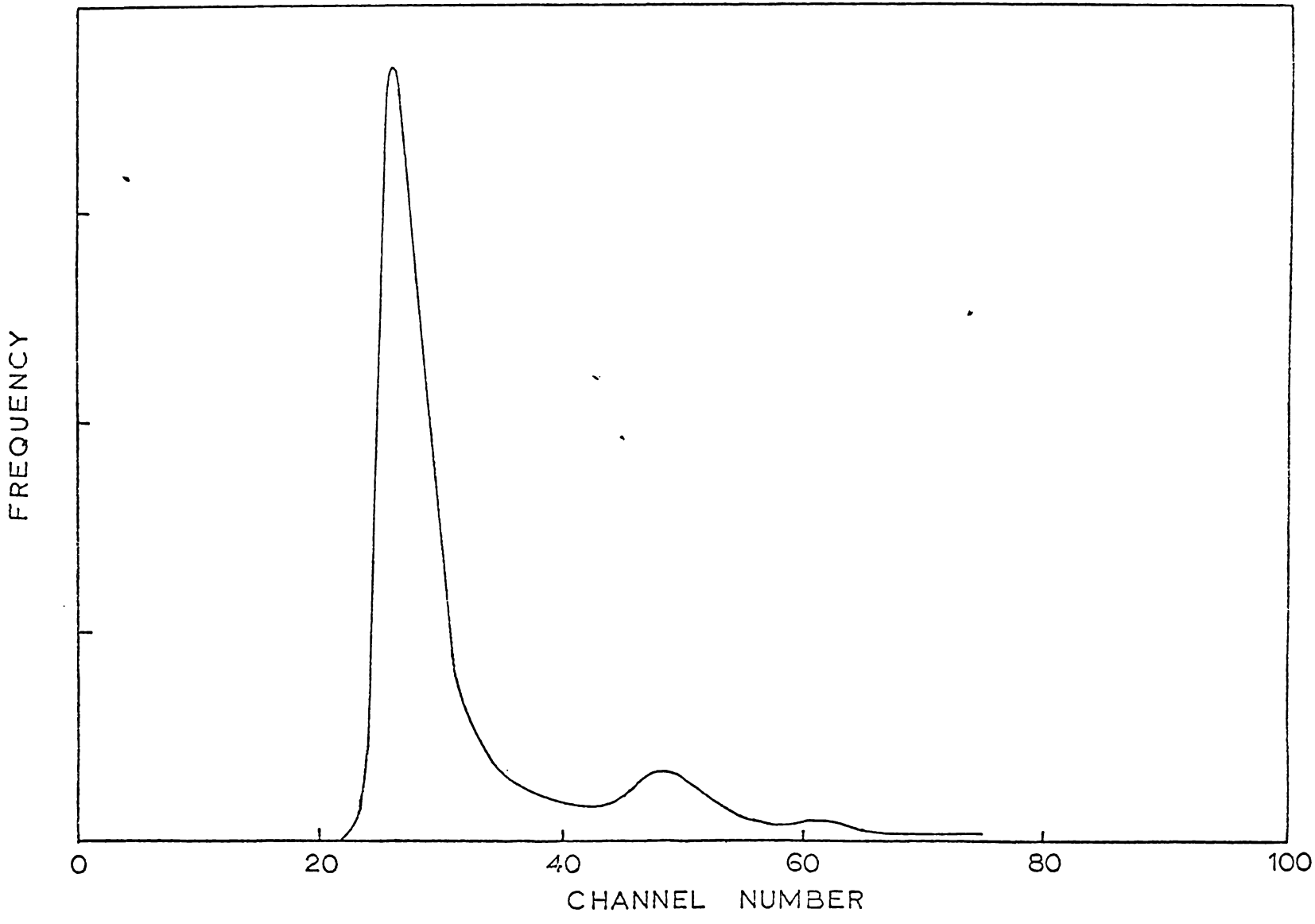

Fig. 4.5 Volume distribution of latex spheres obtained with the Coulter counter - pulse height analyzer system. Channel number is proportional to particle volume.

Both saline (0.9%) and hydrochloric acid (0.1 M) were considered as electrolytes for use with the Coulter counter. The addition of formaldehyde, at a concentration of 0.5% to the saline prevented further bacterial growth in diluted samples. Cell size distributions were observed to have the same form in either saline or HCl, as revealed in Fig. 4.6. The machine calibration of the channel number in terms of volume would be slightly different for the two electrolytes so that a shift of one distribution relative to the other is expected. Approximately 2×10^4 *E. coli* cells in mid-exponential phase were sized to obtain these distributions. Hydrochloric acid has several advantages over saline which led to its exclusive use in this work. It is easily prepared and remains sterile and particle-free when stored in plastic containers. Prevention of further growth or metabolism of microorganisms is ensured and, when used with the Coulter counter, the signal-to-noise ratio is improved. Suspensions of cells in saline plus formaldehyde have been observed to show a slight decrease in cell numbers and size over periods of time of the order of an hour or so (Rye & Wiseman, 1967). Some cultures suspended in 0.1 M HCl also exhibited these effects and it was deemed necessary to count the sample suspensions immediately. This requirement is without doubt important to the success of the experiments. In all the synchronous culture experiments reported here samples were counted immediately after dilution.

Visual observation of the pulse display on the oscilloscope screen of the Coulter counter usually enabled the threshold level to be set quite accurately, so that all

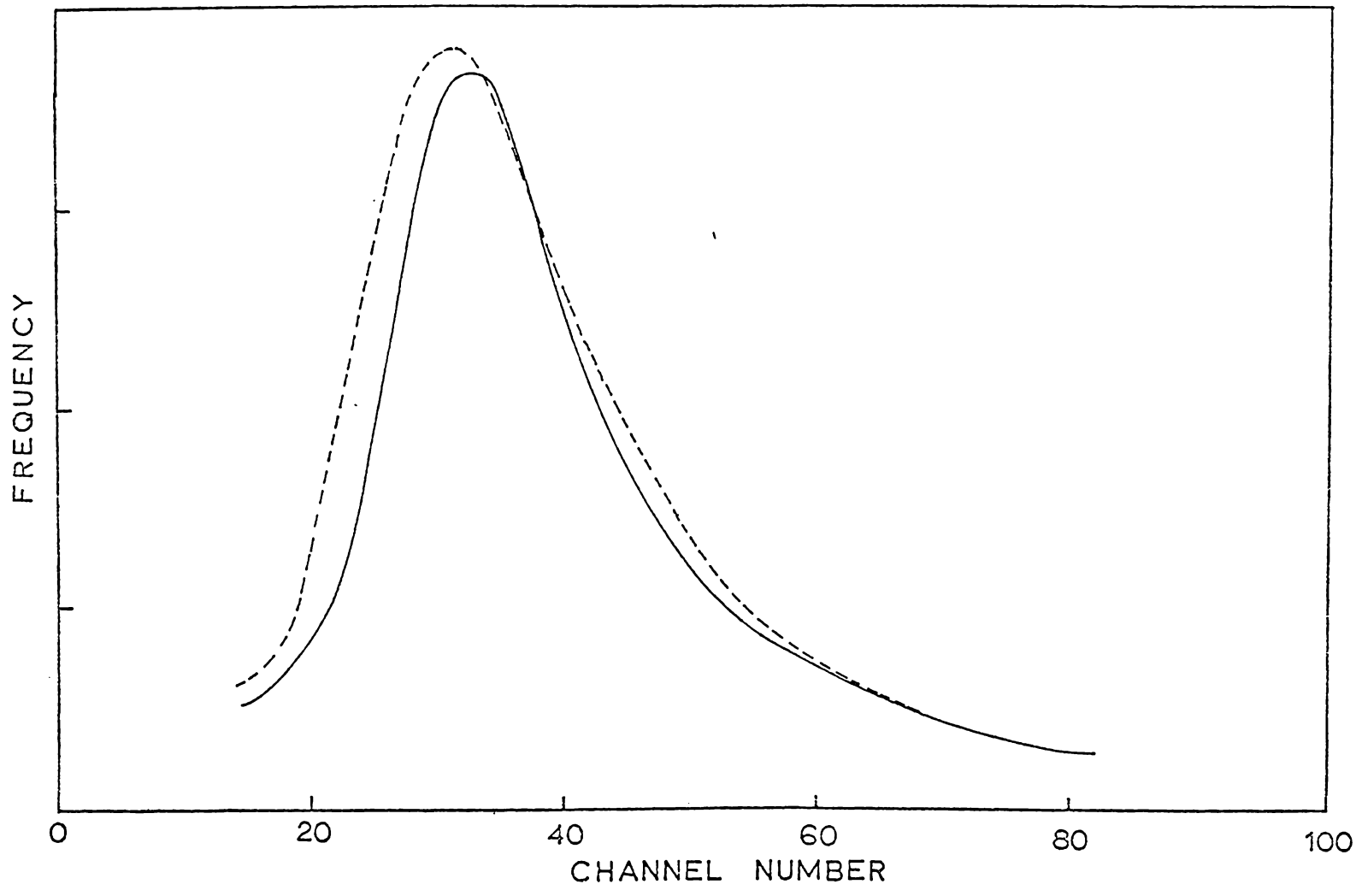

Fig. 4.6 Size distributions of *E. coli* B diluted in saline (solid line) and hydrochloric acid (dashed line). Coulter counter reciprocal aperture current setting = ½, reciprocal amplification setting = ¼. Channel number is proportional to particle volume.

cells were counted and electronic noise was gated out. This level could be confirmed by observation of the pulse height distribution on the multichannel analyzer or by checking the constancy of counts taken at threshold levels above and below that selected. This latter procedure essentially constructs part of a discriminator curve of which a complete example is shown in Fig. 4.7. To obtain this curve, counts were taken of a suspension of late exponential phase *E. coli* cells (grown in glucose-salts medium) in 0.1 M HCl. The presence of a broad plateau shows the adequate separation of noise and cell pulses. A threshold level of 16 was used to obtain total counts of cells grown under these same conditions with the amplification and aperture current settings mentioned in the figure caption.

We have already stressed that the value of the experimental results for our purposes, assuming we have a satisfactory method of synchronization, is largely determined by the precision and frequency of the determinations of cell numbers during the period of synchronous growth. Within the limit imposed by the size of the gradient used, the final yield of cells for the synchronous culture effectively determines these two factors. If sampling is too frequent the culture will be depleted before completion of the experiment and if sampling volumes are reduced to allow higher sampling rates we sacrifice precision in the cell counts. Because of the necessity for counting samples as soon as possible after dilution, practical considerations also limit the sampling frequency. Two minutes was found to be the minimum feasible sampling interval. As doubling times increased it was

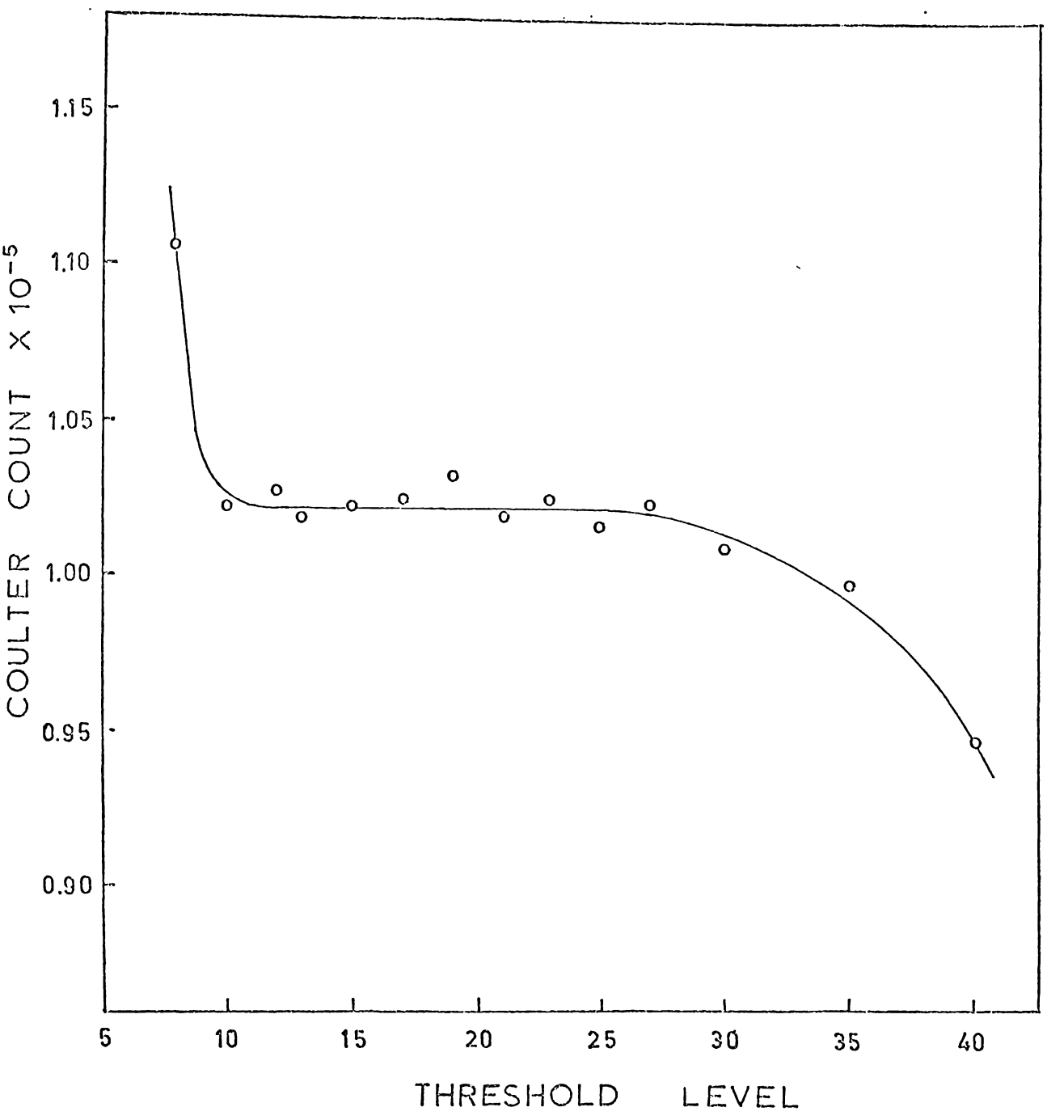

Fig. 4.7 Total counts of a bacterial suspension at various threshold levels. Glucose-grown *E. coli* cells were suspended in 0.1 M HCl. Coulter counter reciprocal amplification setting = ¼, reciprocal aperture current setting = 0.354.

necessary, of course, to use longer intervals. A compromise has to be reached, therefore, between precision and frequency. In all the experiments described here, culture samples of 0.2 ml were removed with a micropipette* having a quoted reproducibility of ± 1%. These were diluted 50-fold with 0.1 M HCl for counting and sizing. This dilution factor brings the cell concentration into the useful range of the Coulter counter, which is set at the upper extreme by coincidence considerations and at the lower by statistical fluctuations. This range was wide enough to encompass several generations of growth using the single dilution factor. A change in dilution factor was to be avoided, as a further source of error would be introduced. Reliable corrections for background level and coincidence are implicit in these considerations; such corrections are discussed in the next section.

4.2.5 Corrections for Background and Coincidence

In order to reduce the background count to a reasonable level several precautions must be observed in handling all solutions and glassware associated with the experimental procedure.

Repeated filtration of the diluent (0.1 M HCl) through 0.22 μm pore size membrane filters served to reduce background levels to 700-800 counts per 0.1 ml at the usual settings of the Coulter counter (reciprocal gain setting = ¼, reciprocal aperture current setting = 0.354, threshold level = 16). It

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*Scientific Manufacturing Industries, Emeryville, California.

has been noted already that all media were subjected to filtration through 0.22 μm filters. These membrane filters were washed before use to rid them of substances which may affect cell growth. Kubitschek (1969a) has pointed out that they may contain detergents to reduce capillary forces. All culture glassware was thoroughly rinsed in filtered, distilled water before sterilization. Erlenmeyer flasks in which cultures were grown were closed with rubber bungs wrapped in aluminium foil. Plugs containing cotton wool were avoided since these were found to shed fibres causing aperture blockages in the Coulter counter. Difficulty was experienced in reducing the background count to low levels when using glassware which had been used and cleaned in the laboratory. For this reason, multiple step dilutions were to be avoided and a single dilution was performed in the counting vessel. As mentioned earlier, single 50-fold dilutions were normally satisfactory when determining cell numbers in the synchronous cultures. These were achieved by diluting 0.2 ml culture samples to 10 ml using a graduated pipette to transfer the diluent. Blow-out pipettes were not satisfactory and it was found necessary to use a cotton wool plug in the pipette to keep background counts down. New Coulter accuvettes* were used as the counting vessels. These are obtained virtually dust free and, if necessary, several could be used during an experiment in full confidence that the background level would not be altered as a result.

For any particular synchronous culture experiment the

Coulter Electronics Ltd, Harpenden, England.

background levels were estimated for the synchronous and control cultures by carrying out the dilution operation on the conditioned medium just prior to inoculation. The graduated pipette was well rinsed in diluent before commencing. It was assumed that the contribution of unwanted particulate matter from the gradient would be negligible as such particles suffer a dilution of the order of 1000-fold in the operation. Background counts thus estimated were generally in the range 1000 to 1500 in 0.1 ml. This is to be compared with a total count of the order of 1.5×10^4 at the beginning of any experiment. This level was considered satisfactory and could be reasonably assumed to remain constant throughout an experiment.

Count rates near the end of some experiments approached around 5000 particles per second. This level corresponds to approximately 1.3×10^5 counts in 0.1 ml. At these extreme counting rates a significant loss in count occurs owing to coincident passage through the aperture of two or more particles. Such coincidences result, of course, in one count being recorded for two or more particles. A reliable formula for estimating the correction to the machine count was necessary; at least 6% correction is needed at the highest count rates. Coincidence losses may be ascertained experimentally by counting an increasingly dilute suspension until losses become very unlikely. By drawing a straight line through these points and extrapolating to the regions of higher concentration the loss of counts in these regions may be estimated. The Coulter ZBI manual provides an empirical equation, from such an experimental analysis, which is a

function of the aperture diameter and the count volume. This equation is of restricted application because it is independent of aperture length. It was derived for a length to diameter ratio of 0.75 which is roughly half the value usually observed for 30 μm apertures (Allen & Marshall, 1972). In addition, Allen & Marshall point out that the true diameters of these small apertures may differ by up to 12% from their nominal values.

Princen & Kwolek (1966) have given a simple derivation for count loss in a particle suspension, which we present here. We assume that the particles are randomly distributed in the solution and that N of these particles are present in the volume V that passes through the aperture in a counting period T. If S is the volume of the sensing zone in and around the aperture, the time, t, spent by each particle in this sensing volume is given by

$$t = \left(\frac{S}{V}\right) T .$$

The mean time interval between the entrance of one particle and the entrance of the next into the sensing volume we denote by $\bar{\tau}$. We must have

$$T = N\bar{\tau}.$$

Due to the random distance between particles, the fraction of particles with intervals between them less than t will be $t/(2\bar{\tau})$. Therefore, the total number of particles not counted will be

$$\frac{Nt}{2\tau} = \left(\frac{S}{2V}\right) N^2 .$$

Hence, of the N particles in volume V, the Coulter counter will only register

$$n = N - \alpha N^2 \qquad (4.1)$$

where we put $\alpha = S/2V$

Since the number of particles, N, in the metered volume, V, is proportional to the concentration of solids, C, we may write

$$N = KC$$

with proportionality constant K. Substituting in equation (4.1) and rearranging gives

$$\frac{n}{C} = K - \alpha K^2 C \qquad (4.2)$$

so that a plot of n/C against C should be a straight line of negative slope $-SK^2/2V$. If we denote a dilute concentration as C = 1, double this concentration as C = 2, and so on, this straight line will intercept the n/C axis at the value n/C = N, the coincidence corrected number count for the initial concentration. By fitting a straight line to real data by least squares we can extract a value for α; it is given by the slope divided by the intercept squared.

Three determinations of α were made by this method, corresponding to the three different gain settings used on the Coulter counter during the course of our synchronous culture experiments. A linear relationship between n/C and

C is observed and the straight lines fitted to the data points by least squares are shown in Fig. 4.8. Bacterial suspensions were used and in all experiments the reciprocal aperture current setting on the Coulter counter was 0.354 (corresponding to an aperture current of 1.414 mA). Dilutions with 0.1 M HCl were accurately determined gravimetrically and a background correction was applied which allowed for the varying contribution to the background by the diluent as the dilution is varied. The least squares estimates of α, together with their standard errors, are:

Reciprocal Gain Setting	α
⅛	$(4.86 \pm 0.20) \times 10^{-7}$
¼	$(5.00 \pm 0.36) \times 10^{-7}$
½	$(5.43 \pm 0.48) \times 10^{-7}$

Within experimental error, α may be deemed to be constant with gain, as expected, since the former parameter depends only on manometer volume and sensing volume of the aperture. All coincidence corrections in this work were evaluated using for α the value 5.0×10^{-7} in equation (4.1).

It should be remarked that the Coulter formula* applicable to the 30 μm aperture and 0.1 ml metering volume, *viz*,

$$N = n + 3.375 \times 10^{-7} n^2$$

clearly underestimates the coincidence correction required.

Instruction Manual, Coulter Counter Model ZBI (Biological) (Coulter Electronics Ltd, Harpenden, England, 1973), p.27.

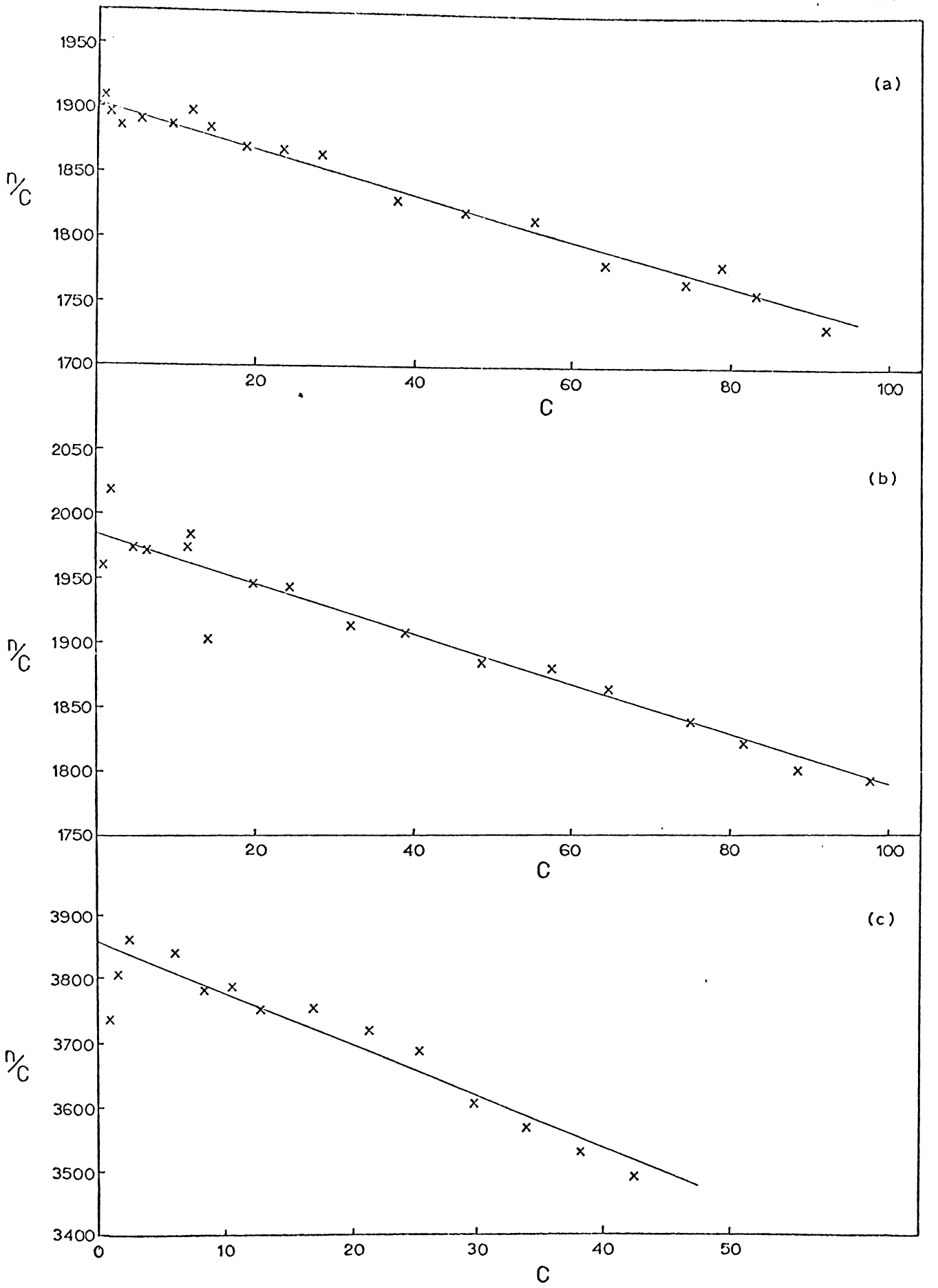


Fig. 4.8 Coincidence analysis. Determination of α (see text for details) at three different reciprocal gain settings in the Coulter counter: (a) 1/8, (b) 1/4, (c) 1/2. The straight lines shown have been fitted by least squares to the data points.

Corrections based on formula (4.1) and those derived from the Coulter expression will differ by about 1% at count levels of 60,000, this error increasing with higher counting rates.

4.3 RESULTS

4.3.1 Constant Temperature (37°C)

By supplementing the basic M9-salts solution with various carbon sources in turn, a range of growth rates was obtained for *E. coli* at 37°C. Table 4.2 lists the approximate doubling times for batch cultures with these energy sources.

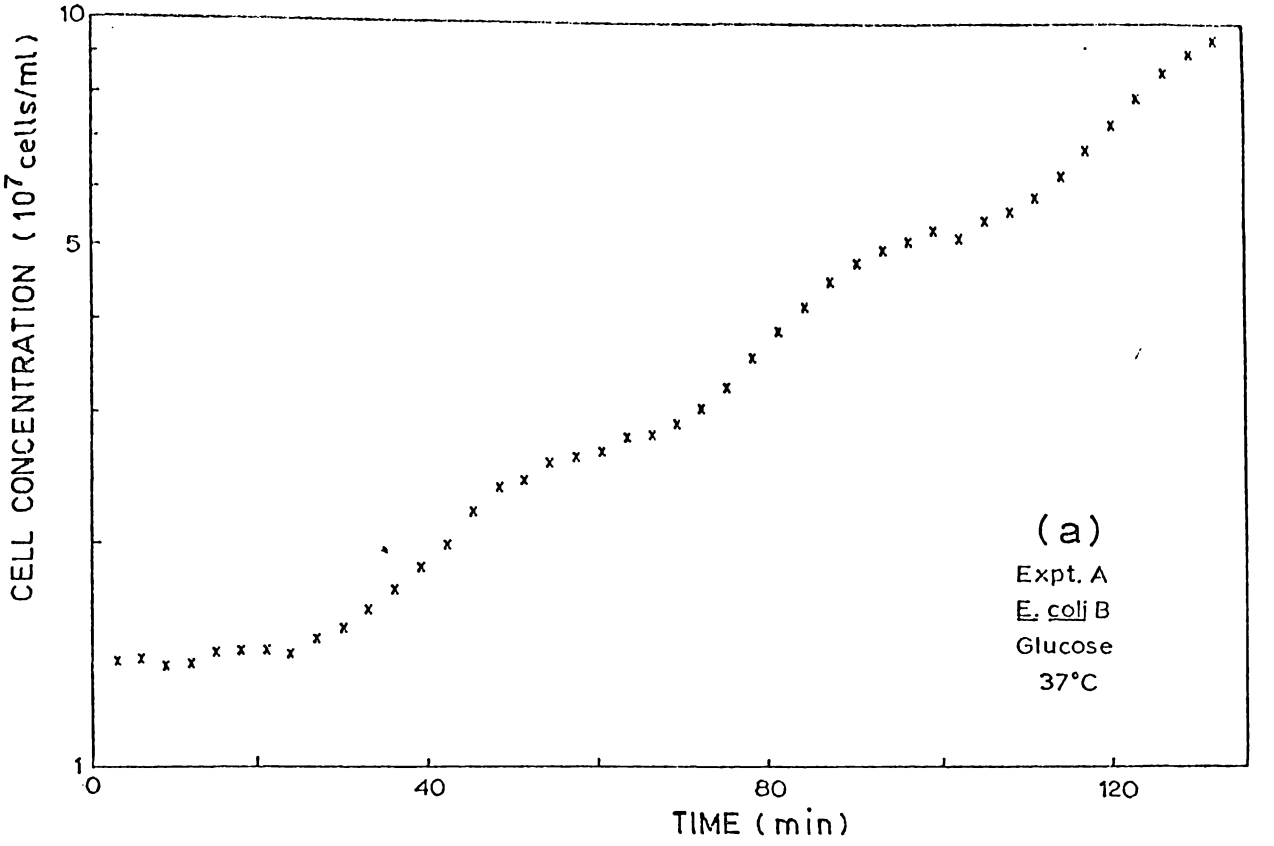
TABLE 4.2 Approximate Doubling Times for Batch Cultures of *E. coli* B at 37°C.

Carbon Source	Doubling Time (min)
glucose	40
sucrose	45
glycerol	48
succinate	80
glucose + methionine + histidine	34

We discuss the results for each carbon source under separate headings since experimental conditions differed slightly for the various growth rates. This is especially true as regards the duration of centrifugation and the gradient material used.

(a) Glucose

Figs. 4.9(a) to (e) show the growth curves for five different synchronous cultures of *E. coli* in glucose-salts media at 37°C. Information on the conditions under which these experiments were performed is tabulated in Table 4.3. The experiments are labelled A to E for reference purposes. Apart from experiment B, all involved banding in 5-30% sucrose gradients. A glucose gradient of 5-30% concentration range was used in this one exception. All gradients were made up in water with the exception of experiment E, in which the sucrose was dissolved in the glucose minimal medium prior to formation of the gradient. All operations in this latter experiment were performed at 37°C, in contrast to the others in which centrifugation in the gradient was carried out at room temperature (approximately 20°C). The entire procedure of concentrating the parent culture, layering the cells on the gradient, and subsequent banding took 20-25 minutes. As the growth curves show, experiments D and E were extended to cover about four generations of growth. The zero on the time scale for each of these curves corresponded to the time of inoculation of the sample from the gradient into the prewarmed conditioned medium. An asynchronous control culture was prepared in experiment E and it is clear from Fig. 4.9(e) how both the asynchronous and synchronous cultures show the same parallel trends in average growth. Samples were removed at three minute intervals from all five synchronous cultures. Background levels estimated by the methods described earlier were usually close to 1000 counts, about 5% of the initial total



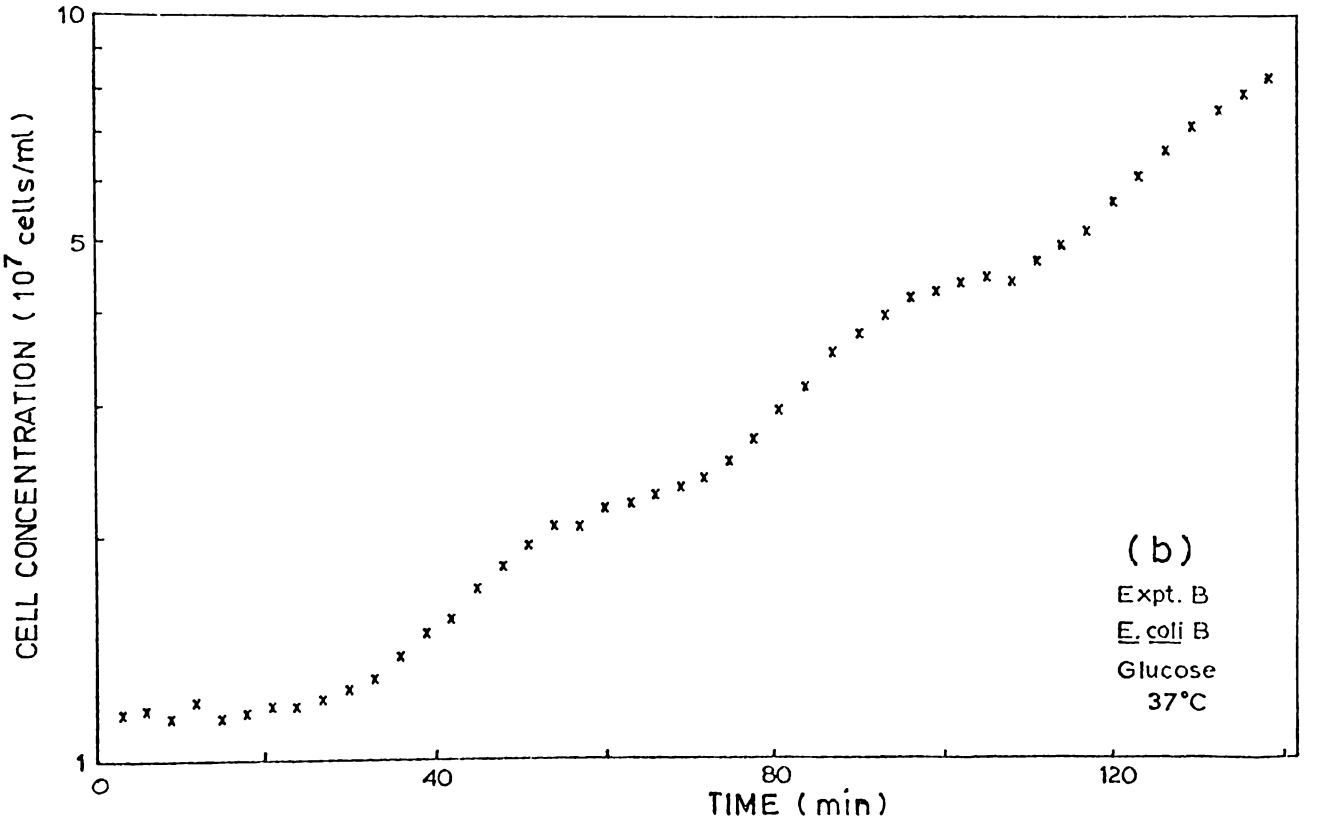
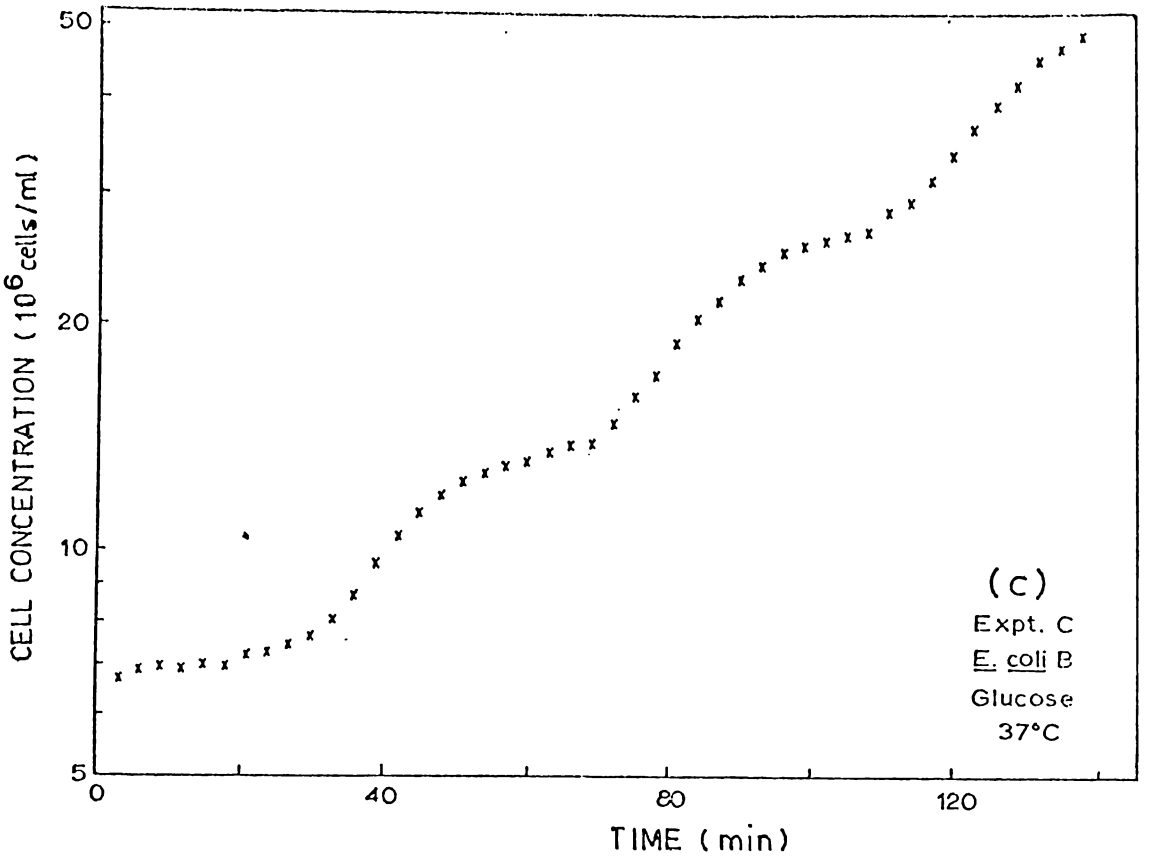
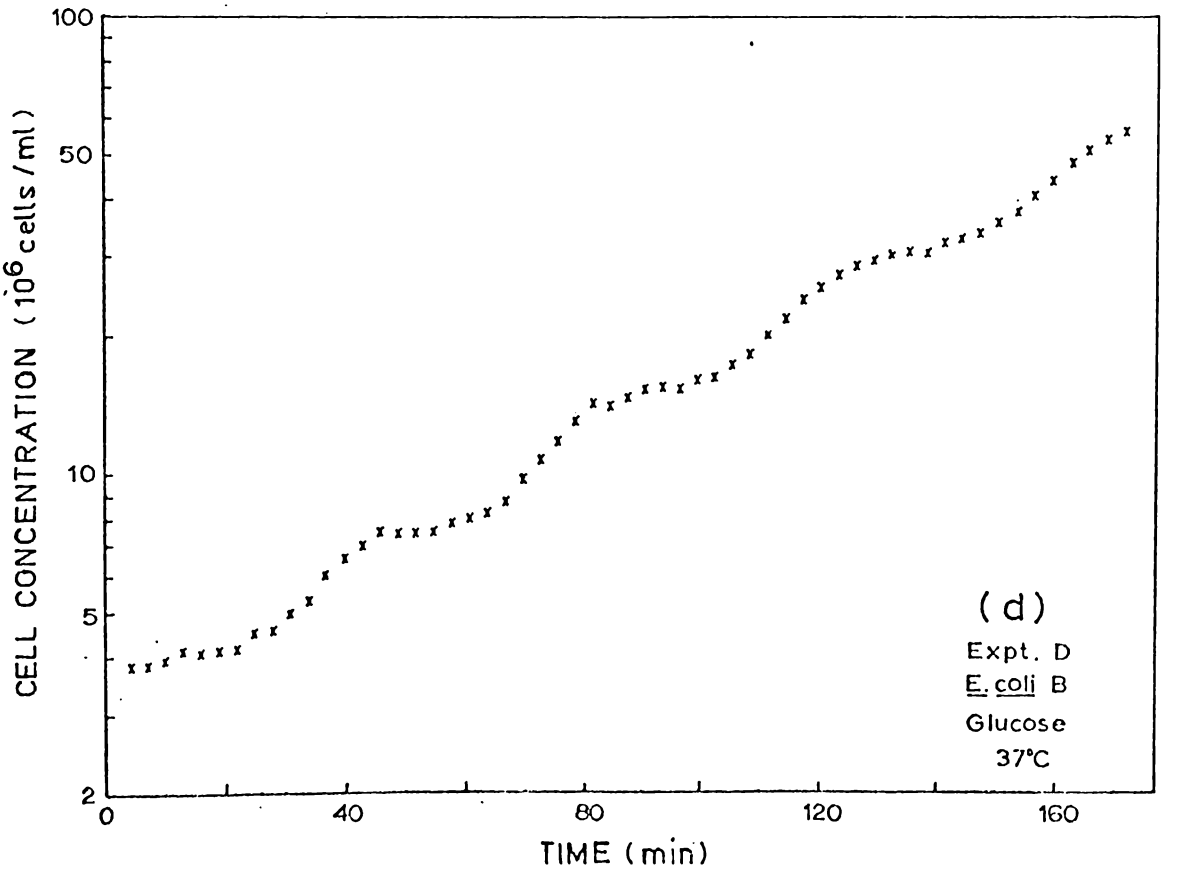
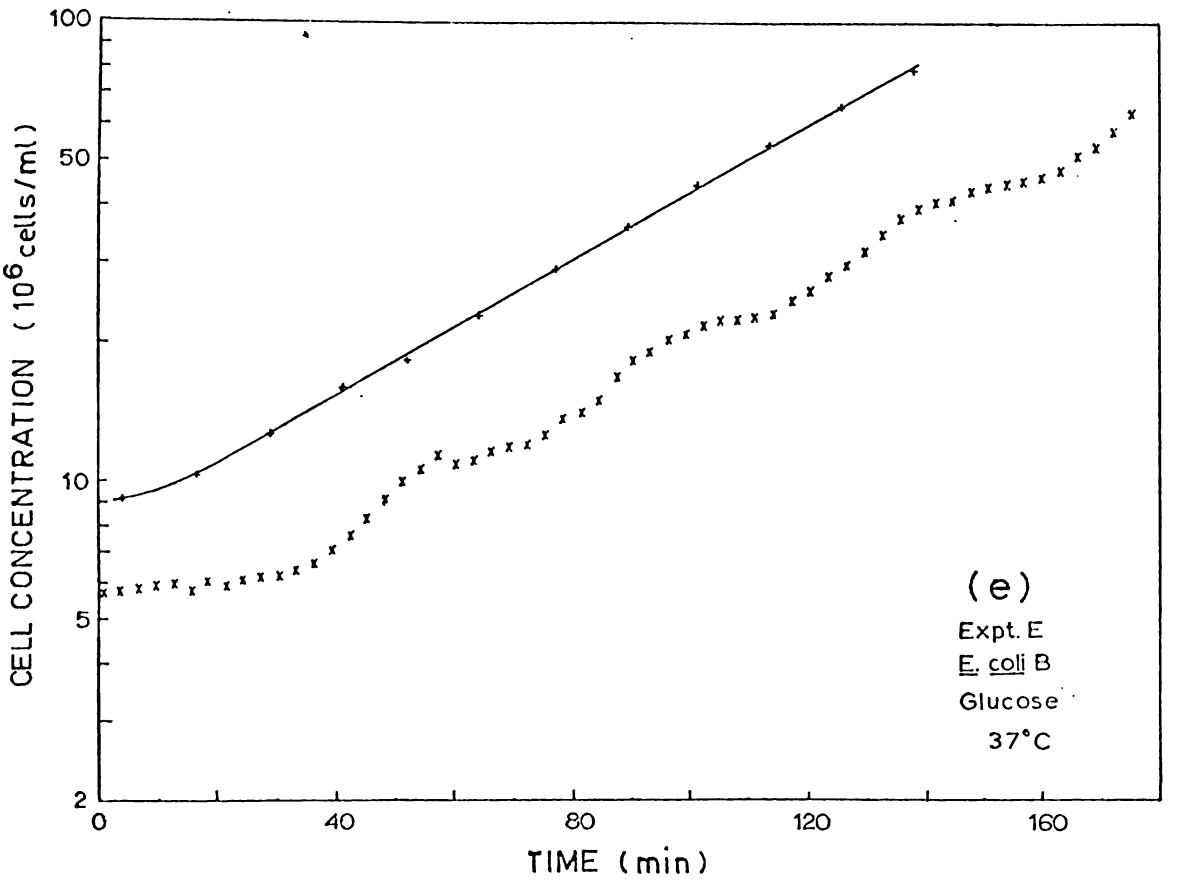


Fig. 4.9(a)-(e) Logarithmic plots of the cell density in synchronous cultures of *E. coli* B growing in glucose-salts media at 37°C. Growth of an asynchronous control culture is plotted in (e) (+).


50
20
10
5
0
40
80
120
CELL CONCENTRATION (10^6 cells/ml)
TIME (min)
(c)
Expt. C
E. coli B
Glucose
37°C


100
50
10
5
2
0
40
80
120
160
CELL CONCENTRATION (10^6 cells/ml)
TIME (min)
(d)
Expt. D
E. coli B
Glucose
37°C


100
50
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5
2
0
40
80
120
160
CELL CONCENTRATION (10^6 cells/ml)
TIME (min)
(e)
Expt. E
E. coli B
Glucose
37°C

TABLE 4.3 Experimental Conditions for Glucose-grown Synchronous Cultures of *E. coli* B at 37°C.

Experiment	Parent Culture Density cells/ml	Gradient	CENTRIFUGATION CONDITIONS Duration min	Force g	Temperature[a]	Recovery[b] %
A	1.8×10^8	Sucrose	12	1500	R.T.	1.6
B	1.0×10^8	Glucose	12	1500	R.T.	2.0
C	1.2×10^8	Sucrose	12	1500	R.T.	1.1
D	1.0×10^8	Sucrose	12	1500	R.T.	0.7
E	1.0×10^8	Sucrose	9	2100	37°C	1.1

[a] Room temperature (R.T.) was in the range 20-23°C.

[b] The yield of cells from the gradient expressed as a percentage of the cells loaded.

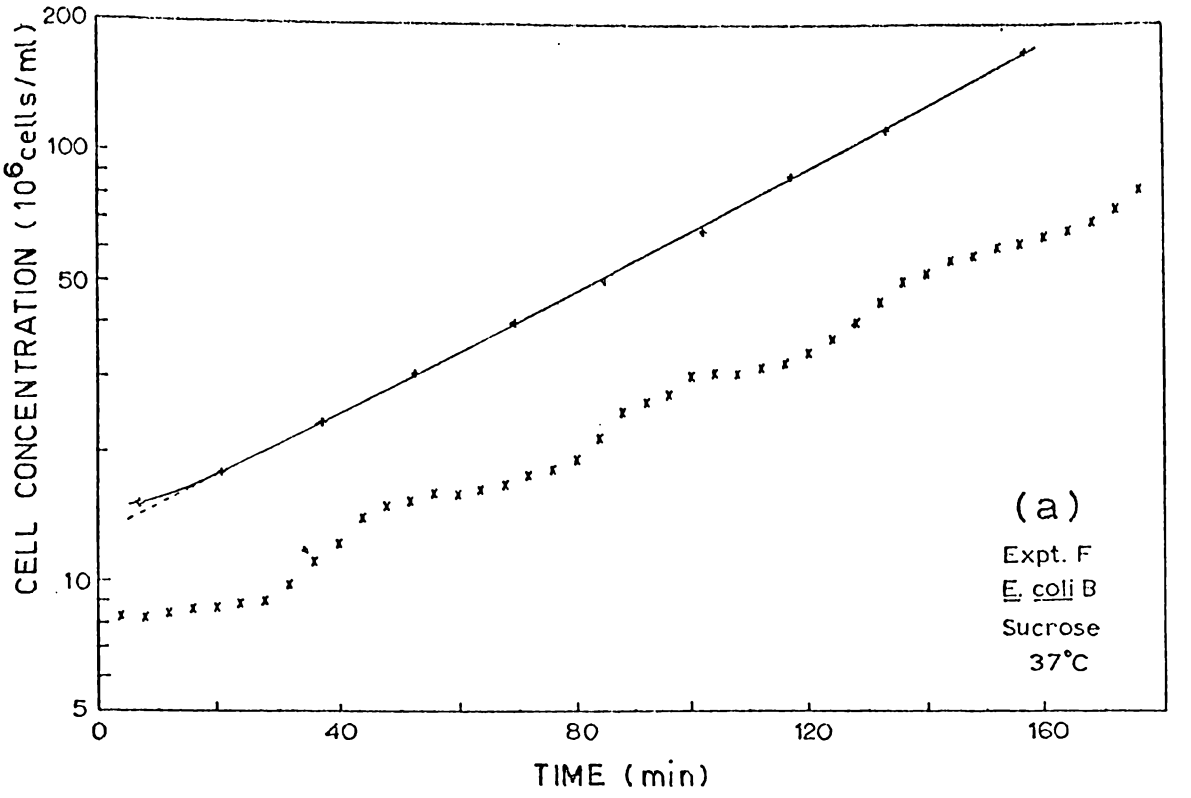
count in the synchronous cultures. Experiments D and E were begun with somewhat lower cell concentrations so that the background level rose to nearer 10% in these two cases.

(b) Sucrose

Two experiments were carried out with cultures grown in M9-salts plus sucrose (0.5%), with the results shown in Fig. 4.10. Gradients of sucrose (5-30%) were made up in M9-salts (experiment F) or water (experiment G). Cells were loaded on the gradients from parent cultures at densities of 1.1×10^8 cells/ml (F) or 5×10^7 cells/ml (G). Banding was accomplished at 1500 g for 15 minutes and approximately 2% of the cells loaded were recovered in each case. Asynchronous controls were prepared in both experiments and samples were withdrawn every 4 minutes from the synchronous cultures.

(c) Glycerol

Since our strain of *E. coli* utilizes sucrose, an energy source on which its growth rate is faster than on glycerol, centrifugation in a sucrose gradient would cause a nutritional shift-up in cells that had been transferred from glycerol media. A 10-50% glycerol gradient was therefore used to obtain the synchronous culture of Fig. 4.11 (experiment H). Cells were loaded onto this gradient from a parent culture at a density of approximately 2×10^8 cells/ml. After centrifuging at room temperature for 15 minutes at 1500 g a band of cells had formed about one-half of the distance down the gradient. Of the cells loaded, about 2.4% were recovered to yield the synchronous culture; samples were removed from this at four minute intervals.



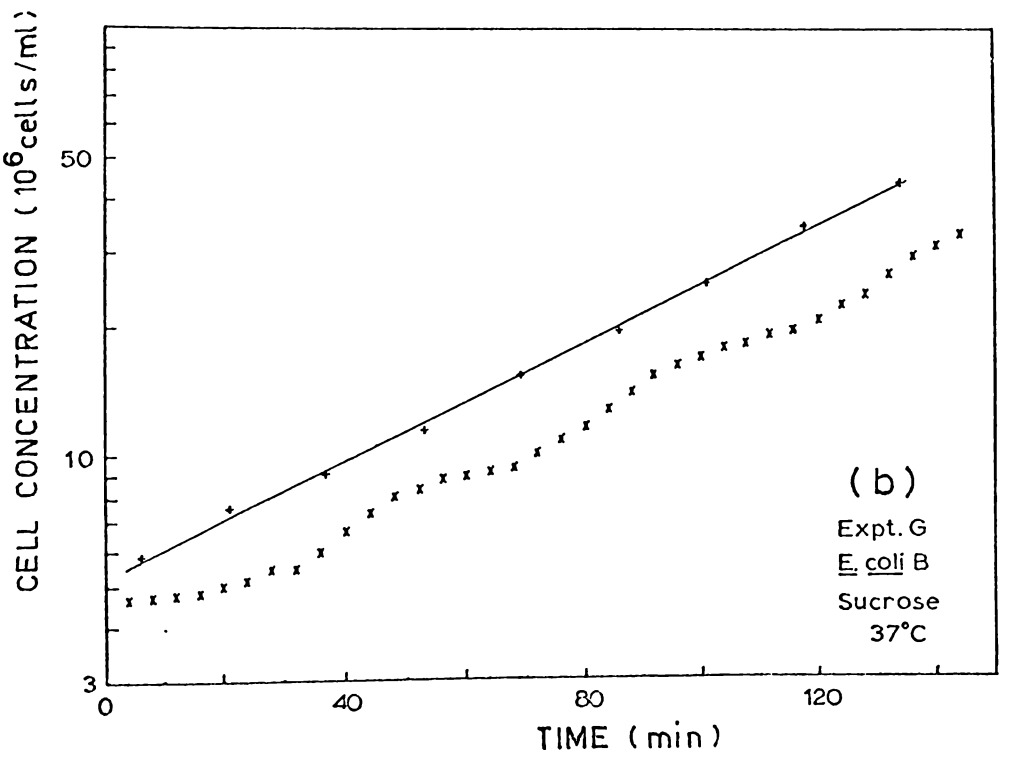


Fig. 4.10 a & b Logarithmic plots of cell concentration in synchronous cultures (x) and asynchronous control cultures (+) of *E. coli* in sucrose-salts media at 37°C.

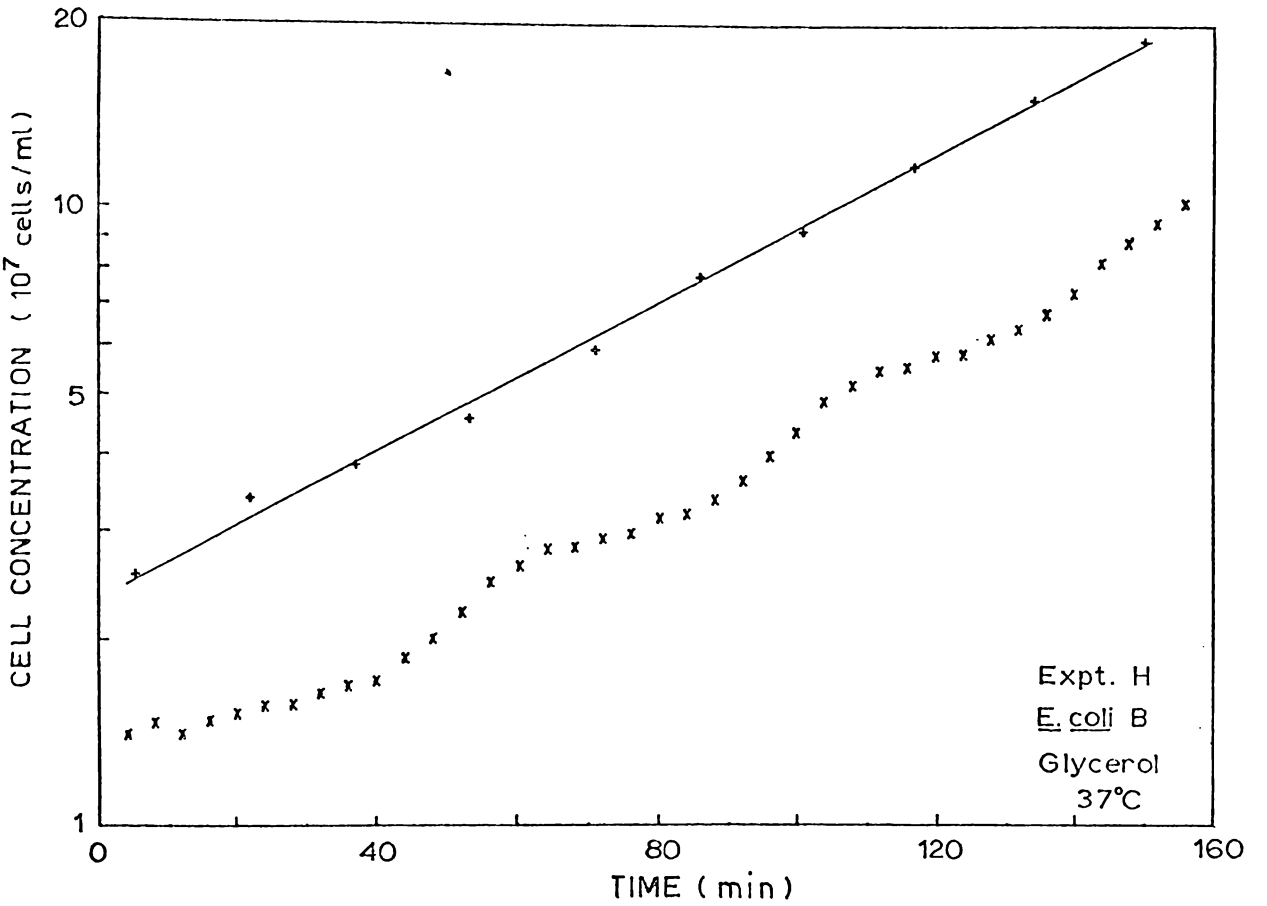


Fig. 4.11 Logarithmic plot of cell concentration in a synchronous culture (x) and an asynchronous control culture (+) of *E. coli* B in glycerol-salts medium at 37°C. Cell concentrations for the control should be divided by two to obtain the experimental values.

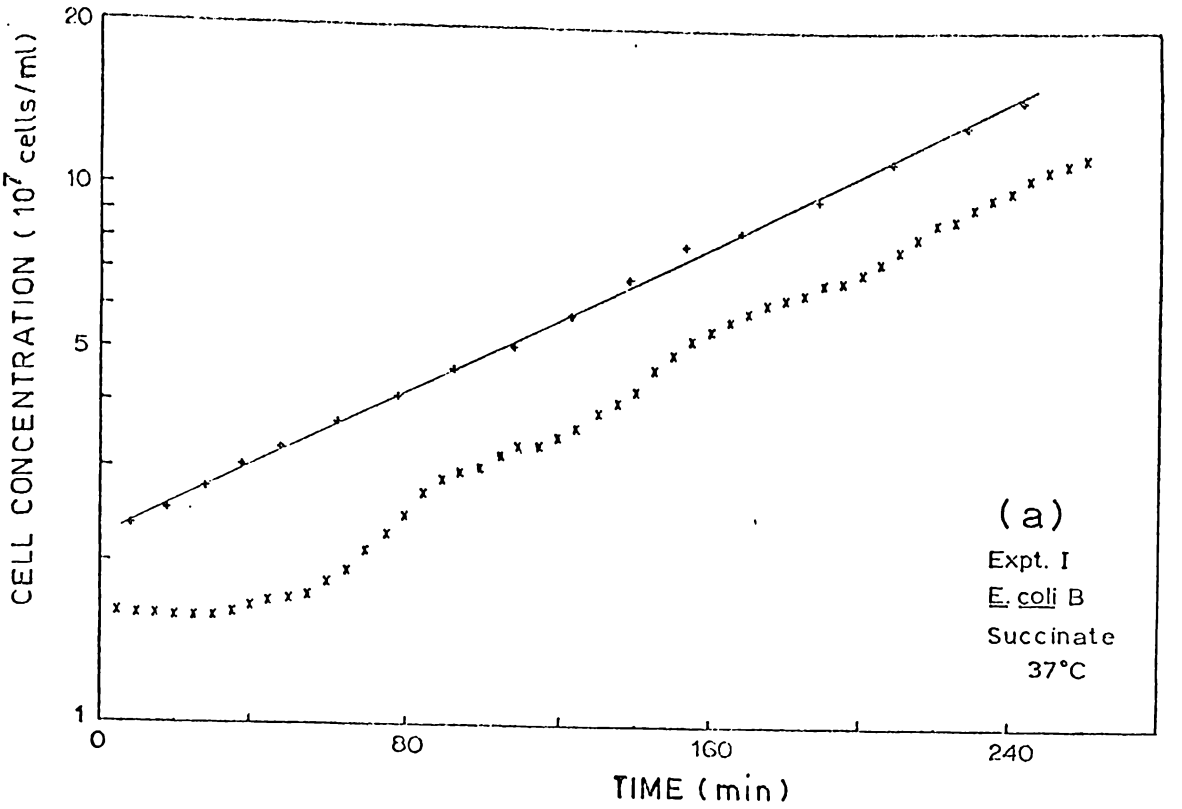
(d) Succinate

Again, to avoid a nutritional shift-up, succinate-grown cells were banded in 5-15% gradients of Ficoll Type 70 which were made up in water. Centrifuging was performed at 2100 g for 10 minutes at room temperature after which time the band had formed about one-third to one-half of the distance down the gradient. Samples were removed from the synchronous culture at five minute intervals yielding the results depicted in Fig. 4.12 (experiments I and J). In both experiments, 2% of the cells loaded onto the gradient were recovered in the synchronous cultures.

(e) Glucose + Methionine + Histidine

These cells have a higher growth rate than those on glucose only and it is desirable to reduce the time which they must spend in the gradient. A higher centrifugal force of 2200 g allowed banding in a 5-30% sucrose gradient to be completed in 6 minutes at room temperature. The gradient was prepared using growth medium as solvent. Samples were removed from the synchronous culture at two minute intervals and counted immediately, giving the results in Fig. 4.13 (experiment K). In this experiment, cells were loaded on the gradient from a parent culture at a density of 7.8×10^7 cells/ml and 2% of these cells were recovered from the synchronous culture.

The experimental conditions for experiments F to K are collated in tabular form in Table 4.4.



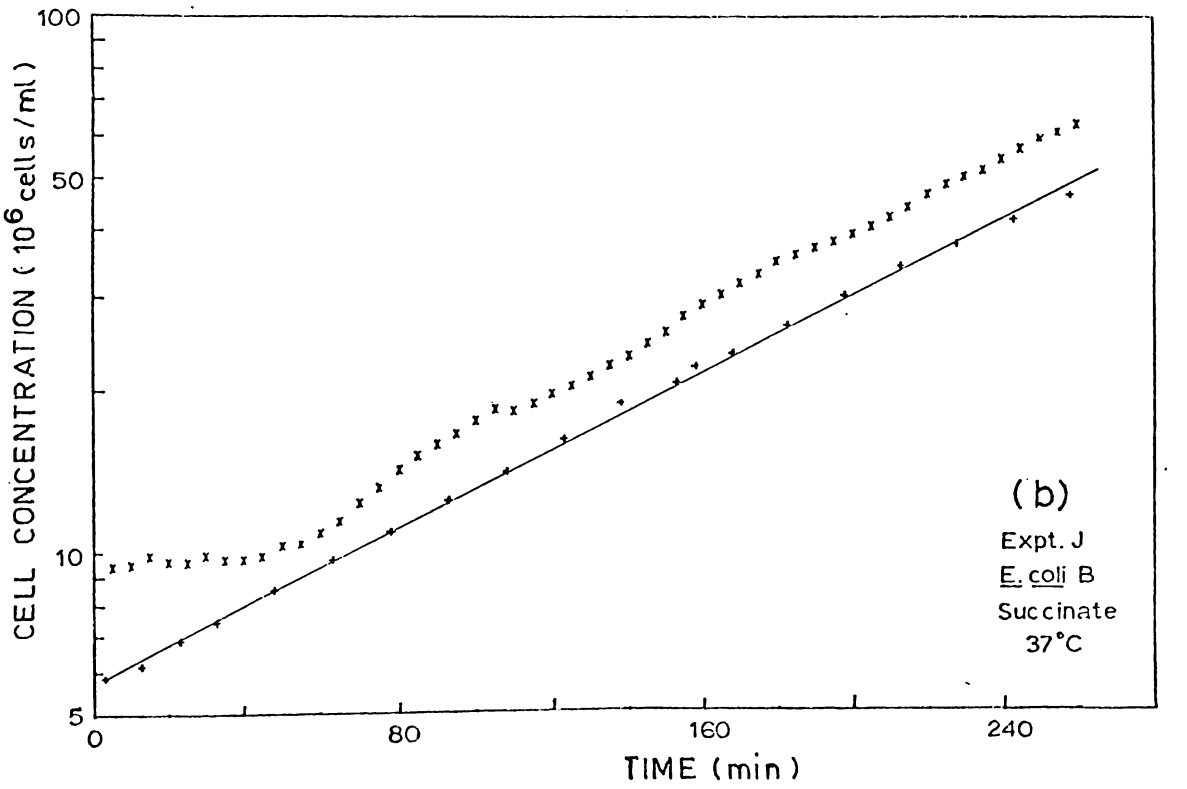


Fig. 4.12a & b Logarithmic plots of cell concentration in synchronous cultures (x) and asynchronous controls (+) of *E. coli* B in succinate-salts media at 37°C.

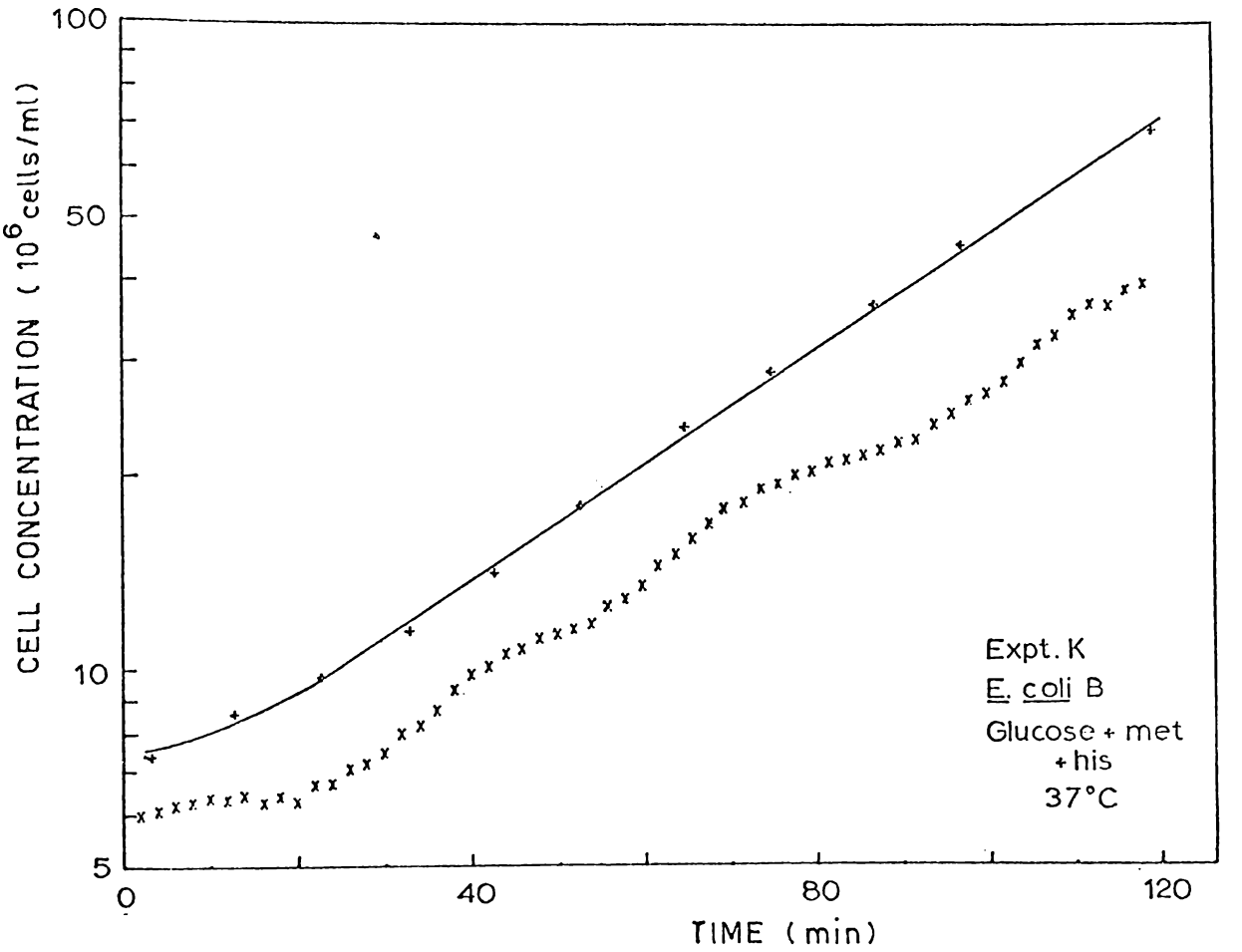


Fig. 4.13 Cell concentration during growth of a synchronous culture (x) and an asynchronous control culture (+) of *E. coli* B in M9-salts containing glucose, methionine and histidine.

TABLE 4.4 Experimental Conditions for Synchronous Cultures of *E. coli* B on Various Carbon Sources at 37°C.

Experiment	Carbon Source	Parent Culture Density cells/ml	Gradient[a]	CENTRIFUGATION CONDITIONS Duration min	Force g	Temperature	Recovery %
F	Sucrose	1.1×10^8	5-30% Sucrose (M9 salts)	15	1500	R.T.	2.0
G	Sucrose	5.0×10^7	5-30% Sucrose (Water)	15	1500	R.T.	2.4
H	Glycerol	2.2×10^8	10-50% Glycerol (Water)	15	1500	R.T.	2.4
I	Succinate	2.2×10^8	5-15% Ficoll (Water)	10	2100	R.T.	2.0
J	Succinate	1.1×10^8	5-15% Ficoll (Water)	10	2100	R.T.	2.0
K	Glucose/met/his	7.8×10^7	5-30% Sucrose (Medium)	6	2200	R.T.	2.0

[a] Gradients were prepared using the solvents given in parentheses.

4.3.2 Variable Temperature

With glucose as the carbon source, synchronous cultures were obtained at various temperatures within the range 26°C to 37°C. Sucrose gradients (5-30%) were used for all experiments and banding was accomplished at 1500 g. The results for the four experiments carried out at less than 37°C are presented in Figs. 4.14 to 4.17. Table 4.5 details the conditions under which these results were obtained. The sucrose gradient for experiment O was prepared in growth medium; all other gradients were prepared in water. A cold gradient (about 10°C) was used in experiment N for the banding operation. The M9-glucose medium used for all experiments was supplemented with 2% sucrose during growth of the parent culture for experiments L and M. This variation was designed to increase the osmotic pressure on these cells so that 'osmotic shock', if any, experienced during banding may be reduced and the relatively high sucrose concentration present in the synchronous culture is maintained in the initial parental culture.

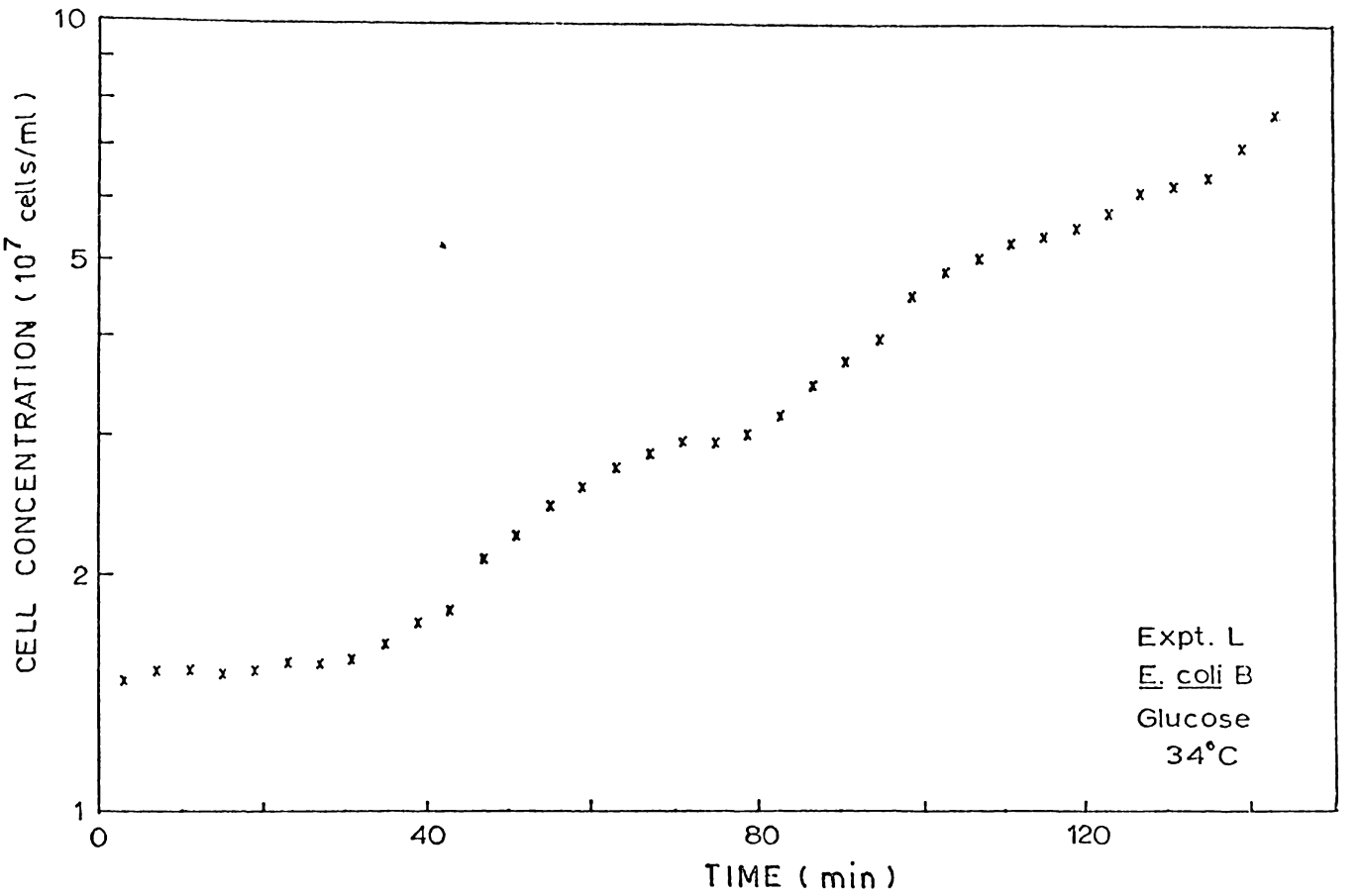


Fig. 4.14 Synchronous growth of *E. coli* B in glucose-salts medium at 34°C.

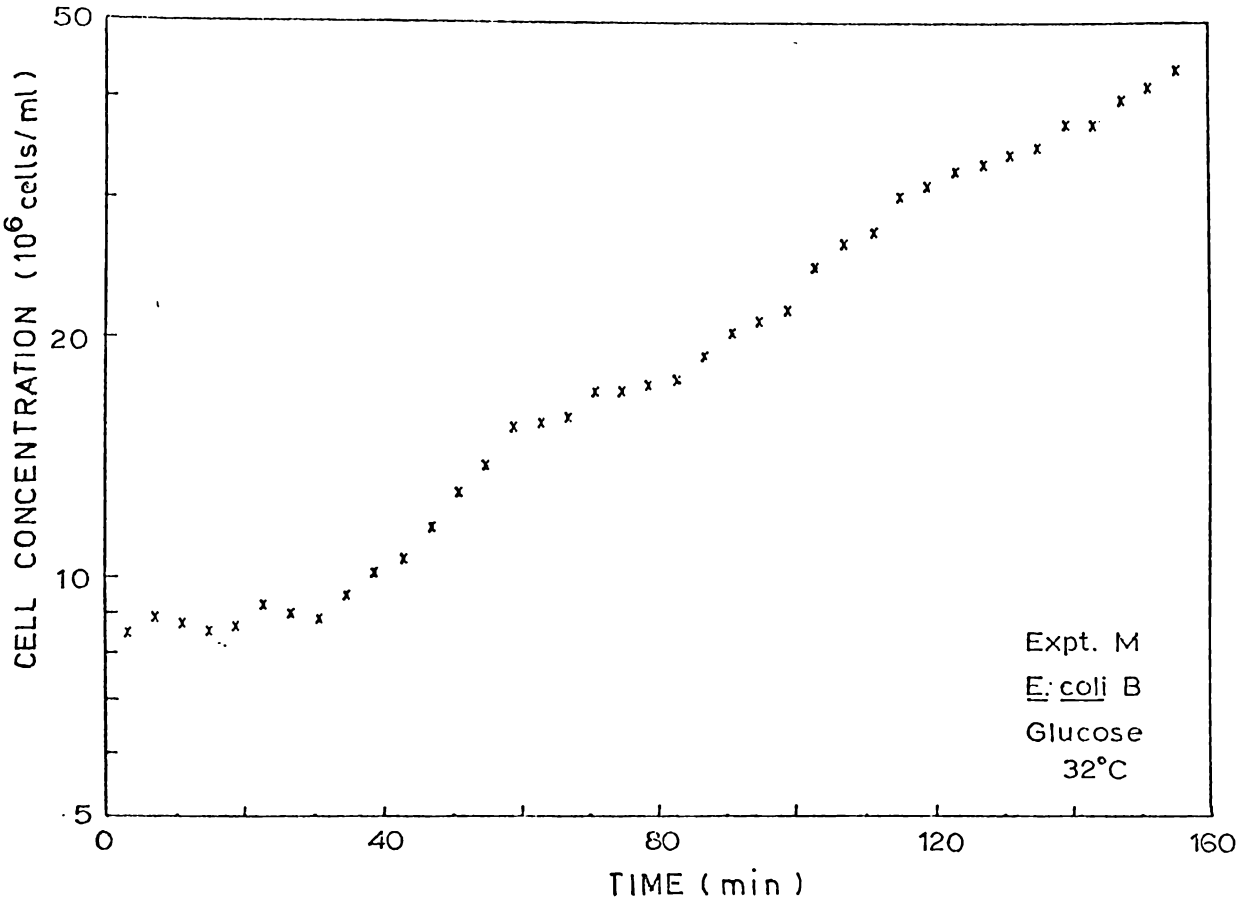


Fig. 4.15 Synchronous growth of *E. coli* B in glucose-salts medium at 32°C.

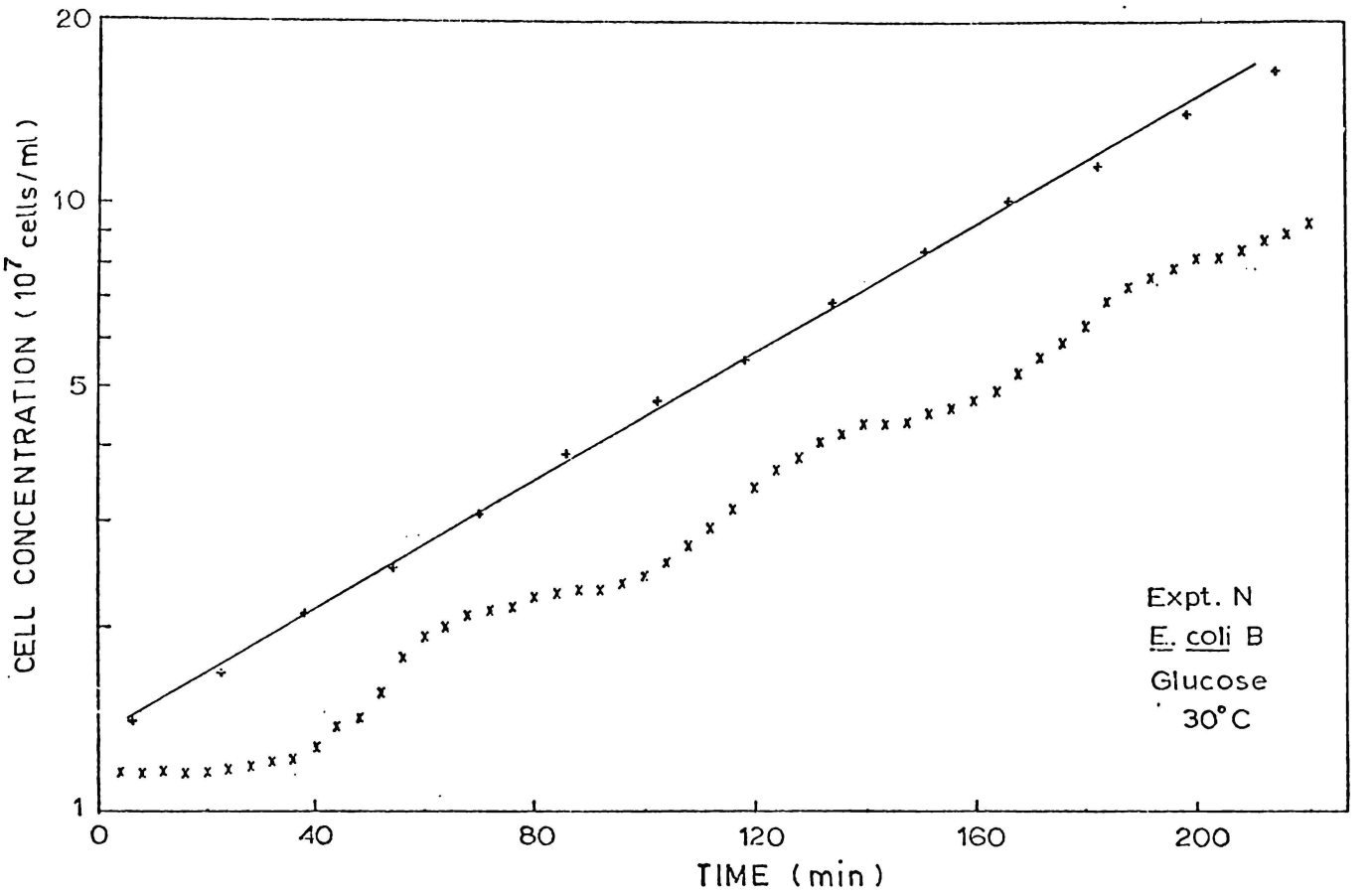


Fig. 4.16 Synchronous growth (x) and asynchronous control (+) for *E. coli* B in glucose-salts medium at 30°C.

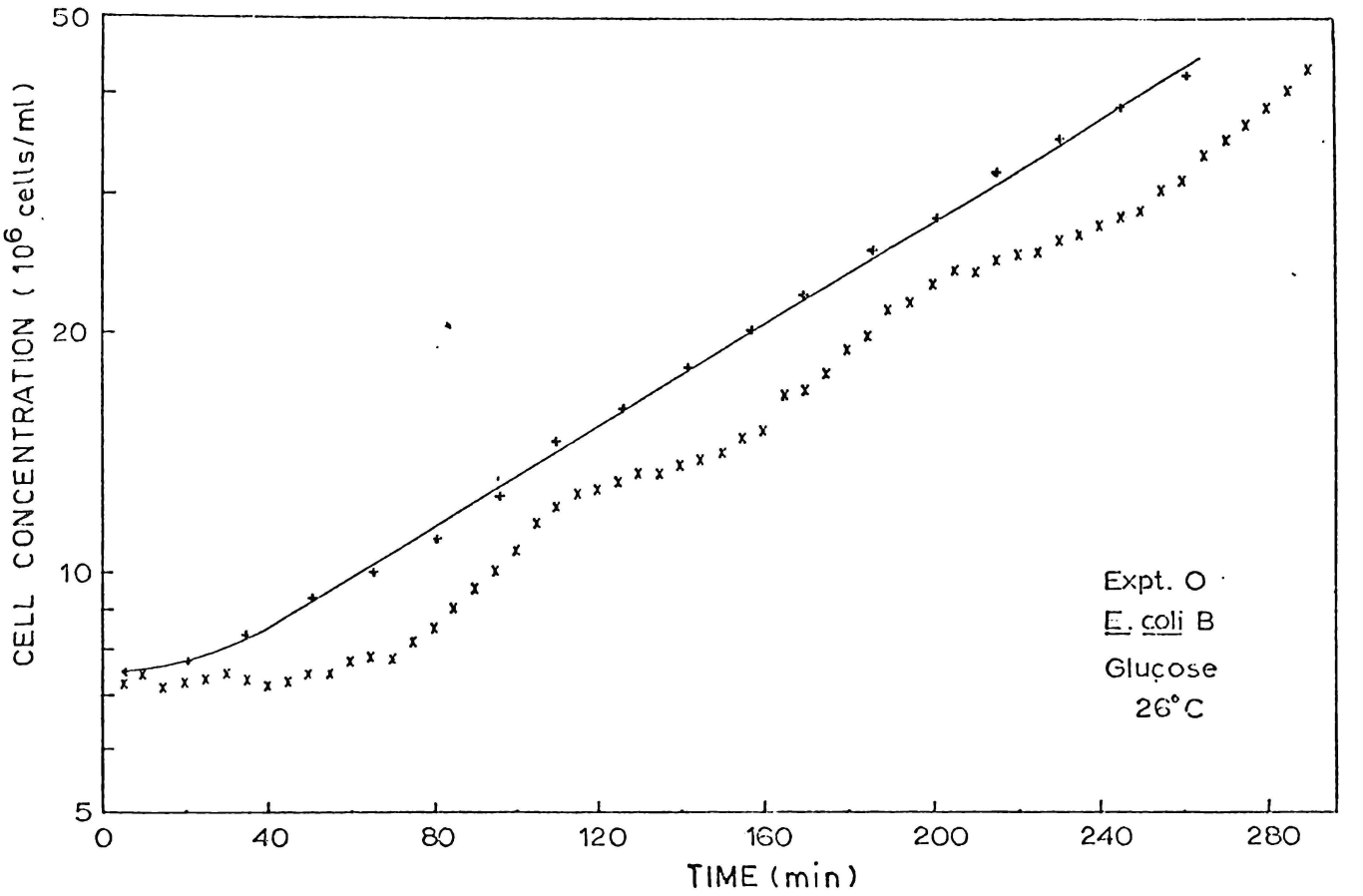


Fig. 4.17 Synchronous growth (x) and asynchronous control (+) for *E. coli* B in glucose-salts medium at 26°C.

TABLE 4.5 Experimental Conditions for Growth of Synchronous Cultures of *E. coli* B at various Temperatures

Experiment	Temperature °C	Approximate doubling time, min	Parent Culture Density cells/ml	CENTRIFUGATION CONDITIONS			
				Duration min	Force g	Temperature	Recovery %
L	34	48	8.5×10^7	12	1500	R.T.	3.2
M	32	54	8.6×10^7	12	1500	R.T.	2.2
N	30	60	2.4×10^8	15	1500	10°C	1.2
O	26	90	9.0×10^7	18	1500	R.T.	2.1

4.3.3 Cell Volume Spectra

Synchronous cultures were prepared from parent cultures having cell densities in the range 5×10^7 to about 2×10^8 cells/ml. It is important, of course, that these parental cultures be in balanced exponential growth at the time of harvesting. Cells become progressively smaller in a culture approaching saturation. This implies that at least some of the cellular components do not double between successive divisions and the cell cycle can no longer, therefore, be considered to be normal. The Coulter counter-multichannel analyzer system enables us to check very easily the volume distributions of cells growing in batch culture as growth proceeds. In the light of our earlier remarks on the measurement of volume distributions, we recognize that distortion of the true distribution may occur. However, marked shifts in the modal volume or the shape of the distributions as cell density increases in batch culture will be indicative of a change away from the steady-state of exponential growth pattern. Reference to Figs. 4.18 and 4.19 shows that, while cell numbers are observed to increase exponentially at least to 8×10^8 cells/ml, a shift in average cellular volume occurs prior to this population density. No significant change in the distributions of cell volume is evident in Fig. 4.19 at least to a population density of 2×10^8 cells/ml. These distributions are represented as smooth curves drawn through the channel frequencies. Approximately 2×10^4 cells were sized for each distribution. It is clear that, as a determinant of balanced growth, the information obtainable from cell volume spectra is important. Cell numbers show no change

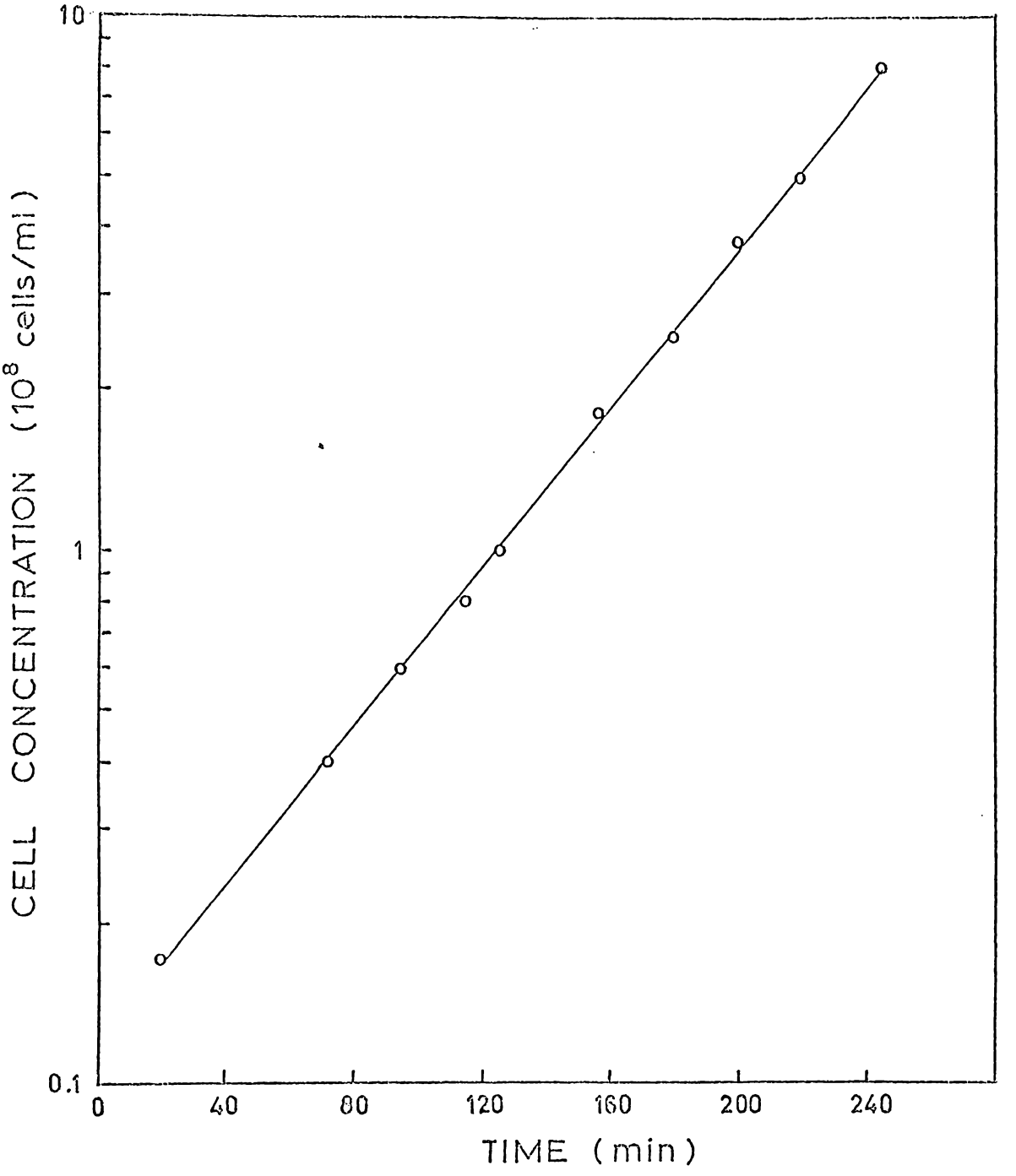


Fig. 4.18 Cell number concentration plotted on a logarithmic scale for batch culture growth of *E. coli* B in glucose-salts medium at 37°C. Time is measured from an arbitrary zero.

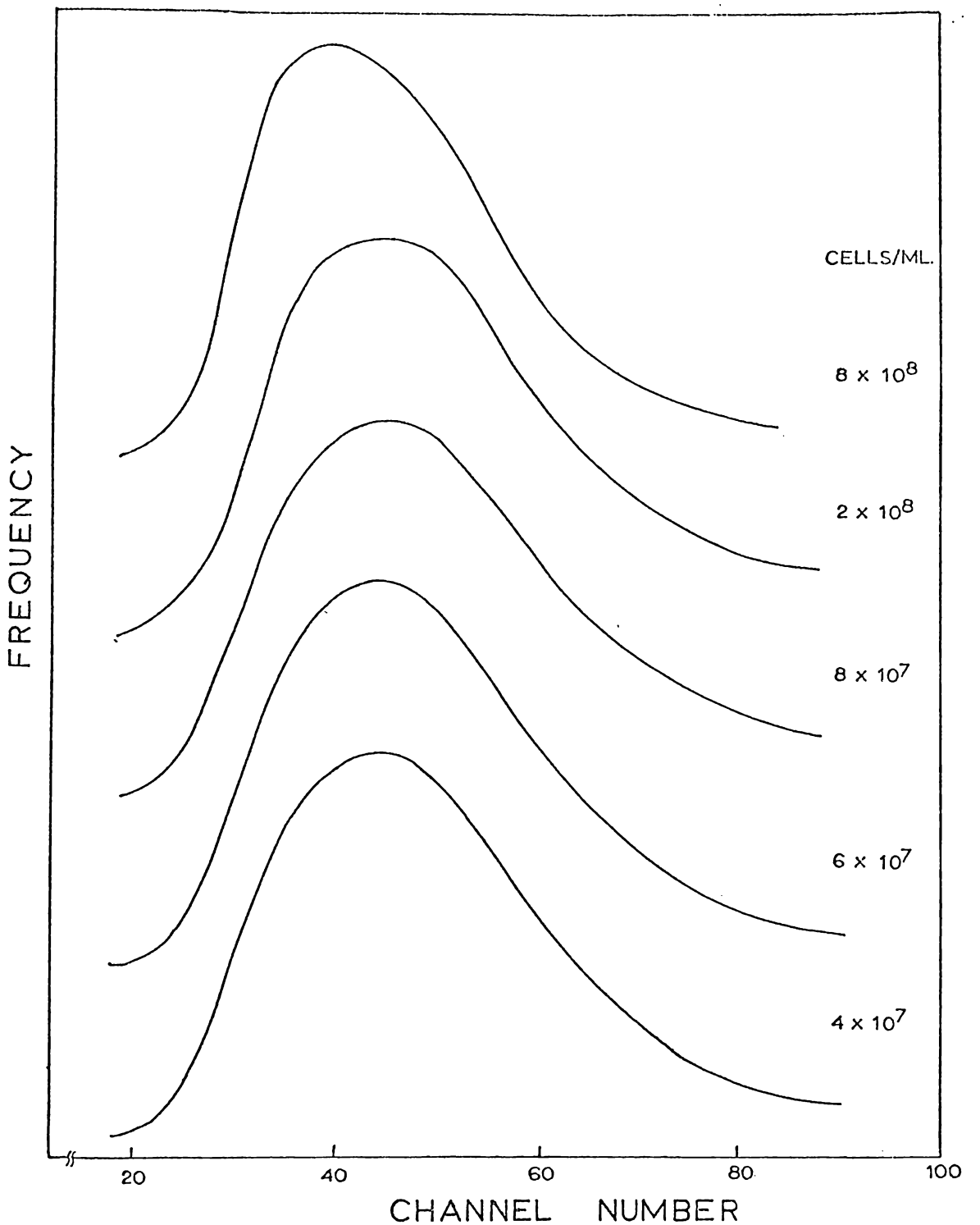


Fig. 4.19 Cell volume distributions at the concentrations indicated in batch cultures of *E. coli* B growing in glucose-salts medium at 37°C.

in rate of increase until a relatively high cell density is reached while a reduction in cell volume may be occurring.

The Coulter counter-multichannel analyzer system was used also to test the efficiency of the gradient centrifugation procedure in separating the cells with respect to their sizes. From a parent culture of *E. coli* B at a density of 2×10^8 cells/ml, 8×10^9 cells were layered on a 5-30% sucrose gradient. After centrifugation, a broad band had formed about half the distance down the gradient. Four fractions were withdrawn from this band at equally spaced intervals from the top to the base. Approximately 0.5% of the cells loaded were removed from the upper region of the band and each of the other fractions constituted about 0.3%. The volume distributions for these fractions are depicted in Fig. 4.20 where we have included, also, the volume distribution of cells in the parent culture. At least 1.5×10^4 cells were sized for each distribution; the distributions have not been mutually normalised to any particular value. The banding procedure has clearly isolated the smaller cells from the parent culture and a marked shift in the mean size of the cells is evident as we go deeper into the band. The resolution, however, falls off for samples taken from progressively deeper regions. The distribution of sizes for the sample taken from the bottom of the band approximates, in fact, that of the parent culture. Wall effects may be important in contributing to this loss in resolution for the deeper samples. Appreciable amounts of cellular material will impinge on the tube walls during centrifugation (Schumaker, 1967) where aggregation of cells may occur with later release into suspension. Kubitschek

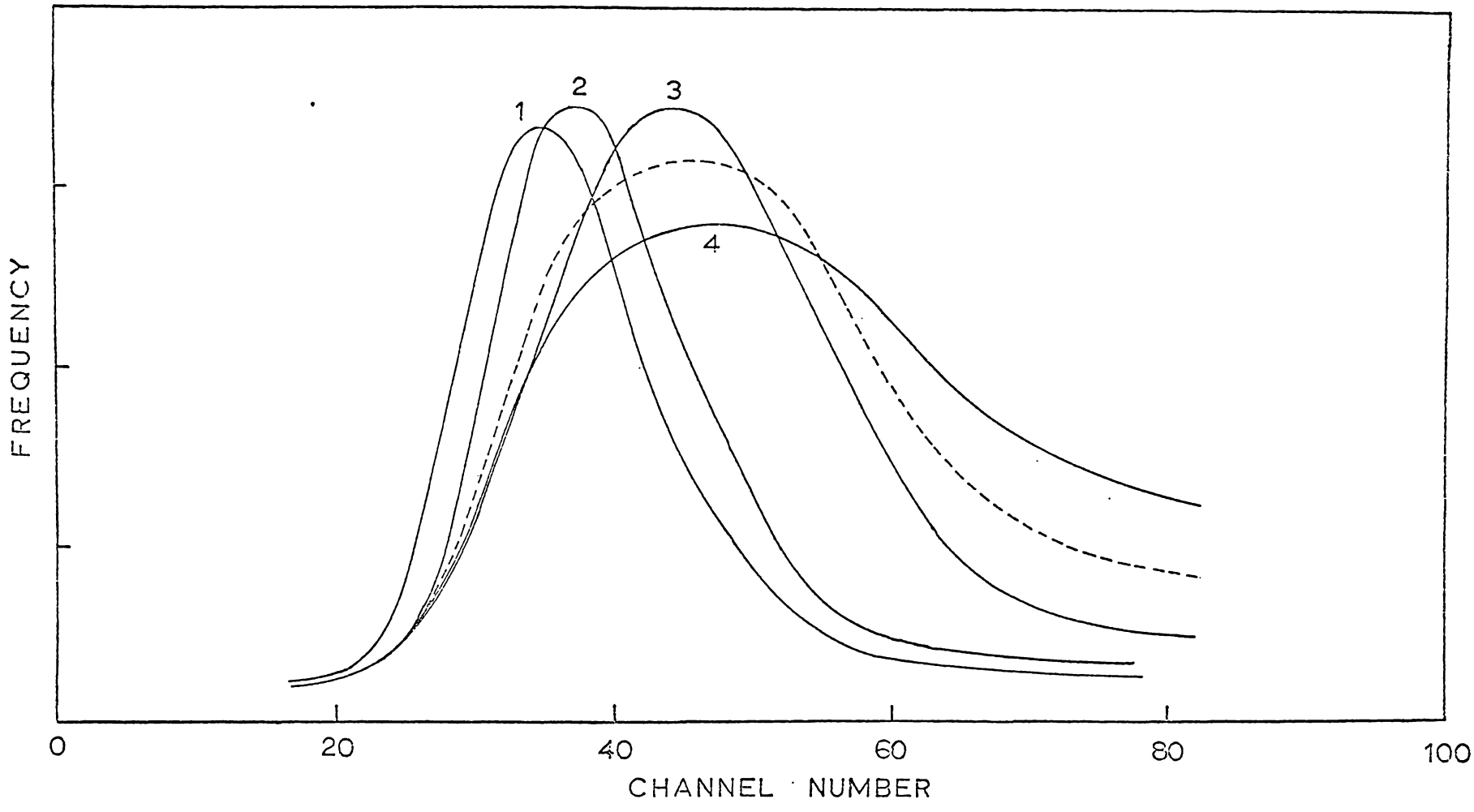


Fig. 4.20 Cell volume distributions for four successive samples removed from a sucrose gradient. The distribution of cell volumes in the parent culture is shown also (dashed line). Channel number is proportional to cell volume.

(1968b) has found evidence of cell clumps in sucrose gradients at the deeper levels of the band which could clearly contribute to the increased dispersion of the deeper samples. A further possible explanation for this loss in resolution is the fact that the larger cells are more rod-shaped (Kubitschek, Bendigkeit, & Loken, 1967).

Some idea of the quality of the separation indicated in Fig. 4.20 can be gained from two approaches. First, we can look at the shift in mean volume. The fraction labelled '1' should approximate the distribution of birth volumes in the parent culture. A calculation puts the mean volume $\bar{V}_b$ of this distribution at about channel 38. Similarly, the mean volume $\bar{V}$ of the parent culture coincides approximately with channel 50. The ratio $\bar{V}_b/\bar{V}$ is therefore 0.76. If we assume that cell size increases linearly with time from birth to division and that there is no variability in cell volume at birth or at division, then (Cook & James, 1964)

$$\bar{V}_b = \bar{V} \ln 2$$

i.e.

$$\bar{V}_b/\bar{V} = 0.69$$

For exponential growth of single cells the ratio becomes

$$\bar{V}_b/\bar{V} = 0.73$$

Results presented by Kubitschek *et al.* (1967) showing various fractions taken from a band of *E. coli* cells in a sucrose gradient indicate a ratio of 0.78. These estimates, although rather crude, suggest that satisfactory separation is being achieved. As a second approach, we can make a direct

comparison of our Fig. 4.20 with the distributions of birth volumes and extant cells obtained for *E. coli* strain B/r/1 by Marr *et al.* (1969). Their results are reproduced in Fig. 4.21. These workers used a modified Coulter counter-pulse height analyzer system to estimate these distributions. Their distribution of birth volumes was obtained from a sample of the effluent stream from a culture growing on a membrane. The majority of these cells would have just been formed by division*. The distribution of birth volumes obtained by Marr *et al.* has a coefficient of variation (CV) of approximately 0.16. Instrumental distortion could contribute to the higher value (CV = 0.22) obtained from Fig. 4.20 for fraction '1'. Comparison of Figs. 4.20 and 4.21 indicates that the starting culture obtained by the gradient-centrifugation method is a suitable sample of young cells. The ratio $\bar{V}_b/\bar{V}$ inferred from Fig. 4.21 is in the range 0.76 to 0.80; it should be noted, however, that since newborn cells are variable in size these comparisons with the distribution of newborn cells of Fig. 4.21 cannot be more than superficial.

In the synchronous culture experiments reported here the yield of cells from the gradient lay approximately between 1% and 3% of the cells loaded. An indication that over this range the dispersion in the cell volumes obtained from the upper region of the band was sensibly constant is provided by Fig. 4.22. Three different fractions are shown amounting respectively to 0.3%, 1.5%, and 4.5% of the cells

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*See the discussion on the membrane-selection technique in Section 3.3.

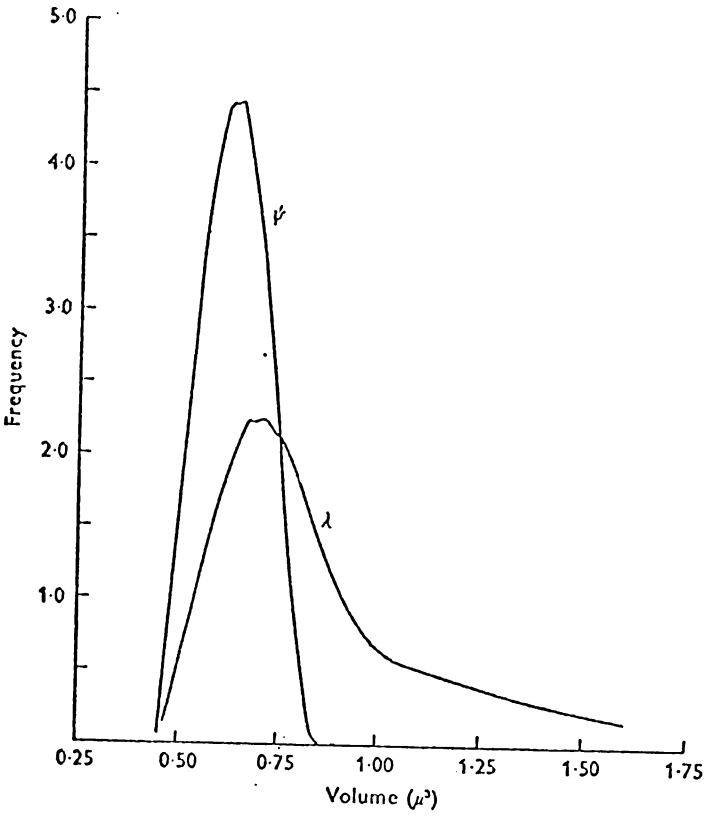


Fig. 4.21 Frequency functions of the distribution of volume of newly formed cells (ψ) and extant cells (λ) of *E. coli* strain B/r/1 growing in glucose minimal medium at 30°C. From Marr, Painter & Nilson (1969).

loaded. For this demonstration, 5×10^9 cells from a parent culture (6×10^7 cells/ml) of *E. coli* B were loaded on a 5-30% sucrose gradient and centrifuged until the band had moved one-half of the distance down the gradient. A slightly higher proportion of larger cells appears to be included in the 4.5% fraction although the dispersion is no greater for this fraction than the others. It is likely that disturbance of the gradient and the fact that 1.8% of the cells had already been removed would contribute to this slight shift in mean volume for the large fraction.

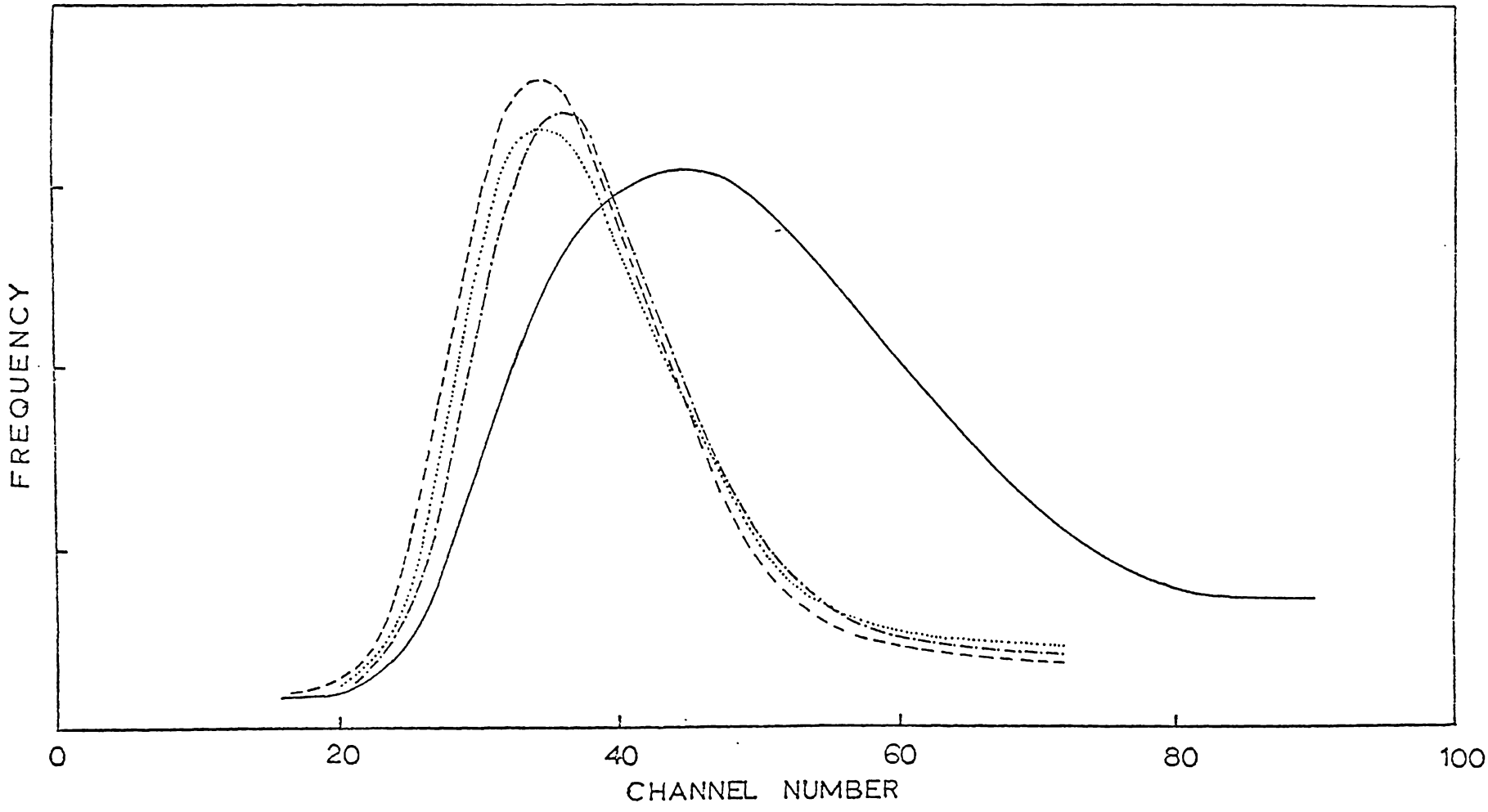


Fig. 4.22 Cell volume distributions for three fractions taken from the top of a sucrose gradient corresponding to 0.3% (---), 1.5% (....), and 4.5% (-·-) of the cells loaded. The solid line indicates the distribution of cell sizes in the parent culture. Channel number is proportional to cell volume.

The Coulter counter-multichannel analyzer system is invaluable for studying synchronous cell cultures, where both numbers and size distributions are required simultaneously for maximum information. Periodic increases in cell numbers are only one criterion for synchrony and a concomitant change in cell size must also be observed. While size distributions were not recorded for all experiments, observation of the pulse display on the Coulter counter readily indicates whether selection had taken place. The subsequent variation in average pulse height reflects the growth in cell mass during synchrony and indicates that the cells are growing in a normal manner while not necessarily always increasing in number. Cell volume distributions taken at intervals of six minutes throughout experiment E (Fig. 4.9e) are shown in Fig. 4.23. This figure demonstrates very effectively the periodic changes in cell size which accompany the cell number changes. While initially the synchronous culture exhibited a unimodal cell size distribution, a second peak emerged as small cells formed by fission of cells in the original peak. Following this bimodal distribution at 43 minutes, similar bimodal curves occur at 85, 127 and 170 minutes. More than four generations of growth are covered in the illustration and significant synchrony is clearly retained throughout this period. The monotonic increase in cell volume (or mass) from the average size at birth to twice that size at division, through each generation, is clear. The shoulder on the right-hand tail of the distribution taken at 49 minutes would be expected to correspond roughly to the average size of dividing cells. Similarly, the left-hand shoulder of the distribution

at 79 minutes is expected to correspond to the average size of newborn cells. The former peak is centred on a volume approximately twice that of the latter, as expected. The size distribution of the parental culture is also illustrated in Fig. 4.23 and this establishes a scale for the location of cells within the interdivision cycle as a function of cell volume.

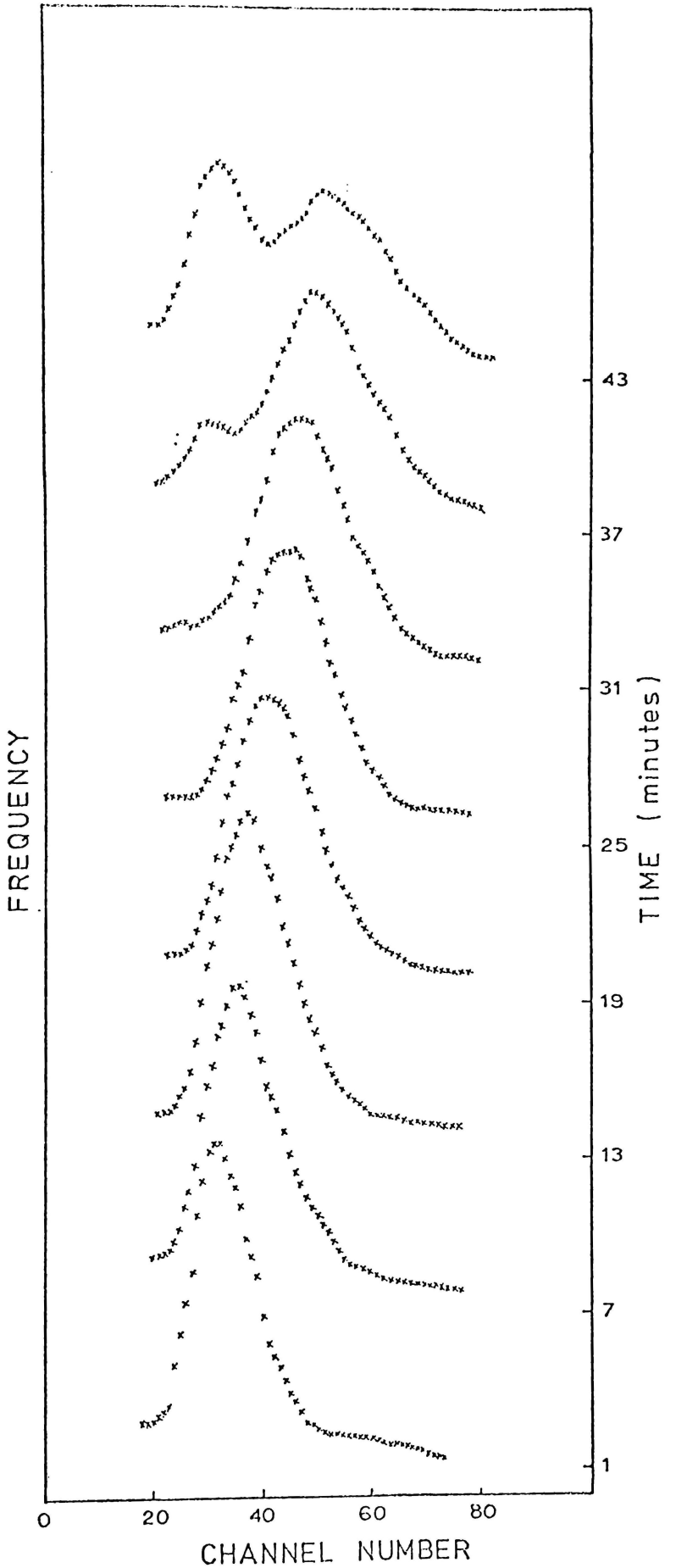
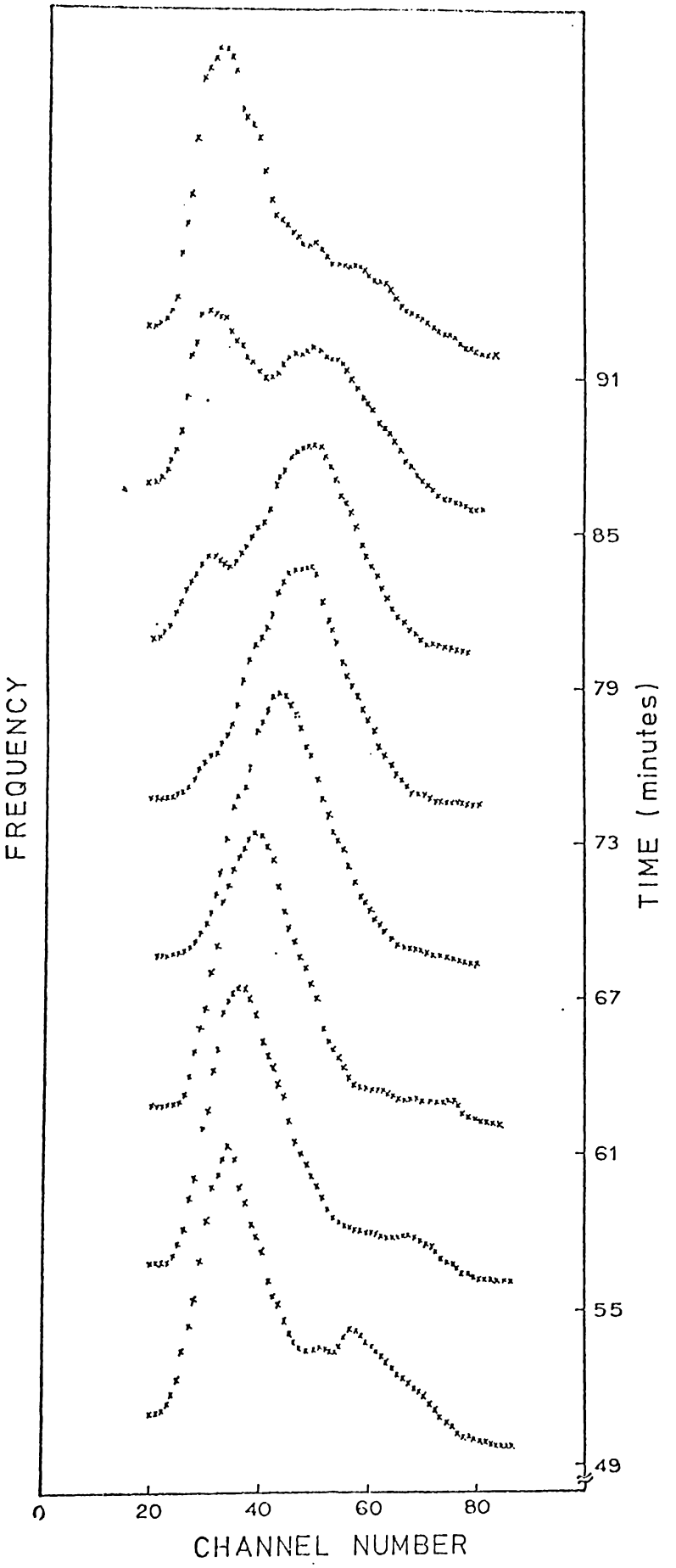
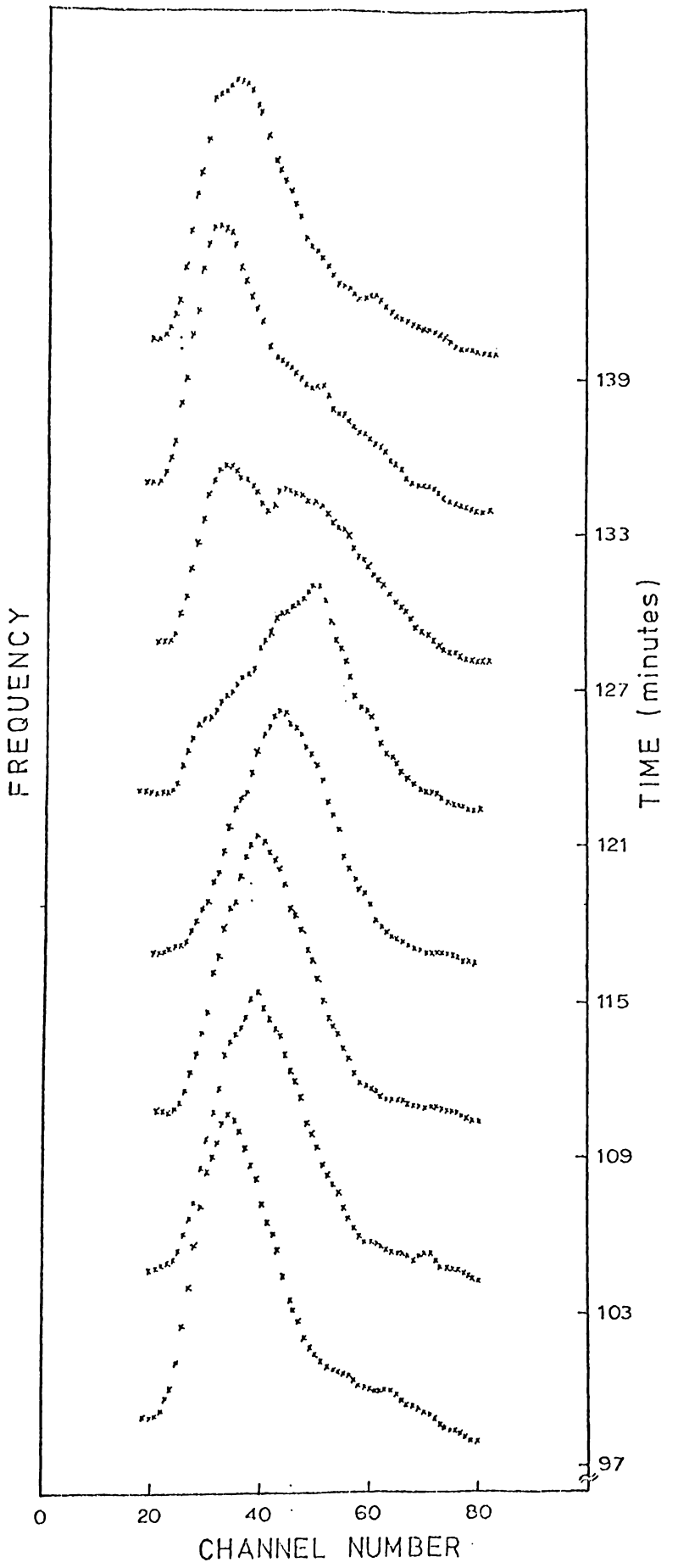
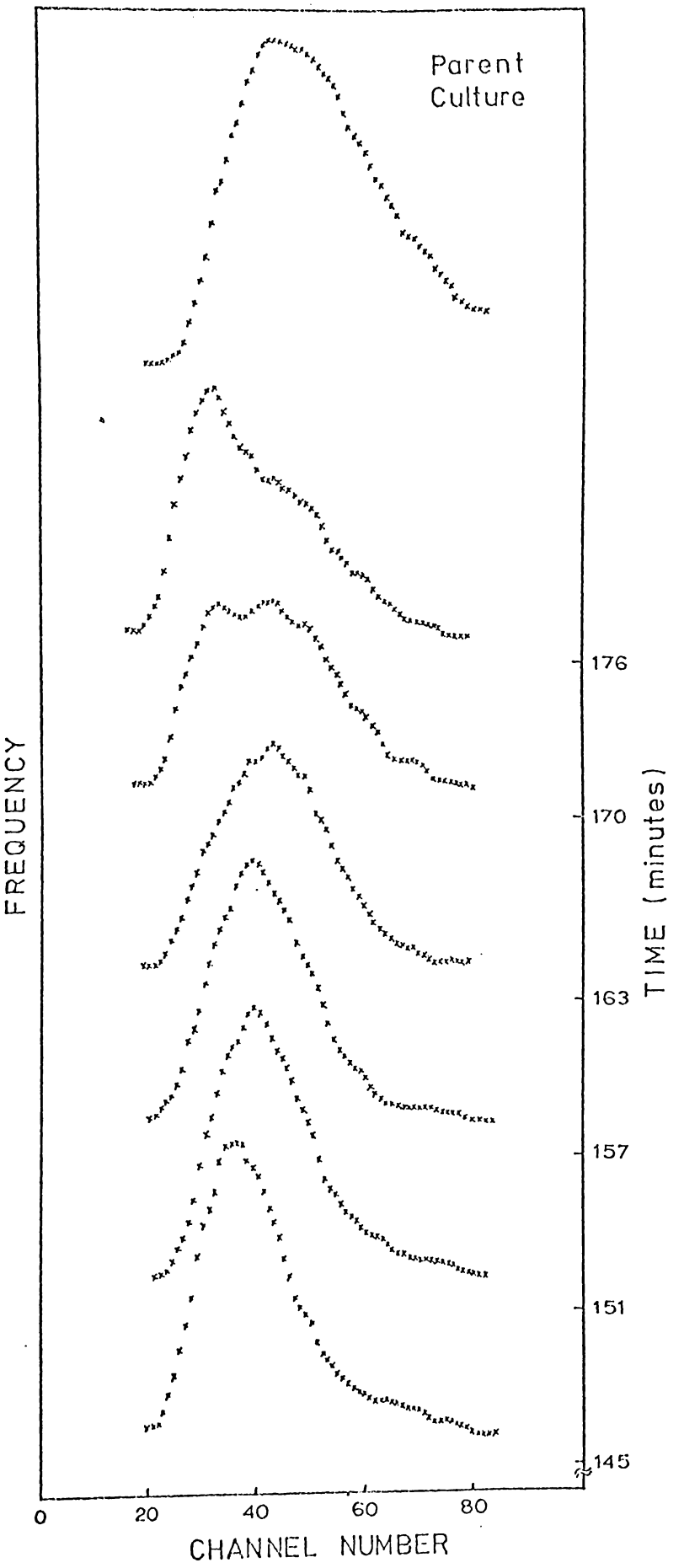


Fig. 4.23 Cell size distributions for a synchronous culture at 37°C (experiment E). Times at which samples were taken are indicated on the right. Channel number is proportional to cell volume.


FREQUENCY
91
85
79
73
67
61
55
49
TIME (minutes)
0
20
40
60
80
CHANNEL NUMBER


FREQUENCY
TIME (minutes)
139
133
127
121
115
109
103
97
0
20
40
60
80
CHANNEL NUMBER


Parent
Culture
FREQUENCY
176
170
163
157
151
145
TIME (minutes)
0
20
40
60
80
CHANNEL NUMBER

CHAPTER 5

DATA ANALYSIS

5.1 INTRODUCTION

We have intimated in the introduction to this thesis how the patterns of growth exhibited by synchronous cultures may provide information on the distribution of generation times of the cells and correlations between the generation times of parent and progeny cells. Before proceeding to a mathematical development for the growth of synchronous cultures some intuitive observations are useful at this stage concerning the features of the synchronous growth patterns depicted in the previous chapter and the sort of information that can be gleaned from these curves.

We consider a culture at zero time consisting only of newborn cells. It is clear that if all of these cells have identical generation times then they will all divide simultaneously and we have a perfectly synchronous culture. Furthermore, such synchrony will persist indefinitely in an ideal situation where overcrowding, nutrients, etc., are not limiting. Such a dispersionless generation time distribution will lead to a growth curve for the synchronous culture which is a series of perfect steps, as shown in Fig. 5.1. Here, all the cells divide at time T_1, again at time T_2, and so on. However, in biological systems, individual cells exhibit some variability in their interdivision times. If this is the case and we adhere to a strict sense of the word

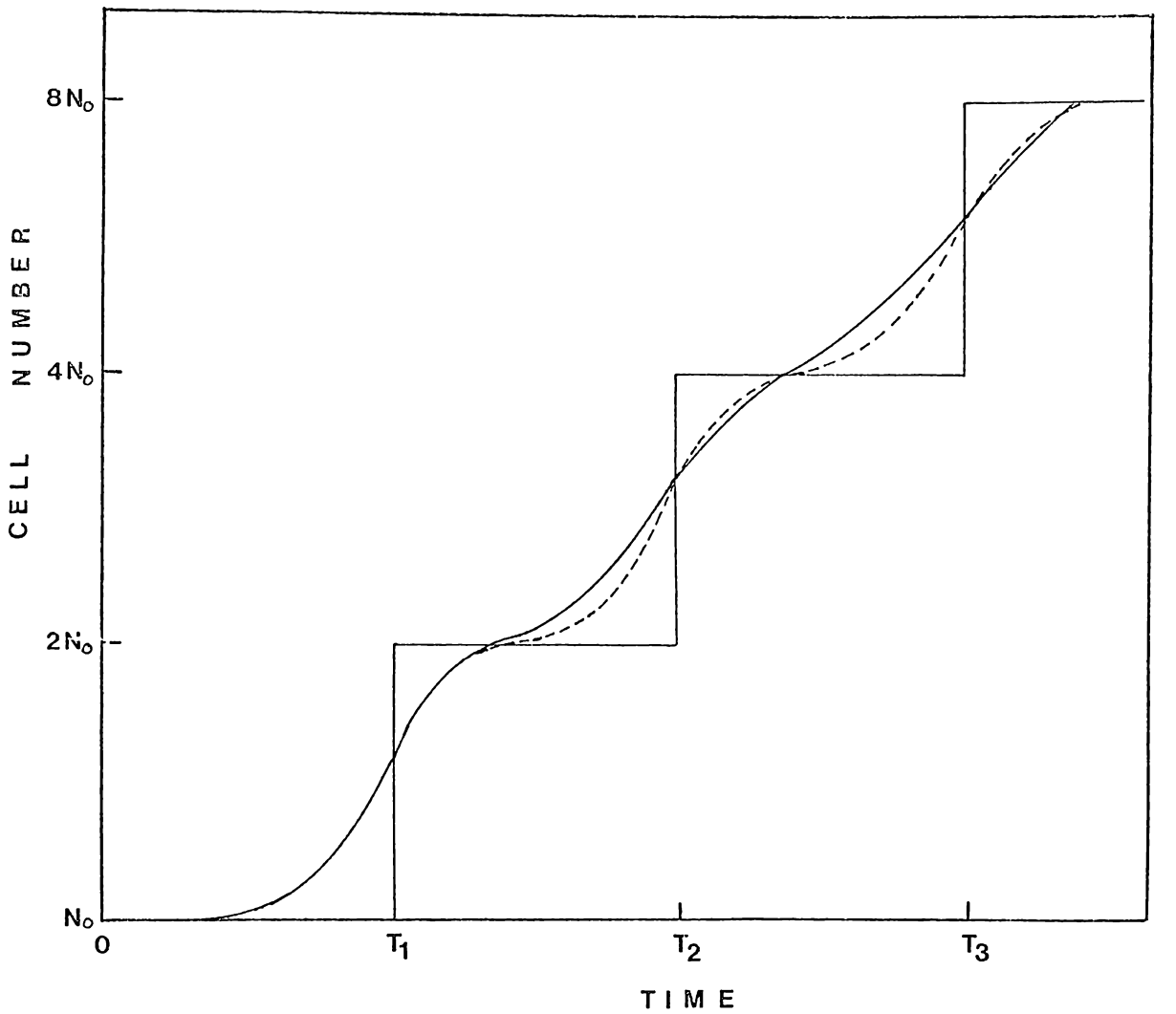


Fig. 5.1 Patterns of growth of cultures with N_0 newborn cells at zero time. Three cases are illustrated:

(1) no variability in the generation times of individual cells (perfect step function),

(2) non-zero dispersion in the generation time distribution and independence of mother and daughter generation times (solid curve),

(3) as for (2), with generation times of mother and daughter cells negatively correlated (dashed curve).

'synchronous', meaning simultaneous, our culture will only be synchronous at zero time. As time advances the cells get progressively out of phase with each other. While there will be a mean generation time characterising the distribution of the individual cell generation times, some cells will divide early and some late and the first step in the growth curve will be smoothed out to some degree (Fig. 5.1). The shape of this smooth increase in cell numbers during the first phase of divisions will obviously reflect the shape of the underlying distribution of generation times. While more cells will divide at T_1 than at any other time during this first phase, T_1 being the instant when the rate of increase of cell numbers is greatest, it does not follow that the mean of all the individual generation times is T_1. Indeed, the majority of cells could have generation times greater than T_1, in which case the mean generation time would also exceed T_1. We have drawn a symmetrical 'S' or sigmoid shaped curve about T_1, implying a symmetrical generation time distribution. If, now, each of these first generation or mother cells gives rise to two daughters which have generation times in no way related to that of their mother it is intuitively clear that the times of division of these second generation cells will be spread over a much broader range than that observed for the first generation. The condition of complete asynchrony in the population, manifested by a straight line, indicating exponential growth, on the semilogarithmic scales in Fig. 5.1, will now be approached fairly rapidly (albeit, asymptotically). If the underlying generation time distribution is fairly broad two or three generations of growth will be all that is necessary before an

essentially asynchronous condition is attained.

Imagine, now, a situation where a negative correlation exists between the generation times of the mother cells and those of their daughters. In other words, mothers which have generation times longer than average tend to give rise to daughters which have generation times shorter than the average, and vice-versa. This compensatory tendency will result in a tightening or narrowing of the distribution of times at which the second generation or daughter cells divide compared to that observed had generation times of daughters been independent of those of their mothers. The dashed line in Fig. 5.1 illustrates the effect. In this way some degree of synchrony could persist for many generations of growth. If, on the other hand, mothers with long or short generation times tend to give rise to daughters also with long or short generation times, respectively, then asynchrony would be washed out more rapidly than if no association existed between mother and daughter cell generation times. In the extreme case, where perfect positive correlation ($\rho = 1$) exists, the daughter cells would have precisely the same generation time as that of their mothers.

The effect of correlations between the generation times of sister cells should also be mentioned. We can consider an imaginary experiment (Harvey, 1972a) in which all the pairs of sister cells formed by division of the first generation cells are separated to form two new cultures. As a consequence of the large number of cells in the cultures we are dealing with, each of these new cultures will exhibit exactly similar

behaviour as time progresses. Likewise, mixing the two cultures together will have no effect on the pattern of growth. Thus, large population synchronous cultures will not yield any information on the correlations between sister cell generation times.

We now wish to put on a firm mathematical footing the way in which the variability in generation times of cells and the correlations between the generation times of related cells influence the patterns of growth in an initially synchronous culture. In doing so, we see how these statistical parameters can be readily extracted from the growth data. A general mathematical description of the growth of such synchronously dividing cultures has been presented by Harvey (1972a) and this development is followed in the next section.

5.2 MATHEMATICAL DESCRIPTION OF SYNCHRONOUS GROWTH

In Fig. 5.2 we illustrate schematically the process of growth from a single individual, newborn at time $T = 0$, as time progresses. This is a binary branching process where each cell gives rise to two new cells at the time of its division. In a real synchronous culture we start at zero time with a large number of newborn cells and each of these gives rise to a 'tree' of the sort depicted. The length of each horizontal line is proportional to the interdivision time or generation time of that particular cell. We have labelled each of these lines with a number, *e.g.* a_{jn}, representing the interdivision time of the n^{th} cell of the j^{th} generation cells. For the j^{th} generation cells, these

numbers are values taken by the variable t_j, a random variable representing the interdivision time of a jth generation cell. We can also write down the times, beginning from $T = 0$ when all cells were newborn, at which individual cells divide. Thus, the individual with interdivision time a_{11} will divide at time $T = A_{11} = a_{11}$, as shown in Fig. 5.2. All the other first generation cells will divide at times at or near this value and this distribution of times will clearly be the generation time distribution of the cells. We let T_1 denote the random variable which takes the values of these division times and we can write $T_1 = t_1$. Similarly, the second generation cells will divide at times A_{21}, A_{22}, and so on for all the second generation organisms in the culture. T_2 is the variable representing these times, and, clearly $T_2 = t_1 + t_2$. The distribution for T_2 can easily be found if the interdivision times t_1 and t_2 are independent. However, we are interested in the general case where the times t_2 may be influenced in some way by the times t_1.

It should also be noted that the distribution of T_1 corresponds to the generation time distribution of the cells only if the initial newborn cells were collected in a random way such that they are free of any influence of the generation times of their mothers. In other words, had they been collected at some instant of time from an exponentially growing culture a biased sample of generation times would result. We have explained already in Chapter 2, in connection with real and artificial distributions of generation time, that this is because a sample of newborn cells at this instant of time in an exponentially growing

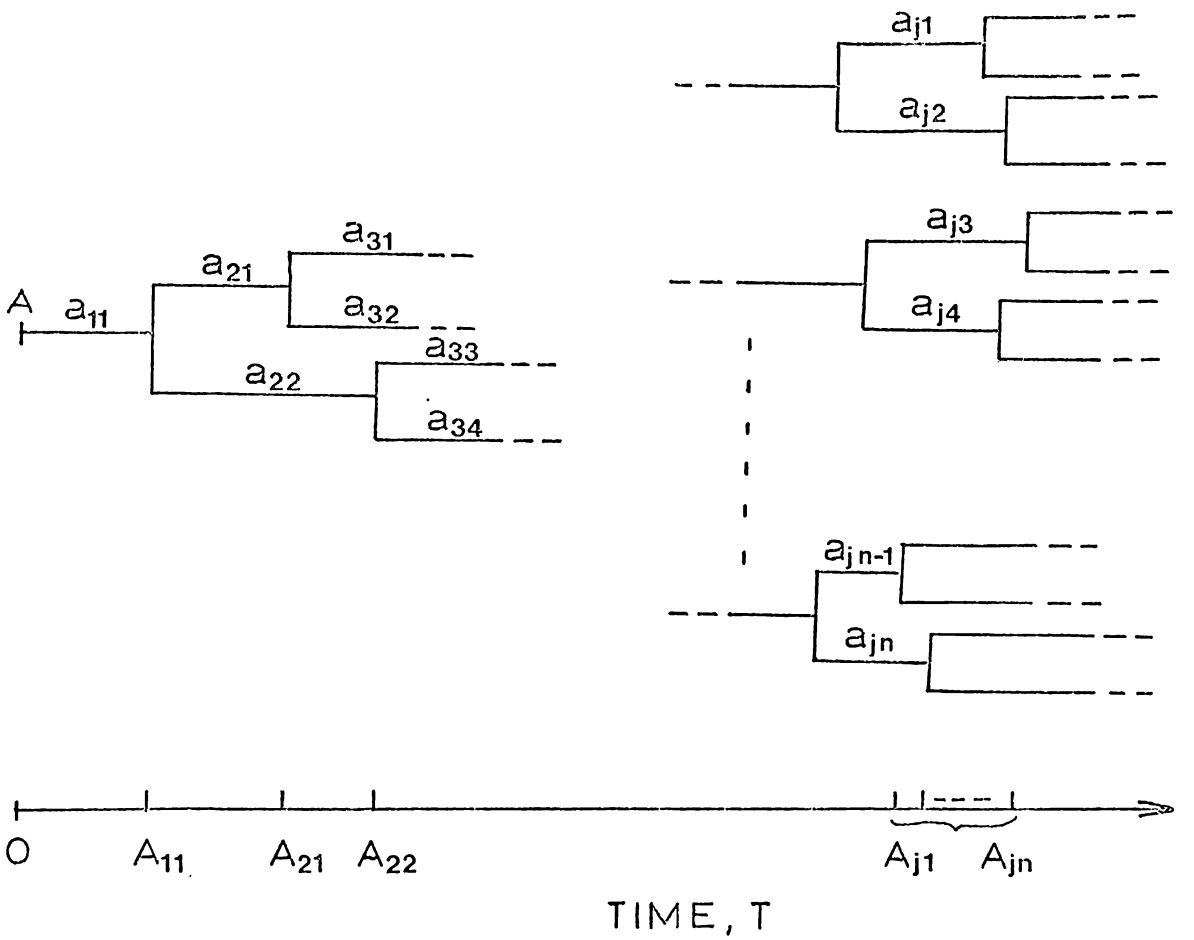


Fig. 5.2 Family tree arising from a single organism A, newborn at T = 0, illustrating notation for division times and interdivision times.

population will contain more cells with short lived mothers than cells with long lived mothers.

We let $G_1(T_1)$ represent the distribution (frequency function) of times T_1. In general,

$$T_j = t_1 + t_2 + \ldots + t_j \qquad (5.1)$$

and we denote the distribution of times T_j by $G_j(T_j)$. We assume, of course, that these distributions satisfy the

normalisation condition,

$$\int_o^\infty G_j\,(t)\,dt = 1\,. \qquad (5.2)$$

This merely expresses the certainty that a jth generation cell will divide at some time after inception of the culture. The probability that a j^{th} generation cell will divide before time T is given by $\int_o^T G_j\,(t)\,dt$. We assume that the culture is established initially with N_o newborn first generation cells. Then, after a time T has elapsed, $N_o \int_o^T G_1\,(t)\,dt$ of these cells will have divided. That is, the number of first generation cells at any time T will be given by

$$N_1\,(T) = N_o\,(1 - \int_o^T G_1\,(t)\,dt)\,. \qquad (5.3)$$

Each of these cells gives rise to two new second generation cells. The number of second generation cells present at any time T in the culture will result from the difference of two terms:

(i) the number of first generation cells that have divided up to this time, $N_o \int_o^T G_1\,(t)\,dt$, multiplied by a factor of 2; and

(ii) the number of second generation cells that have divided up to this time, $2\,N_o \int_o^T G_2\,(t)\,dt$, there being $2\,N_o$ second generation cells produced in the culture.

Thus,

$$N_2\,(T) = 2\,N_o\,\{\int_o^T G_1\,(t)\,dt - \int_o^T G_2\,(t)\,dt\}\,. \qquad (5.4)$$

In general, the total number of j^{th} generation cells ($j > 1$) at any time T will be given by*

$$N_j(T) = 2^{j-1} N_o \left\{ \int_o^T G_{j-1}(t)\, dt - \int_o^T G_j(t)\, dt \right\}. \qquad (5.5)$$

Adding all the contributions, the total number of cells in the culture at any time T will be

$$\begin{aligned} N(T) &= \sum_{j=1}^{\infty} N_j(T) \\ &= N_o \left\{ 1 + \sum_{j=1}^{\infty} 2^{j-1} \int_o^T G_j(t)\, dt \right\}. \end{aligned} \qquad (5.6)$$

We see immediately from equation (5.6) that, if the distributions $G_j(t)$ are peaked at different times, a series of steps will result when we plot the number of cells in the synchronous culture against time. By differentiating this expression with respect to time T we can obtain a relation involving the distributions $G_j(t)$ rather than the cumulative distributions $\int_o^T G_j(t)\, dt$. That is,

$$\begin{aligned} \frac{dN(T)}{dT} &= N_o 2^{j-1} \sum_{j=1}^{\infty} G_j(T) \\ &= N_o \sum_{j=1}^{\infty} F_j(T) \end{aligned} \qquad (5.7)$$

where we put $F_j(T) = 2^{j-1} G_j(T)$. The first term in the sum (5.7) is related to the generation time distribution of the cells in the culture, $G_1(T)$, which we denote by $f(T)$.

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*The corresponding result in Harvey (1972a), equation (4) of that paper, is reproduced incorrectly.

We can now consider the effects of correlations between the generation times of parent and progeny cells and establish how these correlations can be determined from the functions $F_j(T)$.

Considering first the mother-daughter generation time correlation, we introduce the joint (normalised) distribution $H(t_1, t_2)$ of the generation times of the mother (t_1) and the daughter (t_2) cells. The marginal distributions of t_1 and t_2 will, of course, both be the generation time distribution, *i.e.*

$$\int_0^\infty H(t_1, t_2)\, dt_1 = f(t_2) \qquad (5.8a)$$

$$\int_0^\infty H(t_1, t_2)\, dt_2 = f(t_1) . \qquad (5.8b)$$

The distribution of the sum of the random variables t_1 and t_2 is, of course, $G_2(T)$, and may be expressed in terms of the joint distribution function as*

$$G_2(T) = \int_0^T H(t, T-t)\, dt . \qquad (5.9)$$

We wish to find the mean and variance of this distribution. In terms of $F_2(T)$ we have

$$\text{mean} = E(T = t_1 + t_2) = \tfrac{1}{2} \int_0^\infty T F_2(T)\, dT , \qquad (5.10)$$

$$\text{variance} = \text{var}(T) = \tfrac{1}{2} \int_0^\infty \{T - E(T)\}^2 F_2(T)\, dT \qquad (5.11)$$

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*See, for example, Fisz (1963), p.59.

where we realise that the F_j (T) obey the normalisation condition

$$\int_o^\infty F_j (T)\, dT = 2^{j-1} . \qquad (5.12)$$

But, by definition,

$$E (t_1 + t_2) = \int_o^\infty \int_o^\infty (t_1 + t_2)\, H (t_1, t_2)\, dt_1\ dt_2$$

$$= \int_o^\infty t_1 \int_o^\infty H (t_1, t_2)\, dt_2\, dt_1$$

$$+ \int_o^\infty t_2 \int_o^\infty H (t_1, t_2)\, dt_1\, dt_2 -$$

$$= \int_o^\infty t_1 f (t_1)\, dt_1 + \int_o^\infty t_2 f (t_2)\, dt_2$$

$$= E (t_1) + E (t_2)$$

$$= 2 \tau$$

and,

$$\text{var} (t_1 + t_2) = E \{(t_1 + t_2) - E (t_1 + t_2)\}^2$$

$$= E \{(t_1 + t_2)^2\} - \{E (t_1 + t_2)\}^2$$

$$= E (t_1^2) - \{E (t_1)\}^2 + E (t_2)^2$$

$$- \{E (t_2)\}^2 + 2 E (t_1 t_2)$$

$$- 2 E (t_1) E (t_2)$$

$$= \text{var} (t_1) + \text{var} (t_2)$$

$$+ 2 \text{cov} (t_1, t_2)$$

$$= 2 \sigma^2 + 2 \sigma^2 \rho$$

where ρ is the coefficient of correlation between t_1 and t_2. In our usual notation, the mean and variance of the generation time distribution f (t) have been denoted τ and σ^2, respectively. In terms of the joint distribution $H(t_1, t_2)$ we have

$$\sigma^2 \rho = \text{cov}(t_1, t_2)$$

$$= \int_0^\infty \int_0^\infty (t_1 - \tau)(t_2 - \tau) H(t_1, t_2) \, dt_1 \, dt_2. \qquad (5.13)$$

Equations (5.10) and (5.11) now become

$$\tfrac{1}{2} \int_0^\infty T F_2(T) \, dt = 2\tau \qquad (5.14)$$

and

$$\tfrac{1}{2} \int_0^\infty (T - 2\tau)^2 F_2(T) \, dT = 2\sigma^2 (1 + \rho). \qquad (5.15)$$

Independence of the generation times of mother and daughter cells ($\rho = 0$) leads to a standard deviation in $F_2(T)$ of $\sqrt{2}\,\sigma$, while positive or negative values of the correlation coefficient arise when $F_2(T)$ is correspondingly broadened or narrowed, respectively. When $\rho = -\tfrac{1}{2}$, the standard deviation of $F_2(T)$ will be equal to that of the generation time distribution. If we can determine the variance of each of the functions F_1 and F_2, equation (5.15) will provide a value for ρ.

By introducing the joint distribution of the generation times for the first j generations we can extend the above

treatment to correlations covering more than one generation. The mean of the function $F_j(T)$ will clearly be given by

$$2^{1-j} \int_0^\infty T F_j(T)\, dT = j\tau \ . \qquad (5.16)$$

The general expression for the variance

$$\sigma_j^2 = 2^{1-j} \int_0^\infty (T - j\tau)^2 F_j(T)\, dT \qquad (5.17)$$

has been given by Harvey (1972a). We simply note the result for $F_3(T)$, *viz.*,

$$\sigma_3^2 = 3\sigma^2 + 2\sigma^2 (2\rho^{(1)} + \rho^{(2)}) \qquad (5.18)$$

where we have denoted the mother-daughter generation time coefficient by $\rho^{(1)}$ and the grandmother-granddaughter generation time correlation coefficient by $\rho^{(2)}$*.

The analysis to this point has assumed that the culture is perfectly synchronized at time $T = 0$. Harvey (1972a) considered the effects of imperfect synchronization by imagining that new organisms are introduced over a period of time with a certain distribution having variance σ_o^2. This situation would essentially apply to the gradient-centrifugation procedure when the fraction of smallest cells selected exhibits some dispersion in cell ages. The effect of this imperfect selection is to add the constant, σ_o^2, to the variances of the $F_j(T)$. Explicitly,

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In the Introduction to this thesis these coefficients were denoted $\rho(H)$ and $\rho(H_2)$, respectively, in common with the usual notation in the literature.

$$\sigma_1^2 = \sigma^2 + \sigma_o^2 \tag{5.19}$$

$$\sigma_2^2 = 2\sigma^2(1 + \rho^{(1)}) + \sigma_o^2 \tag{5.20}$$

$$\sigma_3^2 = 3\sigma^2 + 2\sigma^2(2\rho^{(1)} + \rho^{(2)}) + \sigma_o^2 \tag{5.21}$$

and so on. Thus, experimental determinations of the σ_i provide unique values of σ, $\rho^{(1)}$ and $\rho^{(2)}$, only if σ_o is known or neglected. As Harvey (1972a) points out it is definitely preferable to reduce σ_o to a negligible value by experimental design.

Summarising, equation (5.7) expresses the time derivative of the total number of cells in a synchronous culture in the form of a series of functions having properties related to those of the generation time distribution of individual cells and to the correlations between the generation times of these cells. The synchronous culture growth curves that we have found can therefore yield, in principle, these correlation coefficients and the generation time distribution. A numerical differentiation will be required, and, as the concentration measurements are subject to experimental errors, the series of points will require smoothing before this differentiation can be carried out.

5.3 DATA SMOOTHING AND DIFFERENTIATION

We expect, on physical grounds, that in a system containing a large number of cells the concentration should vary smoothly with time. In practice, the concentration measurements have associated experimental errors and some scatter is observed in the series of points obtained. Without adequate smoothing these experimental errors will strongly influence the results of a numerical differentiation carried out on the data points.

The two methods of smoothing considered by Harvey (1972b) were a polynomial curve fitting approach and a digital filtering approach. In the former, a least squares fit to the data points is performed with a polynomial curve. It has been pointed out by Harvey (1972b) that the choice of polynomial used can introduce a subjective bias.

The second approach (reviewed by Wood, 1968) is a numerical analogy to the operation of an electrical low pass filter in which all components, of an oscillating input voltage, with wavelengths less than some cut-off wavelength would be rejected. In fact if such a voltage exhibited in the time domain precisely all the fluctuations of the concentration curve we could then obtain a smoothly varying signal by passing such a voltage through a low pass filter having a cut-off wavelength of a few data point spacings. In the frequency domain, the operation essentially involves multiplication of the Fourier components of the input wave form with the transfer function of the filter (in this case,

a step function). Equivalently, we can view the filtering operation in the time domain as the convolution of the input wave form with the Fourier transform of a step function*, *viz.*, sin (ax)/x. We can perform this convolution numerically with the observed data points as the input signal. To reduce errors due to the discontinuity introduced by the truncation of the data points the convolution was performed with sin(ax)/x multiplied by a Fejér linear weighting function (Papoulis, 1966). This serves to favour linearly the low frequency Fourier components.

Harvey (1972b) has shown that results are obtained with less ambiguity using the digital filtering technique than with the polynomial fitting procedure. The former method is used exclusively in this work.

Following this smoothing operation the derivatives were calculated using a six-point numerical differentiation formula (Henrici, 1964). FORTRAN routines accomplishing these operations are included in the computer listings in Appendix C. Computations were performed on a Burroughs B6700 digital computer.

A typical experimental concentration curve is shown in Fig. 5.3a. The result of smoothing and differentiating according to the procedure outlined produced the curve in Fig. 5.3b, where digital filter cut-off wavelengths of 7, 8 and 9 data point spacings have been used. Over this range

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**See, for example, Papoulis (1962) for a discussion on Fourier transform techniques.*

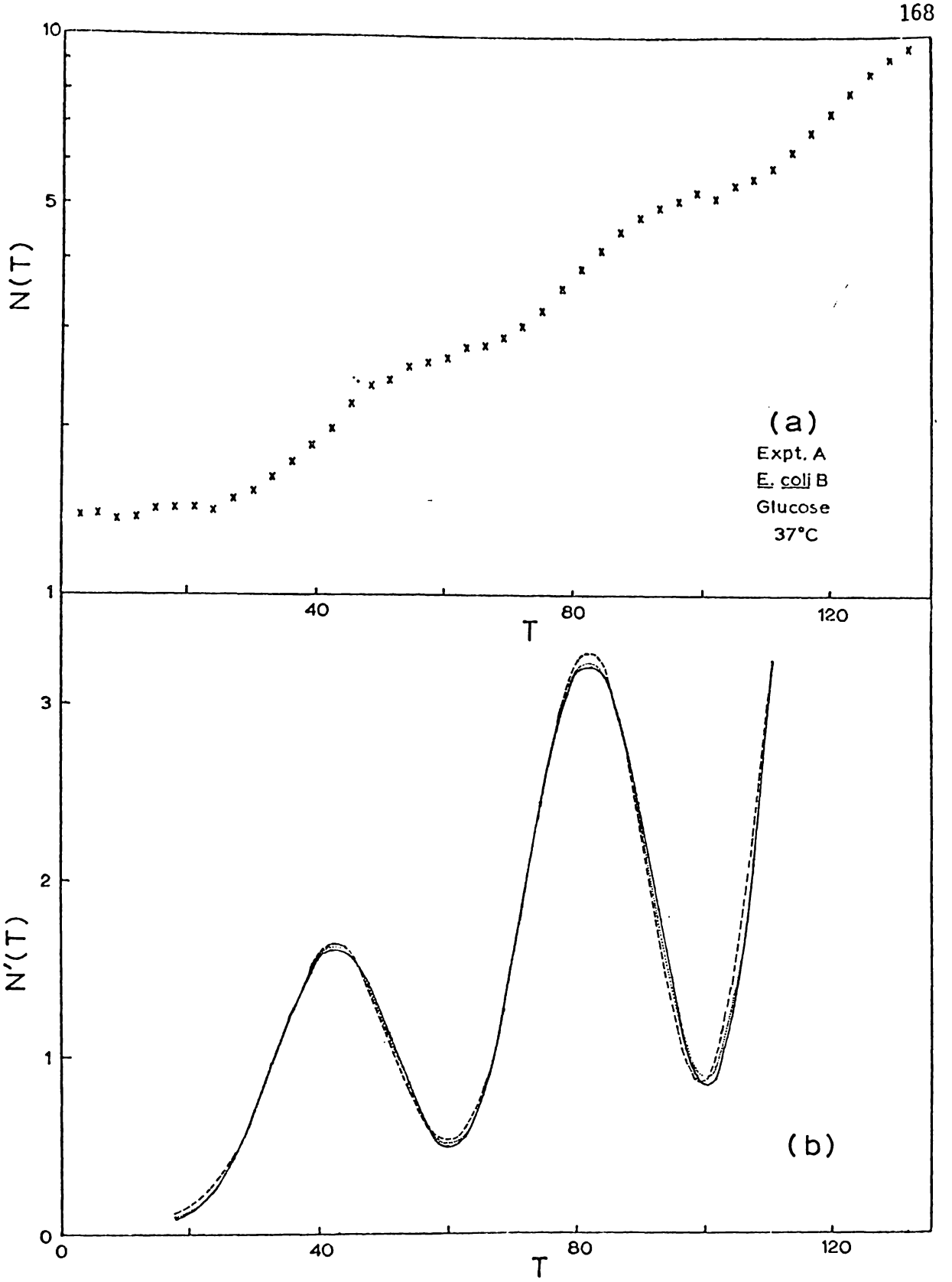

Fig. 5.3 a: Logarithmic plot of the cell concentration in a synchronous culture.

b: Derivative of the cell concentration extracted by digital filtering, with cut-off wavelengths of seven (dashes), eight (dots) and nine (solid line) data point spacings.

Time (T) is measured in minutes and the ordinates are in arbitrary units.

of cut-off wavelengths little change is seen to result in the derivative curves.

5.4 EXTRACTION OF THE PARAMETERS OF THE GENERATION TIME DISTRIBUTION

Fig. 5.3b illustrates the typical form assumed by the functions $F_j(T)$ defined by equation (5.7). The variances of these functions are required in order to compute the parent-progeny generation time correlation coefficients. In regard to the generation time distribution itself, given by $F_1(T)$, two parameters receive predominant mention in the literature. These are the coefficient of variation (CV), a measure of the dispersion of the distribution, and the skewness (α_3) which indicates any departure from symmetry. In our notation,

$$CV \equiv \frac{\sigma_1}{\tau_1} \tag{5.22}$$

and

$$\alpha_3 \equiv \frac{\mu_3}{\sigma_1^3} \tag{5.23}$$

where τ_1, σ_1, and μ_3 are the mean, standard deviation, and third central moment of the distribution. The skewness, α_3, is positive or negative according as the sign of μ_3 is positive or negative, respectively.

It is apparent from Fig. 5.3b that considerable interference exists between the tails of the functions $F_j(T)$. In order to obtain reliable estimates of the moments the straightforward summation procedure used by Harvey (1972b) will be subject to considerable errors. An alternative

method is therefore desirable. Also noticeable from examination of Fig. 5.3b is the lack of any marked skewness in $F_1(T)$ and $F_2(T)$. The Gaussian nature of these peaks immediately suggests fitting a sum of normal frequency functions to the curve. The non-truncated Gaussian function is, of course, defined over the whole real line and its use here may seem questionable on physical grounds. However, because of the negligible values taken at negative times its application here is justified.

A best fit of a sum of Gaussians to the derivative curve will then provide directly the means and variances of the distributions $F_j(T)$. Observation of the fit will also provide some confirmation of the symmetrical nature of these distributions. A nonlinear least squares fitting routine (called RLQF) was obtained* in order to implement such a fit. This routine performs a linearization of the function with respect to the parameters. In other words, the problem is reduced to a linear case by a suitable Taylor series expansion of the function and by considering only the linear terms of this expansion**. This expansion is performed about the initial estimates of the parameters and, by an iterative process, these estimates are improved upon until the improvements become less than some specified amount. The initial parameter values, provided by the user, are easily estimated in the case of a Gaussian fit. The rapidity with

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*Kindly supplied by C.T. Tindle, Physics Department, University of Auckland. The original was written by A.G. Fowler, University of British Columbia.

**See, for example, Draper & Smith (1966), p.267.

which the program converges to the minimum sum of squares depends, of course, on the 'goodness' of these initial estimates. The reader is referred to Appendix C for FORTRAN listings of this routine together with the sub-programs required. A brief guide to the use of the program and sample printouts are also provided in this Appendix.

Fig. 5.4 illustrates the result of fitting a sum of three Gaussians to the data represented in Fig. 5.3b, for a digital filtering cut-off wavelength of 8 data point spacings. The fit is clearly satisfactory and shows directly the absence of any significant skewness in $F_1(T)$ or $F_2(T)$. The fitted function took the form

$$N_F'(T) = \sum_{i=1}^{3} \frac{N_i}{\sqrt{2\pi}\sigma_i} \exp\{-(T-\tau_i)^2/2\sigma_i^2\} \quad (5.24)$$

where the N_i are normalisation coefficients. The minimum sum of squares was found allowing variation in all nine parameters. We present in Table 5.1 the initial and final estimates of the parameters in this example, together with the standard errors ascribed to each parameter through the fitting procedure. A three minute shift must be added to the estimates of the τ_i in order that correspondence be achieved with the graph of Fig. 5.4. This discrepancy occurs because the first data point, taken at three minutes after synchronization, was equated with zero time in the computer program.

All cultures do not exhibit such a precise doubling of the N_i, as i increases from one to three, as that

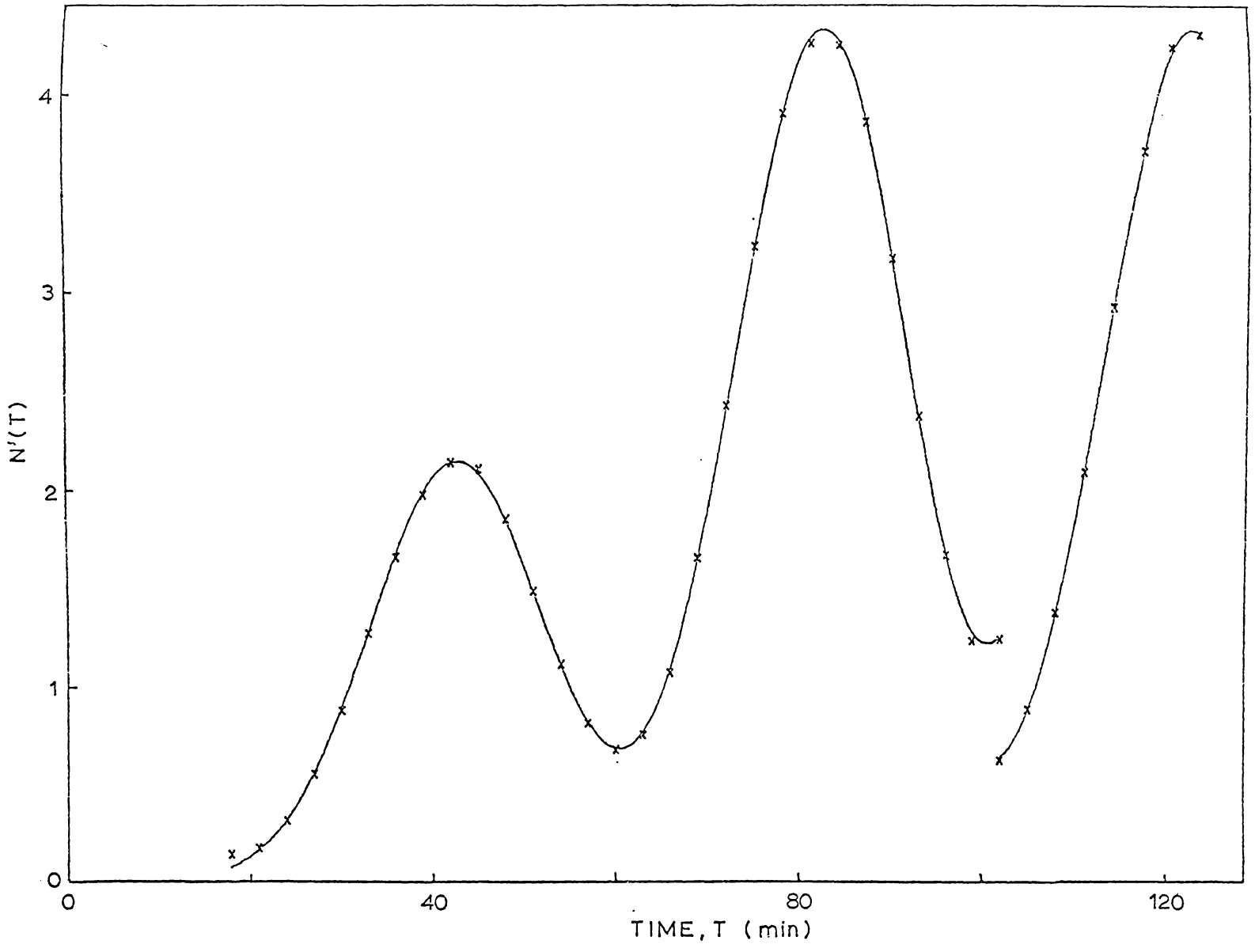

Fig. 5.4 Sum of three Gaussians (solid line) fitted to the data of Fig. 5.3b(x) for a digital filtering cut-off wavelength of eight data point spacings.

indicated in Table 5.1. Ideally, a periodic doubling of cell numbers will occur leading to a doubling of the N_i. However,

TABLE 5.1 Result of a Non-Linear Least Squares Fit of a Sum of Three Gaussian Functions to a Concentration Derivative Curve (Fig. 5.3b)

Parameter	Initial Estimate	Final Estimate	Standard Error
τ_1 (min)	36.0	39.73	0.07
τ_2 (min)	78.0	79.34	0.03
τ_3 (min)	120.0	119.10	0.07
σ_1^2 (min^2)	60.0	90.8	1.3
σ_2^2 (min^2)	80.0	90.1	0.7
σ_3^2 (min^2)	120.0	83.6	1.0
N_1	1.3	1.29	0.01
N_2	2.6	2.58	0.01
N_3	5.0	4.99	0.03

deviations may arise through one or more of the following causes:

(i) the presence of dead or non-proliferating cells in the culture;

(ii) the presence of a background of completely asynchronized cells;

(iii) a discrepancy in the background level (due to dust particles, etc.) in the diluted samples; and

(iv) inadequate correction for the occurrence of coincidence counts in the Coulter counter.

Ascertaining the relative magnitudes of these sources in any particular experiment is difficult but we would expect (iv) to be of minimal importance in the range of count rates used*. Likewise, (ii) could be eliminated if the derivative of the concentration curve were virtually zero for initial times (with due recognition given to any lag in growth that may occur). The results presented in this work do indicate that we are justified in neglecting (ii). Without some alternative means of estimating the percentage of non-proliferating cells in any one culture the effects of (i) and (iii) are practically inseparable. Assuming our estimate of the background level is reasonable* we can determine the proliferative cell fraction, α, by noting that, initially,

$$\alpha x + (1 - \alpha)\, x = N_1 \qquad (5.25)$$

where N_1 is the initial cell concentration and x is the cell concentration at any time. After one doubling

$$2\alpha x + (1 - \alpha)\, x = N_2 . \qquad (5.26)$$

Hence, eliminating x between (5.25) and (5.26),

$$\alpha = \frac{N_2 - N_1}{N_1} . \qquad (5.27)$$

The results in Table 5.1 imply, of course, that α = 100%. This is encouraging, for it suggests that the corrections we have applied to our data points are reasonable. It is expected that values of α in the range 97-100% should be

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*See Section 4.2.5 for a discussion of coincidence corrections and background estimation.

obtained, as these would accord with viability studies of bacterial cultures in balanced growth (Smither, 1975).

The degree of skewness, if any, in the generation time distribution $F_1(T)$ is of considerable interest. In the first two chapters of this thesis mention has been made of various skewed distributions adopted by other workers as suitable functional forms for the generation time distribution of bacteria. Most common among these are the Pearson Type III (gamma), lognormal and reciprocal normal distributions. All of these distributions are skewed to the right whereas visual observation of the fit of a Gaussian to our data for $F_1(T)$ indicates no noticeable skewness. However, it is of value to fit some of these distributions to the data to test the sensitivity of our fitting procedure to various functions and confirm the Gaussian nature of the generation time distribution. Visual observation of the various fits and the measures of the sum of squares of the residuals for the least squares procedure are suitable criteria for judging the sensitivity of the method. The distributions we considered, apart from the Gaussian, with their functional expressions, were:

(1) Gamma Distribution

$$f(T) = \frac{\alpha^{\nu+1}}{\Gamma(\nu+1)} (T-T_o)^{\nu} \exp\{-\alpha (T - T_o)\} \qquad (5.28)$$

$$(\alpha > 0, \nu > -1, T > T_o)$$

(2) Lognormal Distribution

$$f(T) = \frac{\alpha}{(T - T_o)\sqrt{2\pi}} \exp\left[-\tfrac{1}{2} \{\nu + \alpha \ln (T - T_o)\}^2 \right] \qquad (5.29)$$

$$(\alpha > 0,\ \nu < 0,\ T > T_o)$$

(3) Weibull Distribution

$$f(T) = \frac{\nu}{\alpha} \left(\frac{T - T_o}{\alpha}\right)^{\nu - 1} \exp\left\{- \left(\frac{T - T_o}{\alpha}\right)^{\nu}\right\} \qquad (5.30)$$

$$(\alpha > 0,\ \nu > 0,\ T > T_o)$$

(4) Normal Distribution with Adjustment for Skewness

$$f(T) = \frac{1}{\sqrt{2\pi}\,\sigma} \left[1 - \frac{\alpha_3}{2} \left(\frac{T}{\sigma} - \frac{T^3}{3\sigma^3}\right)\right] \exp\left\{- \frac{(T - T_o)^2}{2\sigma^2}\right\} \qquad (5.31)$$

The same symbols have been adopted for the parameters in distributions (5.28) to (5.30) purely for ease of tabulation*; no relationships between the distributions are implied. The reciprocal normal distribution was not fitted; it tends to be more highly skewed than the gamma and lognormal distributions. Distribution (5.31) is a second-approximation curve obtained by using the first two terms of the Gram-Charlier series (Johnson & Kotz, 1970). The gamma and lognormal distributions are always positively skewed, although they approach normality in certain regions of their parameter values. The Weibull distribution also shares this latter property but can exhibit positive or negative skewness. This

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*The properties of these distributions are discussed in, for example, Johnson & Kotz (1970).

distribution, like the gamma distribution, has been used in situations requiring adjustments to exponential distributions, as in representing lifetimes or times to failure in quality control work.

In Table 5.2 we present the results of the least squares fits (to the data of Fig. 5.3b) for these functions in terms of the residual sum of squares and the standard statistical parameters. The values for the Gaussian fit are included for comparison. Graphical representations of the fits, in comparison with the Gaussian, are shown in Fig. 5.5. Each of the functions was considered as the sum of a pair in fitting to the data, so that the overlap region between F_1 (T) and F_2 (T) was accommodated. The fits were extended to the peak of F_2 (T). Eight parameters were estimated in each case, since normalisation parameters were included in the fitted functions.

TABLE 5.2 Statistics of Various Functions Fitted to Generation Time Distribution Data

Function	Mean τ_1 min.	Standard Deviation σ_1 min.	Skewness α_3	Mode min.	Residual Sum of Squares x 10^5
Gaussian	42.7	9.55	0	42.7	0.453
Gamma	46.4	10.4	0.47	42.0	7.34
Lognormal	44.8	10.5	0.60	41.8	9.79
Weibull	42.5	9.3	-0.10	43.2	1.10
Second Approximation	42.7	9.52	0.00	42.7	0.615

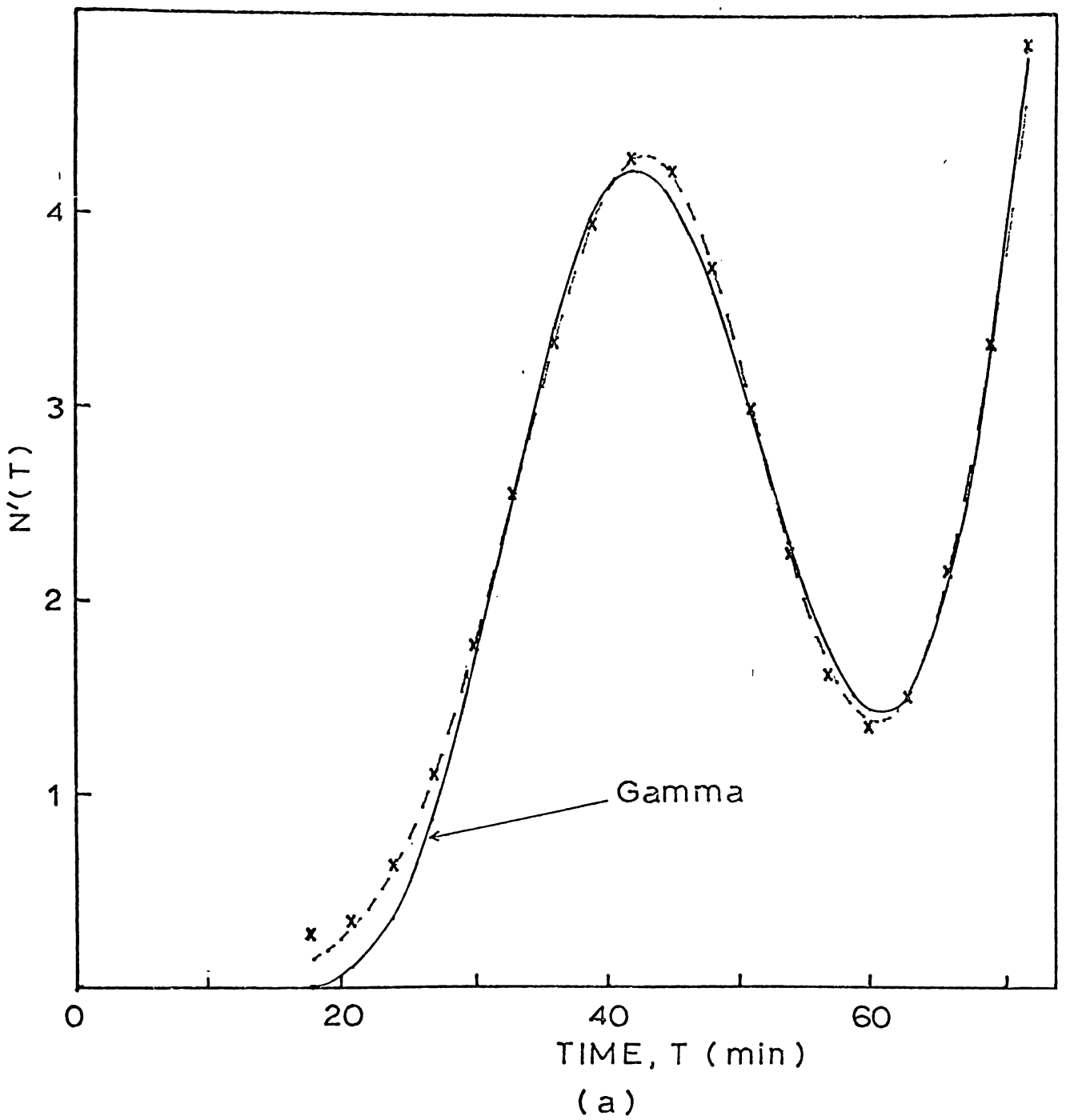
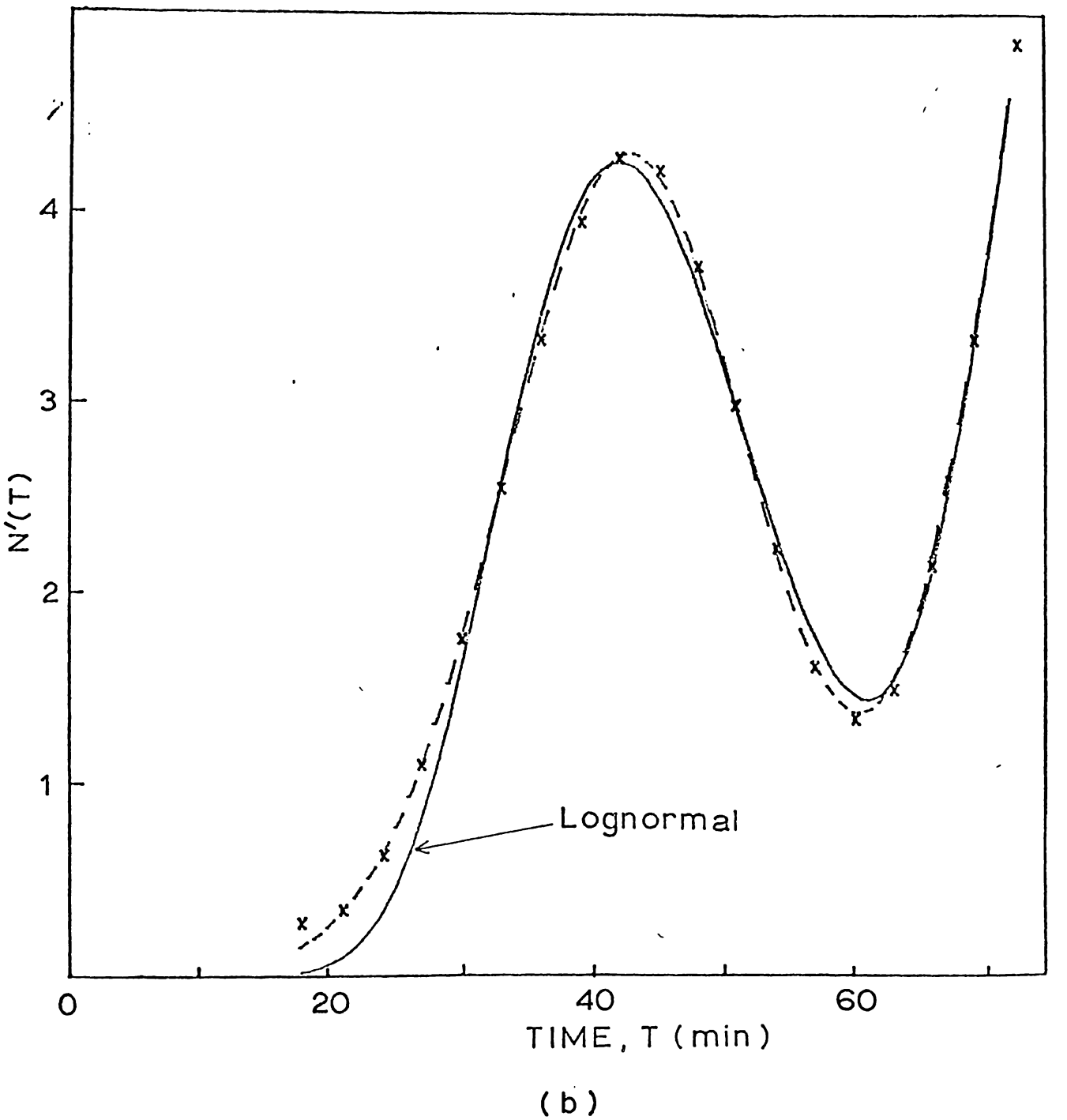


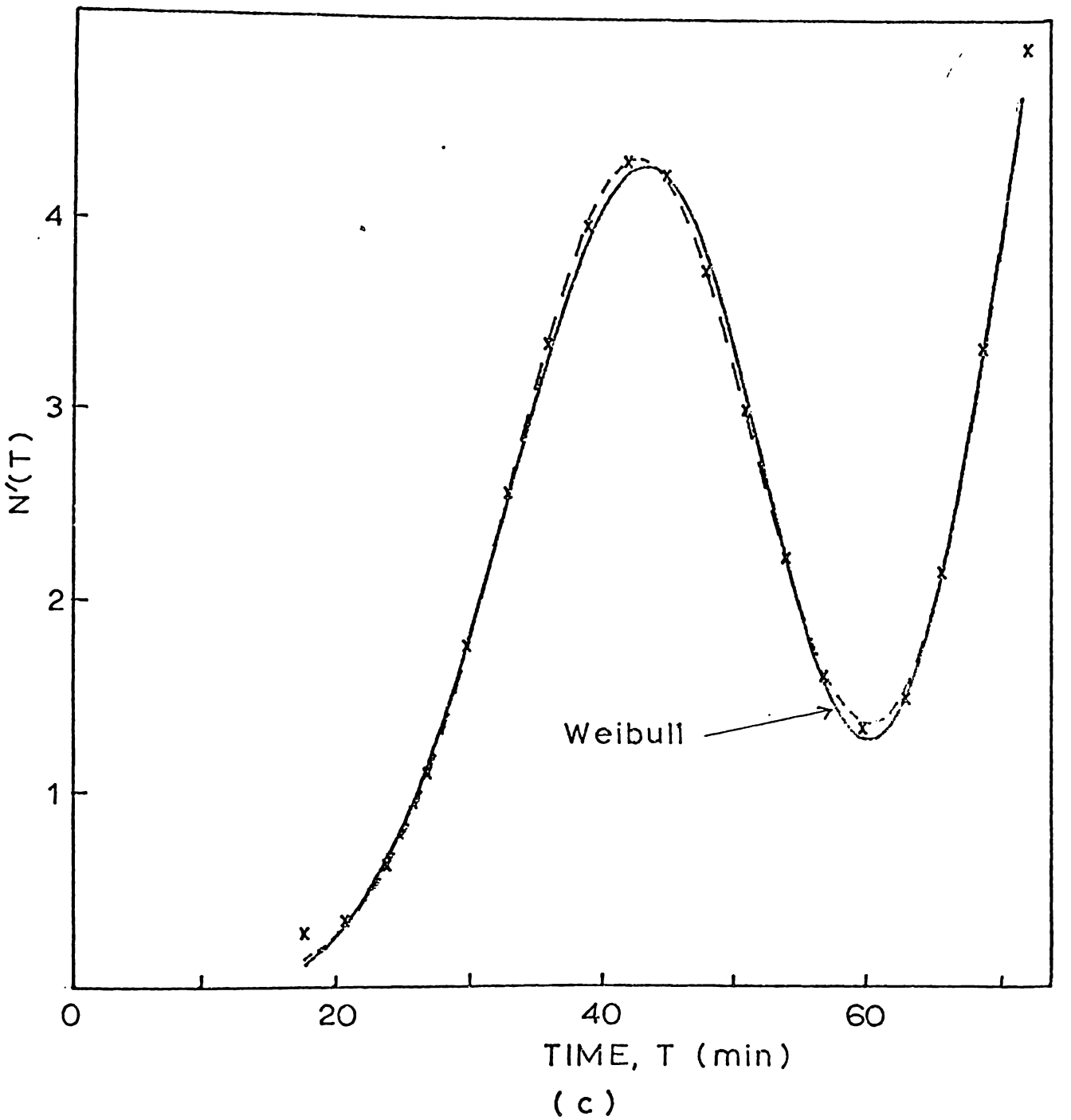
Fig. 5.5 Comparative least squares fits of the Gaussian distribution (dashed line) and

(a) the gamma
(b) the lognormal, and
(c) the Weibull distributions

to the data of Fig. 5.3b for a cut-off wavelength of eight data point spacings (x).



(b)



(c)

Looking at the relative magnitudes of the sums of squares in Table 5.2 it is apparent that the gamma and log-normal distributions do not fit the data nearly as well as the normal distribution. This is borne out by the graphical comparisons. The Weibull distribution provides a considerably better fit and exhibits a much reduced skewness, although negative, but even here there is a clear discrepancy observable in Fig. 5.5c between the data and the fitted curve. The left-hand tail of the distribution in the case of the gamma and the lognormal functions contributes strongly to the sum of squares in these cases, but in all three distributions plotted it is possible to see that the shape of the distributions around the peak of F_1 (T) is at variance with the data and no improvement to the fit is obtained by employing these functions rather than a pure Gaussian.

The best estimates of the parameter values, provided by least squares, for the gamma, lognormal and Weibull distributions are given in Table 5.3.

TABLE 5.3 Parameter Estimates for Functions Fitted to Generation Time Distribution Data

Function	α	ν	T_o
Gamma	0.418	17.00	1.4
Lognormal	5.07	-20.0	-7.9
Weibull	35.0	3.83	10.9

We can conclude from these results that observation of the fit of Gaussian functions to all the experimental data will provide adequate confirmation of the suitability of such a function in fitting the various derivative curves.

We are now in a position to determine the means and variances of the functions F_j (T) and thus to estimate the mother-daughter cell generation time correlation coefficients, plus the grandmother-granddaughter generation time correlation coefficient in instances where the data have been extended beyond three generations of growth. Clearly, the data of Fig. 5.3b will yield a negative parent-daughter generation time correlation coefficient since the variance of F_2 (T) is less than twice that of F_1 (T). Before discussing the significance of these parameter values we need, of course, some estimates of their error bounds, and the next section deals with this aspect of the analysis.

5.5 EXPERIMENTAL ERRORS

The errors associated with the experimental sample counts arise predominantly through fluctuations in the dilutions. With practice, a reproducibility approaching ± 0.1% is readily obtained with the 10 ml graduated pipette used for transferring the diluent. The 0.2 ml micropipette used for sampling has a quoted reproducibility of ± 1% (coefficient of variation) or better. This latter variation is the principal source of uncertainty. The counts registered by the Coulter counter follow, ideally, a Poisson distribution.

Two counts were taken per sample and initial counts were generally close to 2×10^4, rising thereafter. Thus, errors contributed from this source are expected to be well under 1%.

Practical tests were carried out to determine the magnitudes of the errors attributable to the data points. Replicate dilutions were performed from a stock sample of cells suspended in diluent, and two counts were recorded for each sample. The computed standard deviation for the distribution of sample counts was close to 1.5% at count levels of 2×10^4 and 5×10^4 (see Table 5.4). Hence the sources of error just mentioned appear to provide a reasonable picture of the situation. Initial counts in some experiments were nearer 1.5×10^4 so that a little more scatter may be observed about these points. However, in the final analysis, rather more scatter can be tolerated in the first few points of the concentration curve in terms of the effects on the extracted parameter values.

Making the reasonable assumption, then, that the background level from sample to sample is essentially constant we are justified in assigning a 1.5% uncertainty (standard deviation) to each experimental point.

TABLE 5.4 Results of Two Determinations of the Uncertainty Associated with Diluted Sample Counts Using the Coulter Counter

Sample No.	COULTER COUNTS Experiment A		Experiment B	
1	24180,	24415	52659,	52550
2	24443,	24647	52422,	52602
3	24589,	24557	52135,	51736
4	23973,	23997	50830,	51476
5	23828,	23713	51421,	51914
6	24176,	23997	51652,	52009
7	23844,	23540	50055,	49924
8	23489,	23612	51149,	50526
9	23854,	23897	51414,	51853
10	23543,	23522	52283,	51149
11			51272,	50463
Mean	23992		51523	
S.D.	380		766	
C.V.	1.6%		1.5%	

5.6 ESTIMATION OF UNCERTAINTIES IN THE EXTRACTED PARAMETERS

Having determined a satisfactory uncertainty to assign to each experimental point in the growth data, some method of estimating the resulting error in the extracted parameters is required. It was considered that the most reliable way of accomplishing this was to simulate the effect of a random error applied to each point of a set of 'perfect' data. In other words, the analysis of actual experimental data was simulated as closely as possible with data generated analytically and bearing close resemblance to the former. Such data were generated by integration of a sum of Gaussian functions, the parameters of which had similar values to those found for particular experiments. In most cases, four Gaussians were summed so providing satisfactory data through the third generation. Five Gaussians were necessary in simulating extended data used for the purpose of obtaining estimates of the grandmother-granddaughter generation time correlation coefficients.

Each data point generated by the integration routine was multiplied by a random error; these errors being normally distributed with mean unity and standard deviation 0.015. The smoothing, differentiation, and nonlinear least squares fitting of a sum of Gaussians was then carried out in the normal way. Twenty to thirty repetitions of this process of sampling from a random number generator and subsequent analysis yielded measures of the dispersion in each of the parameters sought. Further repetitions were not

necessary to adequately characterize these dispersions. Different experimental conditions thus required separate treatment so that the generated data conformed reasonably closely to the experimental results in respect of the means and variances of the peaks in the derivative curve, and, of course, the data point spacing.

The analysis was performed on a Burroughs B6700 computer; a FORTRAN listing of the program is reproduced in Appendix C. The machine specific random number generator RANDOM furnishes uniformly distributed random floating point numbers between 0 and 1.0. A subroutine called GAUSS* then uses twelve such random numbers to compute, by the central limit theorem, a random number from a normal distribution**. The result is then adjusted to match the specified mean and standard deviation. Fig. 5.6 illustrates, in histogram form the distribution of a sequence of 500 such random numbers. The calculated mean and standard deviation of this distribution is 1.000 and 0.016, respectively. The theoretical distribution with mean unity and standard deviation 0.015, normalised to 500, is also shown. Processing times of, typically, about five minutes were necessary for a complete run at each cut-off wavelength.

An example will clarify the procedure. Fig. 5.7 shows the result of generating a set of noisy data by integration of a sum of four Gaussian functions, these functions having means and variances corresponding closely to

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IBM 1130 Scientific Subroutine Package, 3rd edition, 1968; International Business Machines, New York.

**See, for example, Hamming (1962).*

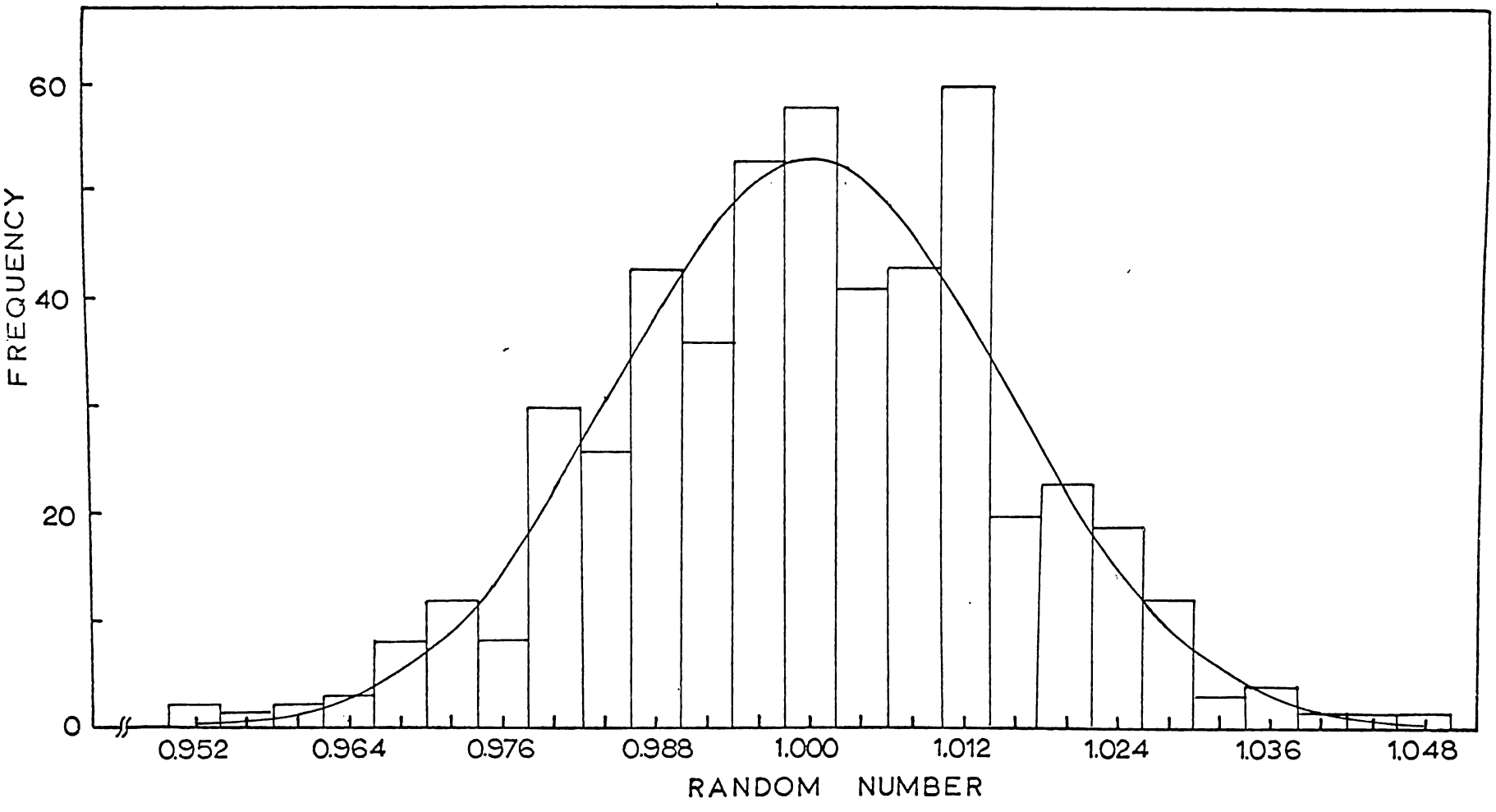


Fig. 5.6 Distribution of a sequence of normally distributed random numbers generated by computer (histogram) and theoretical population normal distribution (solid curve).

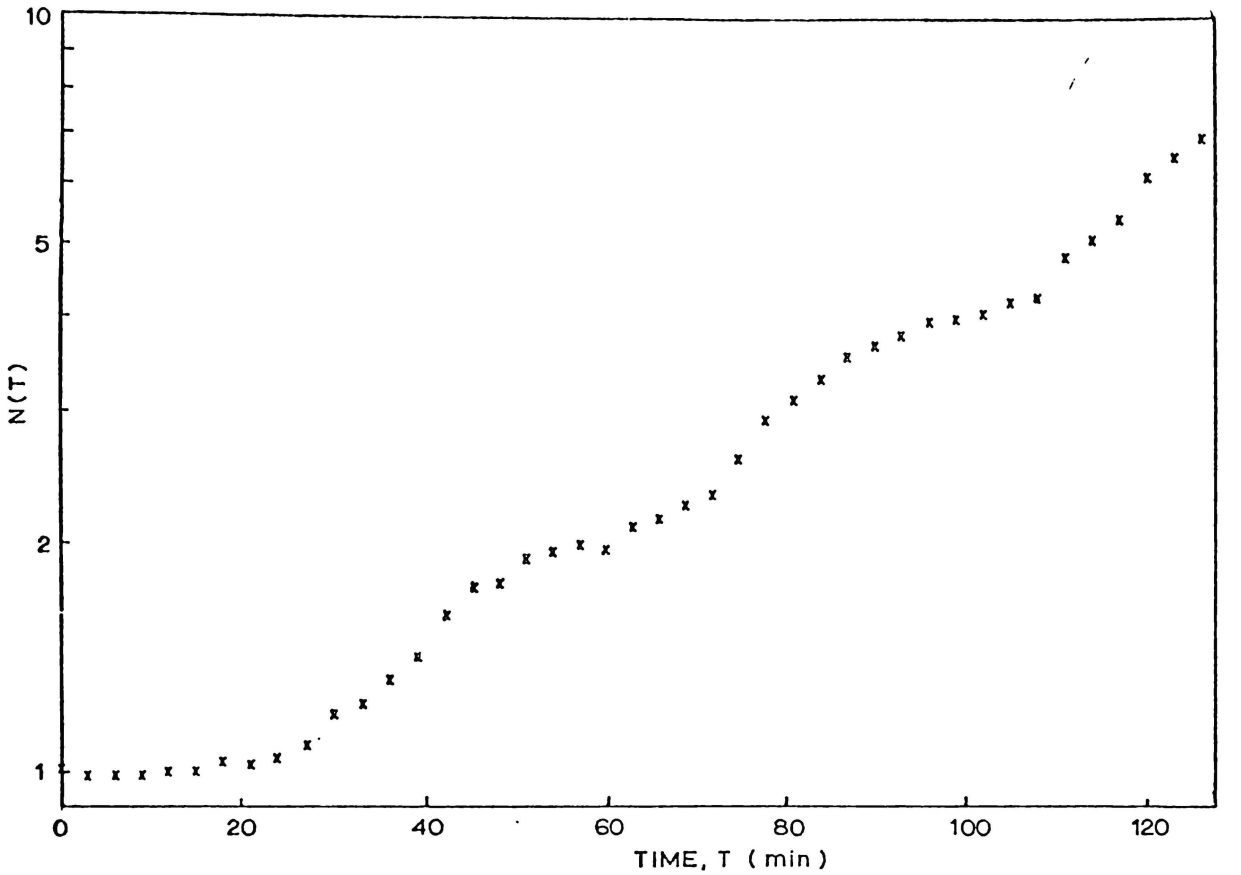


Fig. 5.7 Set of data generated by integration of a sum of four Gaussian functions and a random error (1.5% standard deviation) then impressed on each point.

those found for the data of Fig. 5.3a and listed in Table 5.1. After this integration is completed each data point is multiplied by a random number simulating a small error, as we have described. A number of repetitions of the process of applying noise to the data is carried out and each set of noisy data, of which Fig. 5.7 is representative, is then subjected to the smoothing, differentiation and least squares fitting operations. Fitting a sum of three Gaussians to each data set will yield estimates of the means and variances of these Gaussians which will vary slightly from one set to another. The extent of this variation can be represented by the standard deviation of the distribution of parameter values obtained. This standard deviation will be a measure of the uncertainty attached to any single determination of the means and variances of the functions F_j (T) from one set of experimental data. Table 5.5 lists the results of such a determination of the dispersions in the parameter values for two different cut-off wavelengths in the smoothing operation. For a cut-off wavelength of 8 data point spacings the results for both 20 and 30 repetitions of the analysis are shown. The distribution of the means, τ_1, of F_1 (T) is shown in histogram form in Fig. 5.8 for a set of pooled results using digital filter cut-off wavelengths of 7,8, and 9 data point spacings. For each wavelength 30 parameter estimates were obtained. The mean and standard deviation of this distribution is 40.0 and 0.56, respectively, in agreement with the results in Table 5.5. The normal distribution having this mean and standard deviation is plotted also, indicating that the distribution of parameter values follows Gaussian form.

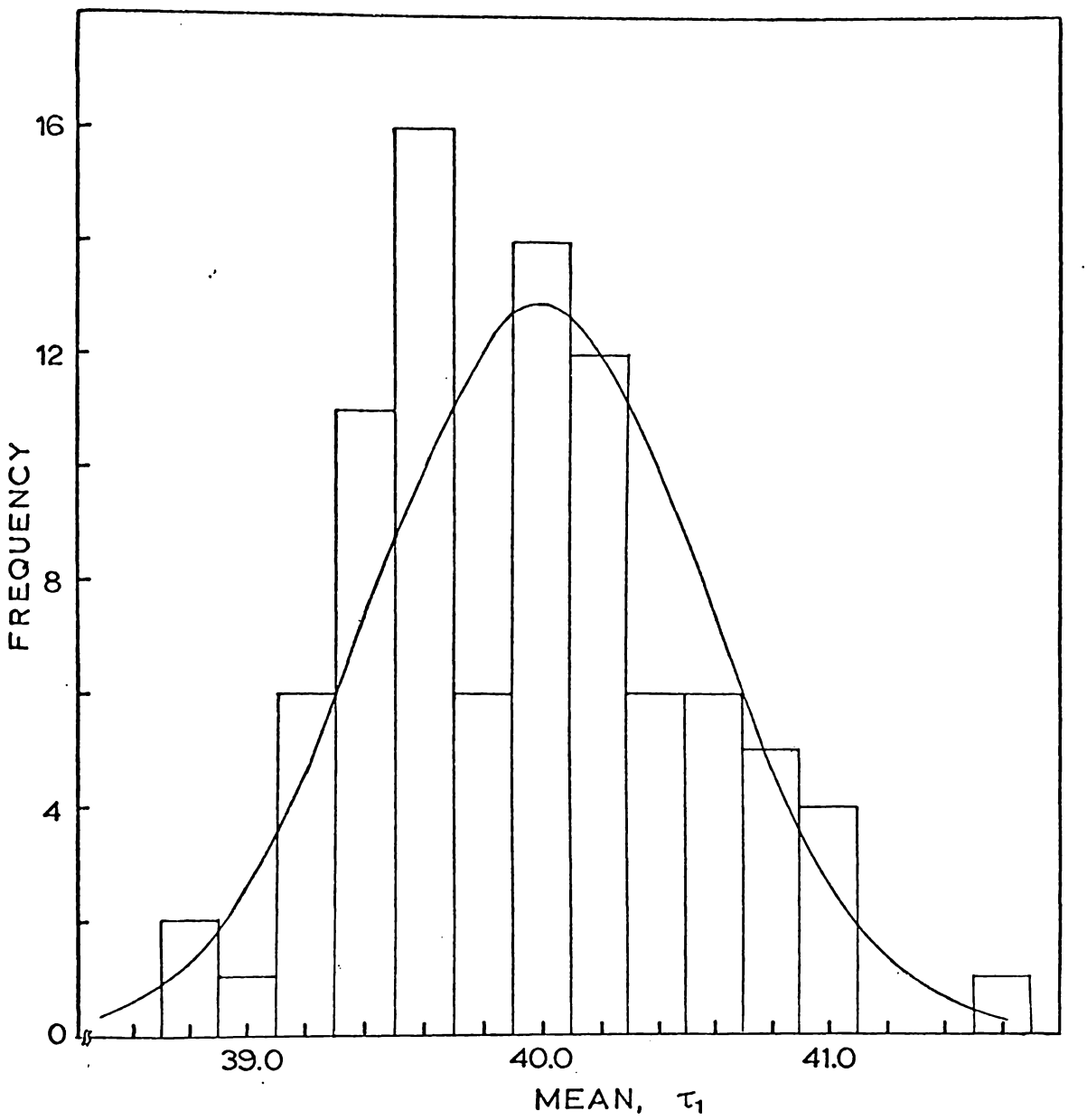


Fig. 5.8 Distribution of the means of $F_1(T)$ (histogram) estimated from simulated noisy data. Results for digital filtering cut-off wavelengths of 7, 8, and 9 data point spacings are pooled. The solid curve shows the normal distribution having the same mean and variance.

TABLE 5.5 Example Illustrating the Standard Deviation (S.D.) in Parameters Estimated from Simulated Noisy Data

Cut-off Wavelength:		8		8		7	
Number of Repetitions:		20		30		30	
Parameter[a]		Mean	S.D.	Mean	S.D.	Mean	S.D.
τ_1	(40)	40.2	0.5	40.1	0.5	40.1	0.5
τ_2	(80)	80.4	0.5	80.4	0.5	80.1	0.6
τ_3	(120)	117.3	0.6	117.4	0.6	122.1	4.1
σ_1^2	(90)	96	15	94	14	94	15
σ_2^2	(90)	95	13	99	14	96	17
σ_3^2	(90)	55	7	53	7	97	42
$\rho(1)$		-0.48	0.15	-0.45	0.15	-0.47	0.16

[a] Data was generated using the values given in parentheses alongside each parameter.

Noticeable from Table 5.5 are the large discrepancies in the values for τ_3 and σ_3^2, the mean and variance of the function $F_3(T)$. These are expected since the generated data extended to only 43 points, roughly corresponding to the peak of $F_3(T)$. As such, this peak was not well-defined and, in addition, the smoothing operation considerably distorts the last few points of a data set. Results presented in Table 5.6, in fact, provide evidence of this effect of smoothing on the last few points. Data was generated as before but noise was not introduced. This perfectly smooth data was then subjected to the digital filtering routine prior to differentiation. The results of a least-squares estimation

of the parameters is given in the right-hand column of Table 5.6. The middle column gives the results of this estimation when the digital filtering operation is bypassed. Iteration was halted in the least squares fitting routine when, in successive iterations, the parameter values changed by less than 1%. Within this range, therefore, the correct parameter values have been realised when digital filtering is not applied. The large discrepancy in the estimate of σ_3^2, after digital filtering has been used, indicates the distortion introduced by this operation. The importance of gathering data adequately extended in time is clear. If we are interested in characterising the distribution $F_3(T)$ we must obtain data well into the fourth generation.

TABLE 5.6 Effect of Data Smoothing on Parameter Estimation

Parameter[a]	Parameter Estimates	
	Without Smoothing	With Smoothing[b]
τ_1 (40)	40.0	40.0
τ_2 (80)	80.0	80.0
τ_3 (120)	120.1	121.3
σ_1^2 (90)	90.0	91.8
σ_2^2 (90)	89.9	93.4
σ_3^2 (90)	90.9	102.7

[a] The parameter values given in parentheses were used to generate the data.

[b] A digital filtering cut-off wavelength of 8 data point spacings has been used.

5.7 ANALYSIS OF EXPERIMENTAL RESULTS

5.7.1 Constant Temperature

(a) Glucose

Applying the analysis discussed in the previous sections to the glucose-grown culture of Fig. 5.3a (experiment A) produced the results shown in Table 5.7. The cut-off wavelengths for the digital filtering procedure are selected such that adequate smoothing is achieved (see Fig. 5.3b) and no gross changes in the parameters are observed. At 10 data point spacings a significant shift in the values of the estimated parameters is noted as appreciable rounding of the steps on the concentration curve begins to occur. The mean and variance of F_3 (T) is included in Table 5.7 although little reliance can be placed on these values as only half

TABLE 5.7 Extracted Parameters for a Synchronous Glucose-Grown Culture (Experiment A) at 37°C

Cut-off Wavelength[a]	Means (min) τ_1	τ_2	τ_3	Variances (min^2) σ_1^2	σ_2^2	σ_3^2
7.0	42.8	82.1	122.3	92	84	103
7.5	42.7	82.2	122.0	92	86	97
8.0	42.7	82.3	122.1	91	90	84
8.5	42.7	82.4	120.7	91	90	79
9.0	42.7	82.4	120.4	91	91	75
Mean	42.7	82.3	121.5	91	88	88

[a] in units of data point spacings.

of this peak has been determined and the reliability of the smoothing process near the end of the data is somewhat reduced. The parameters, averaged over five cut-off wavelengths are given in the Table and the interdivision times $\tau_2 - \tau_1$ and $\tau_3 - \tau_2$ indicate a slight lengthening of the first generation compared to the second and third. This effect will be discussed in detail later in this section.

The average value of $\rho^{(1)}$, the mother-daughter generation time correlation coefficient, derived from the variance of $F_1(T)$ and $F_2(T)$ according to equation (5.20) is (neglecting σ_o^2):

$$\rho^{(1)} = -0.52 .$$

Simulation of these results in the manner described in Section 5.6 allows us to assign uncertainties to the parameter values as given in Table 5.8. We can therefore express $\rho^{(1)}$ and the coefficient of variation for the generation time distribution as

$$\rho^{(1)} = -0.52 \pm 0.14$$

$$\text{C.V.} = 0.24 \pm 0.02$$

TABLE 5.8 Results of Simulation Corresponding to Data in Table 5.7

Parameter	Standard Deviation
τ_1	0.5 min
τ_2	0.5 min
σ_1	0.7 min
σ_2	0.7 min
$\rho^{(1)}$	0.14

where the mean of the generation time distribution has been taken to be the difference between τ_1 and τ_2.

The parameters derived for the five synchronous culture experiments, A to E, conducted with *E. coli* B in glucose-salts medium at 37°C, are detailed in Table 5.9. Figures 5.9a to d show the results of fitting a sum of Gaussians to the derivative curves for experiments B to E. Experiments D and E were extended beyond four generations and a suitable simulation of these results yielded the errors shown for τ_3 and σ_3. Examination of the Table reveals that the interdivision time, $\tau_2-\tau_1$, varies between 39 and 41 minutes. This can possibly be attributed to small differences in the absolute temperature of the incubator between experiments. On the assumption that the five experiments can be treated as identical as far as the coefficient of variation of the generation time distribution and the mother-daughter generation time correlation coefficient are concerned we can

TABLE 5.9 Results of Synchronous Culture Experiments with *E. coli* B in Glucose-salts Medium at 37°C. (Average of five cut-off wavelengths)

Expt.	Fig.	Parameters of F_j (in minutes) τ_1	τ_2	τ_3	σ_1	σ_2	σ_3	$\tau_2-\tau_1$ min	$\tau_3-\tau_2$ min	CV	$\rho'^{(1)}$
A	5.3	42.7±0.5	82.3±0.5	121.5±2.5	9.5±0.7	9.4±0.7	9.4±1.6	39.6±0.7	39.2±2.6	0.24±0.02	-0.52±0.14
B	5.9a	45.2±0.6	85.2±0.6	125.6±2.5	10.8±1.0	9.0±0.6	9.9±1.7	40.0±0.9	40.4±2.6	0.27±0.03	-0.65±0.11
C	5.9b	41.2±0.5	82.3±0.5	125.9±3.5	8.9±0.9	9.8±0.7	10.9±2.5	41.1±0.7	43.6±3.5	0.22±0.02	-0.39±0.13
D	5.9c	36.2±0.3	74.8±0.5	115.6±1.0	7.6±0.4	8.4±0.6	9.3±0.7	38.6±0.6	40.8±1.1	0.19±0.01	-0.39±0.13
E	5.9d	47.3±0.5	88.3±0.6	131.5±0.6	8.5±0.6	9.7±0.7	10.6±0.8	41.0±0.8	43.2±0.9	0.21±0.02	-0.35±0.13

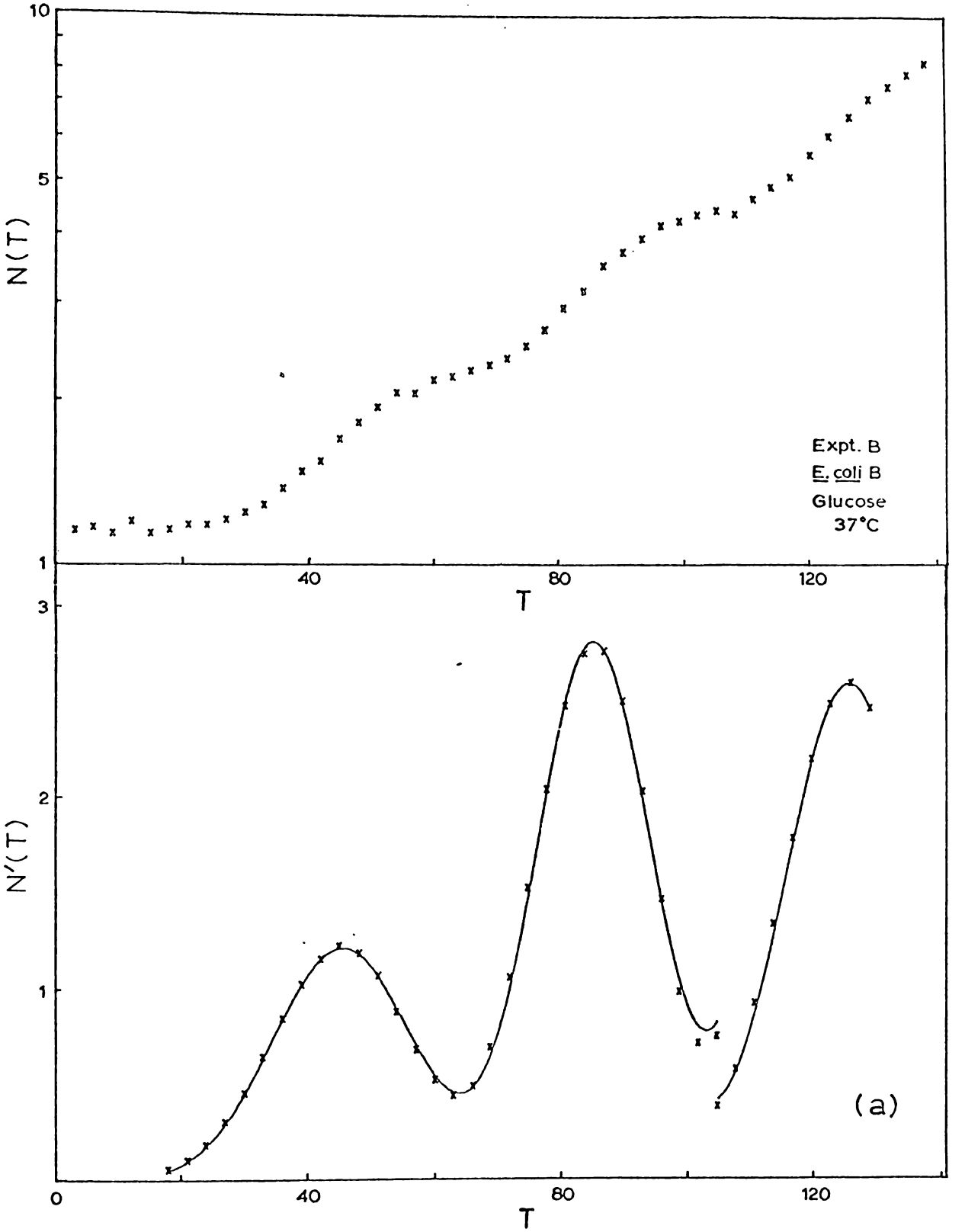
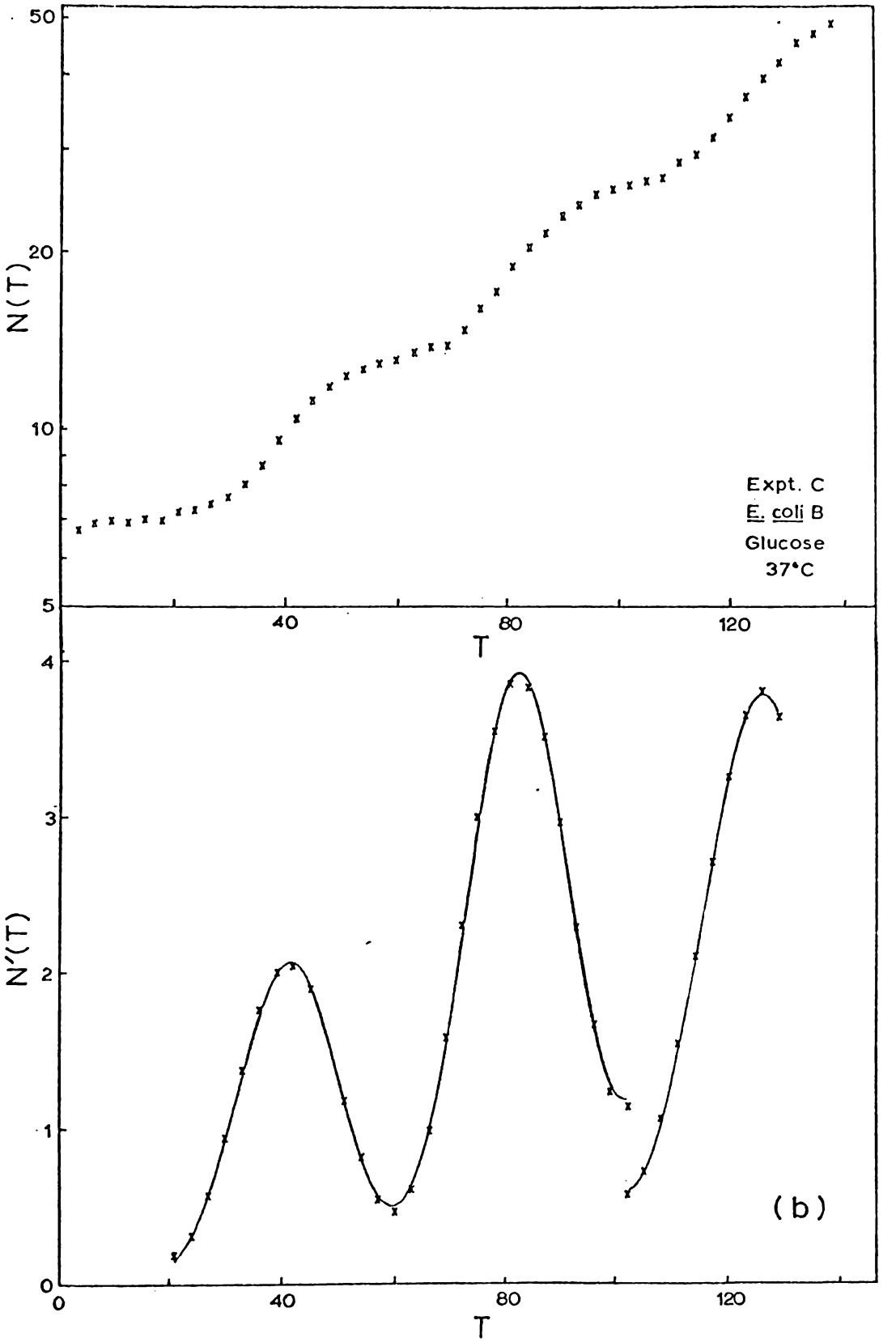
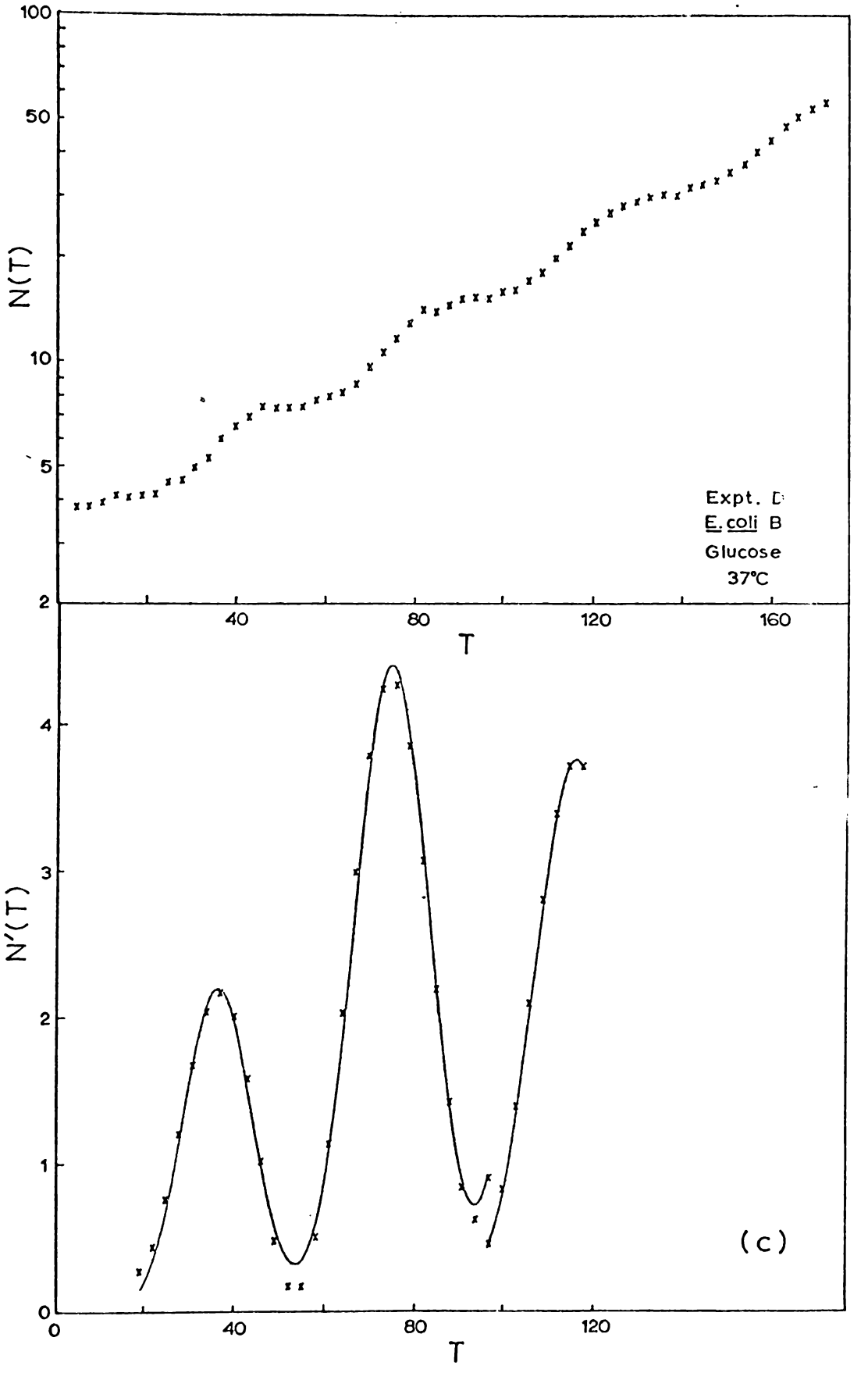
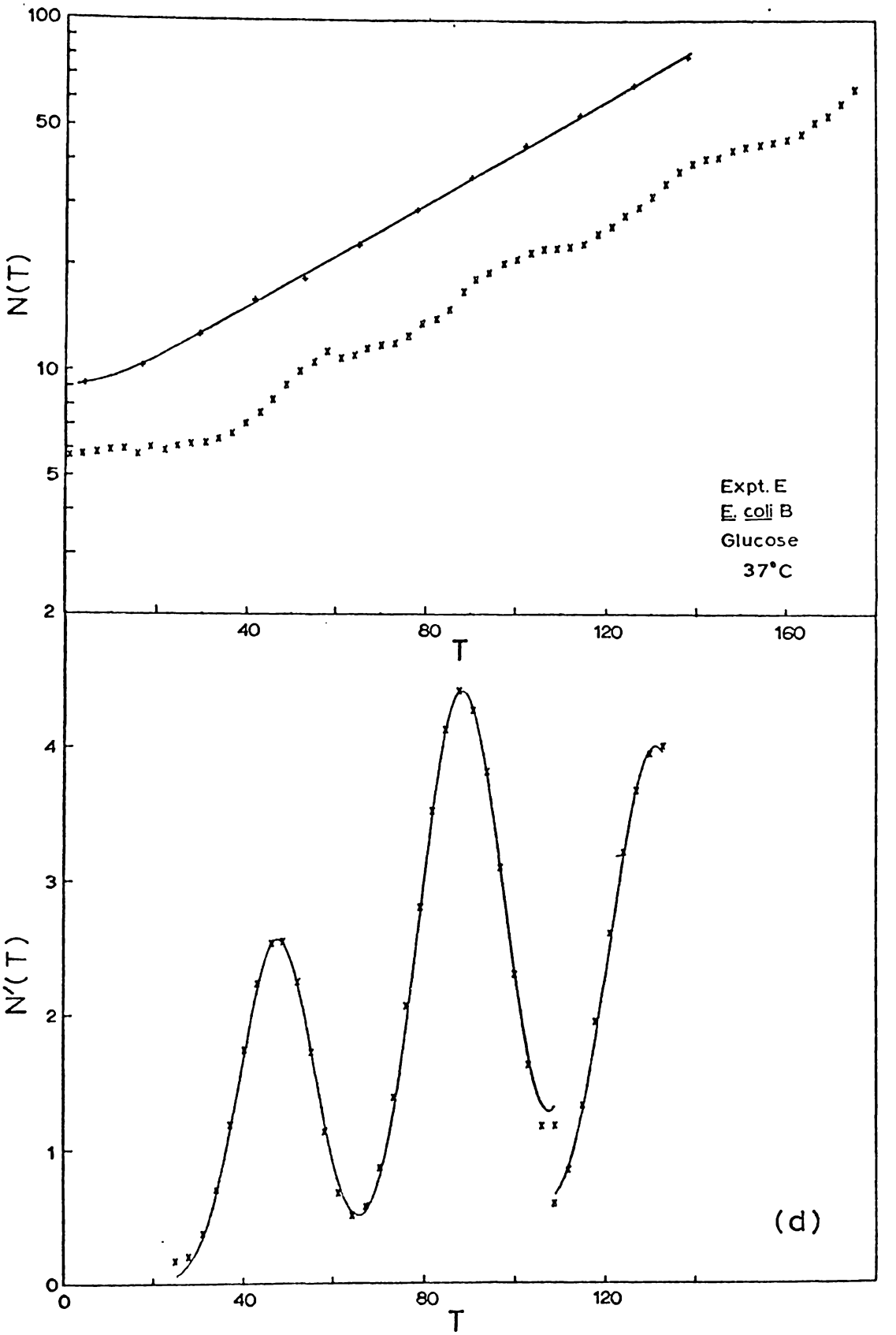


Fig. 5.9a-d Lower: Sum of three Gaussian functions fitted to differentiated data (x) after smoothing with a cut-off wavelength of eight data point spacings. For times greater than 2.5 τ_1 the ordinates have been divided by two. Upper: Experimental cell concentration plot. Time (T) is measured in minutes and the ordinates are in arbitrary units.


50
20
10
5
N(T)
Expt. C
E. coli B
Glucose
37°C
40
80
120
T
4
3
2
1
0
N'(T)
(b)
0
40
80
120
T


100
50
10
5
2
N(T)
Expt. D
E. coli B
Glucose
37°C
40
80
120
160
T
4
3
2
1
0
N'(T)
(c)
0
40
80
120
T


100
50
10
5
2
N(T)
40
80
120
160
T
Expt. E
E. coli B
Glucose
37°C
4
3
2
1
0
N'(T)
0
40
80
120
T
(d)

write down estimates of these parameters averaging over all experiments. The weighted mean of the values of $\rho^{(1)}$ is

$$\overline{\rho^{(1)}} = -0.47 \pm 0.06$$

where we have taken the weight to be assigned to each observation as proportional to the reciprocal of the square of its standard deviation. The standard error in $\rho^{(1)}$ has the same value, 0.06, whether derived entirely from the standard deviations of the separate observations or based also on the differences between the observations. Hence, the observations as a whole show 'internal' and 'external' consistency (Topping, 1962). The weighted mean coefficient of variation is

$$\overline{CV} = 0.21 \pm 0.01$$

where, again, internal and external estimates of the standard error give the same value.

Experiments D and E, having been extended beyond four generations of growth, allow us to estimate $\rho^{(2)}$, the grandmother-granddaughter generation time correlation coefficient. Reliable estimates of the parameters of $F_3(T)$ were obtained by fitting the sum of four Gaussians to the data, but constraining the first Gaussian to have the parameters previously determined for $F_1(T)$. Thus nine parameters were again varied during the least squares routine. This same procedure was followed in obtaining an estimate of the uncertainty in $\rho^{(2)}$ by the simulation method. Fig. 5.10

shows the result of fitting this sum of Gaussians to the data of experiment D for a cut-off wavelength of 7 data point spacings. Using the formulae 5.20 and 5.21, and neglecting σ_0^2, we obtain

$$\rho^{(2)} = +0.03 \pm 0.24 \text{ (expt. D)}$$

and

$$\rho^{(2)} = -0.02 \pm 0.23 \text{ (expt. E)}$$

The precision in the experimental data is thus not sufficient to enable this parameter to be estimated with any great degree of reliability. The values quoted are consistent with the hypothesis that

$$\rho^{(2)} = \{\rho^{(1)}\}^2$$

i.e., $\rho^{(2)}$ arises from the mutual correlation of the grandmother and granddaughter cell generation times with the daughter cell generation time.

Returning to Table 5.9 it appears, on comparing the values of τ_1 and $\tau_2 - \tau_1$ for the five experiments, that a lag in growth occurs at the beginning of experiments A, B, and E. Reference to Fig. 4.9e shows that the control culture for experiment E also exhibits a short lag so that, whatever its cause, this lag is not merely an artifact of the synchronous culture. Cells at all stages of development are affected. Burdett & Murray (1974) also observed this phenomenon when cells were exposed to sucrose. They overcame the problem by using Dextran or Ficoll as the gradient

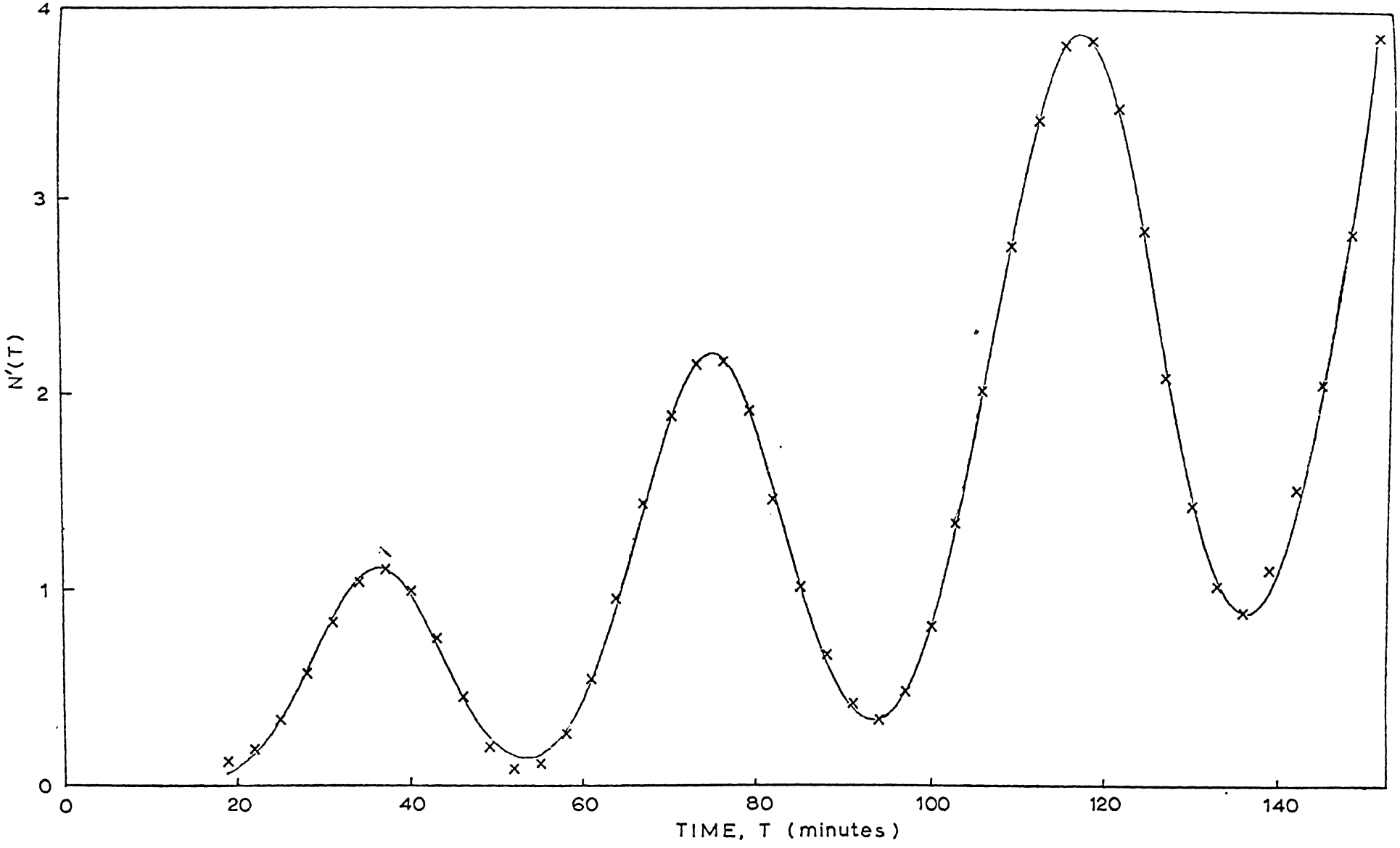


Fig. 5.10 Sum of four Gaussians fitted to the differentiated data for experiment D after smoothing with a digital filtering cut-off wavelength of seven data point spacings. The ordinates are in arbitrary units.

material. In the work here it is clear that the use of glucose gradients, sucrose gradients made up in growth medium, or the performance of all operations at 37°C does not eliminate the lag. Apart from this undesirable effect each of the synchronous cultures exhibits, within experimental errors, the same interdivision time during its second and third generations of growth. This mean generation time for experiment E coincides with the doubling time exhibited by the control culture (41-42 minutes). Thus no serious perturbations to the normal growth of the cultures are apparent. No significant differences between the results for experiments C and D, which lack an initial lag, and the other experiments appear to exist. We can expect that, while a lag is an undesirable feature in that it indicates some initial disruption to the normal growth of the cells, its presence here has negligible effect on the parameters we seek. Although this strain of *E. coli* utilizes sucrose it will preferentially metabolize glucose and similar results are obtained whether gradients employed contain only sucrose, only glucose, or sucrose plus glucose medium. It is noteworthy that experiment D exhibits a shortened mean generation time for the first generation. It is conceivable that the cell sample removed from the gradient in this experiment may have come from a slightly deeper region of the band and consisted of a size fraction of cells slightly larger than newborn cells. This could lead to a reduced variability in the times to division as is observed. It could result also in a smaller value for the magnitude of $\rho^{(1)}$. It should also be remarked that growth of cells may occur in the gradient during the deceleration phase of the

centrifuge so that the zero of the time scale could in fact be in error by a minute or two. This error would act in the opposite sense to a lag. Such growth is only likely to occur in those gradients containing glucose. A combination of the effects of a lag and of the recovery of slightly older cells could lead to a culture showing no apparent lag, such as that of experiment C.

(b) Sucrose

Fig. 5.11 illustrates the result of fitting a sum of three Gaussians to each of the derivative curves obtained from the data of experiments F and G. The case where a digital filtering cut-off wavelength of 8.0 data point spacings was used is shown. The parameters obtained by this analysis with their uncertainties yielded by the simulation procedure are listed in Table 5.10. In both these experiments a shortening of the first generation compared to subsequent generations is evident. It is probable that the discrepancy results from the cells pursuing their growth uninterruptedly during the time of centrifugation in the sucrose gradient. Young cells, initially separated in the gradient, would grow slightly older during the deceleration phase and during the later stages of centrifugation. A sample of these cells would not be expected to exhibit a significantly different generation time distribution than if they had been retrieved four or five minutes earlier in their newborn state. The results for experiment F indicate some disturbance to the growth of the cells, in that a prolonged period of second generation growth occurs.

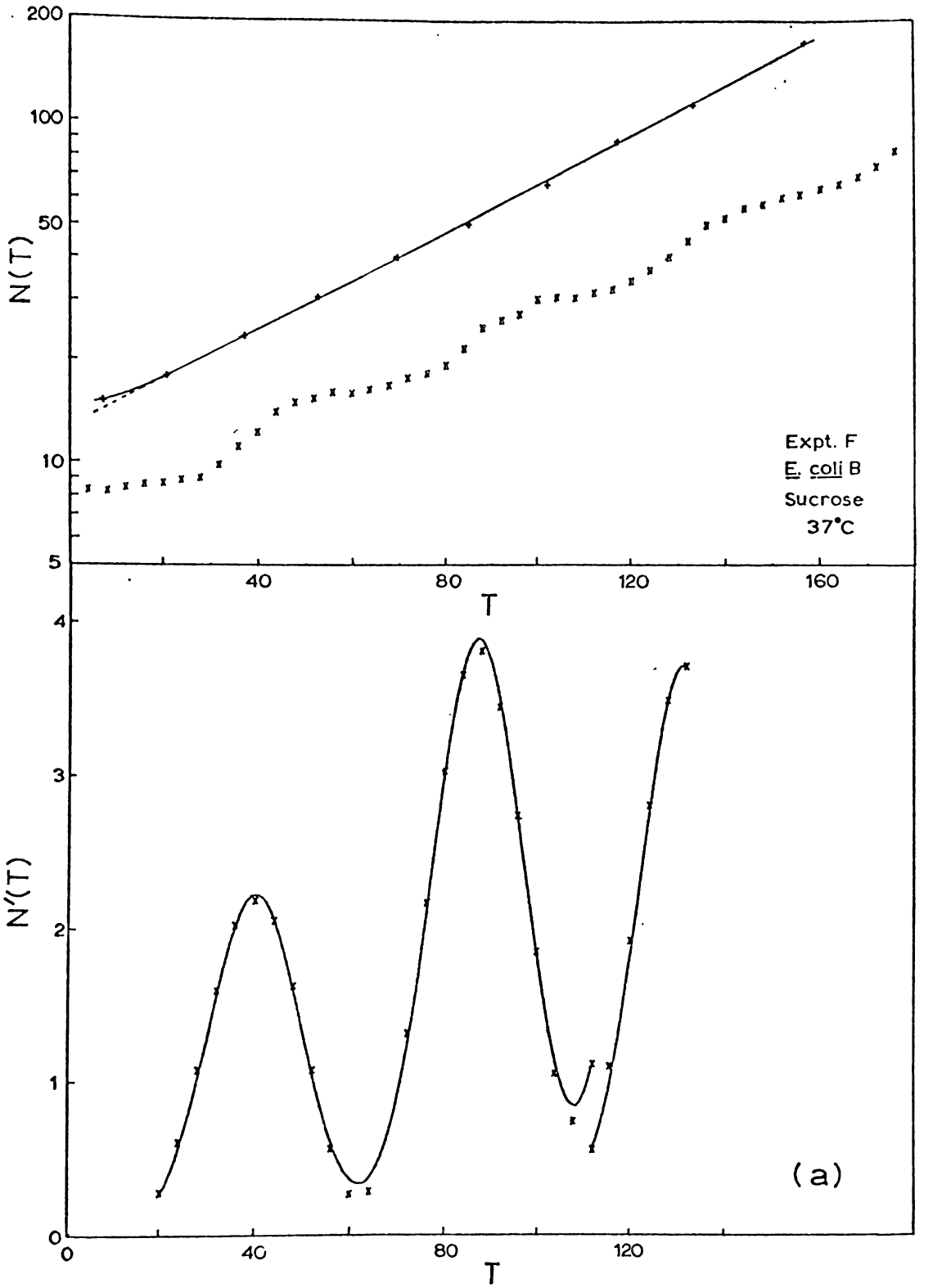


Fig. 5.11 a & b

Lower: Sum of three Gaussian functions fitted to differentiated data (x) after smoothing with a cut-off wavelength of eight data point spacings. Upper: Experimental concentration curve. Time (T) is measured in minutes and the ordinates are in arbitrary units.

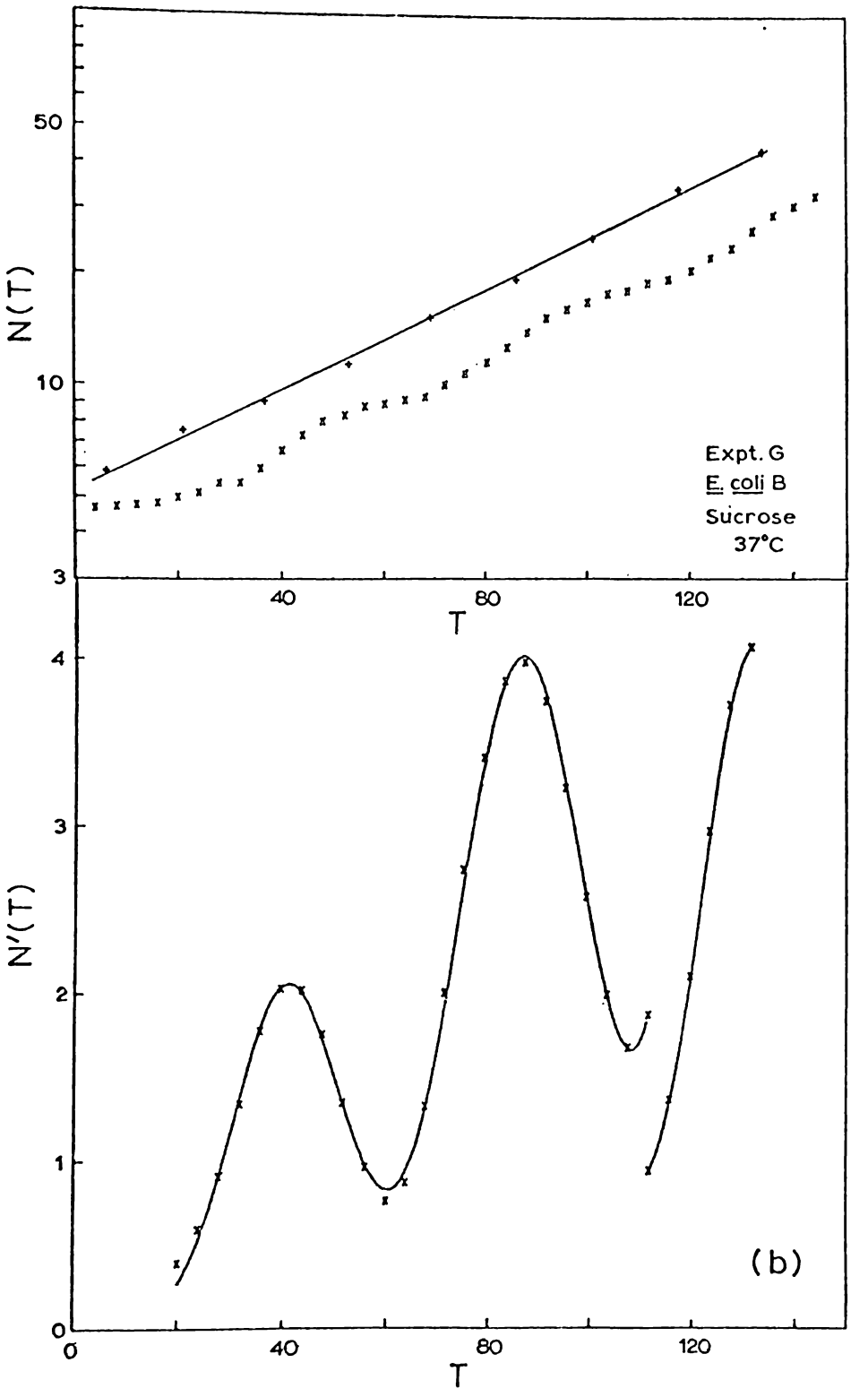

50
10
3
N(T)
Expt. G
E. coli B
Sucrose
37°C
40
80
120
T
4
3
2
1
0
N'(T)
(b)
0
40
80
120
T

TABLE 5.10 Results of Synchronous Culture Experiments with *E. coli* B using Various Carbon Sources at 37°C. (Average of five cut-off wavelengths)

Expt.	Carbon Source	Fig.	Parameters of F_j (in minutes) τ_1	τ_2	τ_3	σ_1	σ_2	σ_3	$\tau_2-\tau_1$ min	$\tau_3-\tau_2$ min	CV	$\rho^{(1)}$
F	Sucrose	5.11a	39.8±0.5	87.2±0.5	131.1±0.6	9.6±0.6	10.2±0.4	9.6±0.6	47.4±0.7	43.9±0.8	0.20±0.01	-0.44±0.09
G	Sucrose	5.11b	41.2±0.5	87.3±0.5	132.4±3.0	10.9±0.7	12.5±0.5	10.8±1.4	46.1±0.7	45.1±3.0	0.24±0.02	-0.35±0.09
H	Glycerol	5.12	52.9±0.5	100.2±0.6	145.0±2.0	11.1±0.6	11.2±0.6	10.3±2.2	47.3±0.8	44.8±2.1	0.23±0.01	-0.49±0.10
I	Succinate	5.13a	79.6±0.5	145.4±1.8	235±20	18.1±0.9	17.6±2.9	-	65.8±1.9	90±20	0.23±0.02	-0.53±0.16
J	Succinate	5.13b	78.7±1.5	155±10	248±20	18.6±1.7	(26)	-	76±10	93±21	0.24±0.02	(0)
K	Glucose/ Met/His	5.14	35.3±0.7	65.9±1.6	107±8	7.6±0.7	9.8±1.0	11.2±2.8	30.6±1.8	41±8	0.25±0.03	-0.18±0.28

The doubling time of the control culture is 42 minutes. Although this control culture shows a slight initial lag, this may not be real since the scatter in the points would also accommodate a straight line. No such lag can be discerned in experiment G. The control in this case has a doubling time of 46 minutes in accord with the lengths of the periods of second and third generation growth. On the basis that experiment F shows some distortion in the pattern of growth the most reliable estimates of CV and $\rho^{(1)}$ would be

$$CV = 0.24 \pm 0.02$$

$$\rho^{(1)} = -0.35 \pm 0.09$$

(Pooling the results of the two experiments gives $\overline{CV} = 0.22 \pm 0.01$ and $\overline{\rho^{(1)}} = -0.40 \pm 0.06$)

(c) Glycerol

The result of the curve-fitting program for the glycerol-grown synchronous culture (experiment H) is shown in Fig. 5.12 and the parameters are tabulated in Table 5.10. The values for τ_1 and τ_2 indicate an initial lag of some five minutes which is to be expected on the basis of the explanation offered in the previous paragraph for the sucrose case. This lag is not readily discernible in the control culture presumably because of the scatter in the points and the fact that the first data point for this culture was taken at five minutes. The control has a doubling time of about 48 minutes which agrees, within experimental error, with the doubling time for the synchronous culture. The

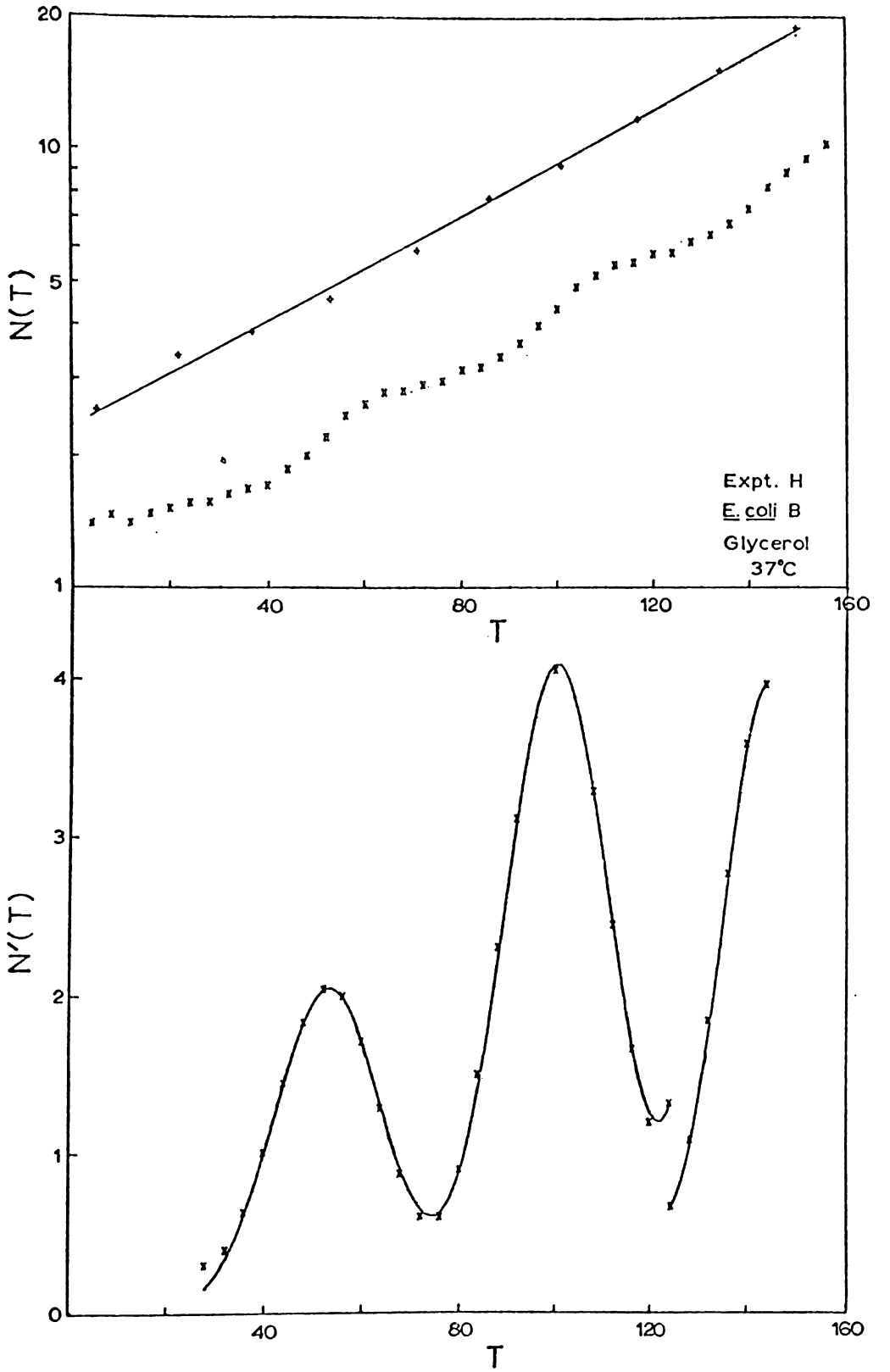


Fig. 5.12 Sum of three Gaussian functions fitted to differentiated cell concentration data after smoothing with a cut-off wavelength of eight data point spacings.

extracted values of CV and $\rho^{(1)}$ are

$$CV = 0.23 \pm 0.01$$
$$\rho^{(1)} = -0.49 \pm 0.10 \ .$$

(d) Succinate

The growth curves for experiments I and J (Fig. 4.12) show a marked difference from those of the earlier experiments. After two generations of growth in these experiments the synchrony, which is clearly established at the first generation, is effectively washed out. This is particularly so for experiment J where only a slight periodicity after the first generation of growth distinguishes the synchronous culture from the asynchronous control. We would expect in this situation that the curve fitting procedure for estimating the parameters would be rather unreliable; small fluctuations in the data points in the regions beyond the first generation will have a significant effect on the trends in the smoothed curve and, hence, on the form of the fitted functions. This is borne out when we attempt to simulate the original noisy data and fit a series of Gaussians to the curve. Large fluctuations in the estimates of σ_2 occur both for the same cut-off wavelength and for different cut-off wavelengths. On the basis of this complete uncertainty in the estimate of σ_2 yielded by this curve fitting procedure we are not able to put an error bound on $\rho^{(1)}$. However, the data for experiment J provide the estimate of σ_2 shown in Table 5.10. The variation in this parameter over cut-off wavelengths in the range 8 to 11

data point spacings leads to a standard deviation of about 10%. Thus while this parameter is reasonably well defined by our curve fitting method for the experimental data, it is difficult to obtain a reliable estimate of its uncertainty. The values given for σ_1 and σ_2 lead to a mother-daughter generation time correlation coefficient very close to zero. Visual observation of the data for experiment J suggests that a negative correlation between the generation times of mothers and daughters is absent. The uncertainties in the means τ_2 and τ_3 are better defined by the simulation procedure, having the values shown in Table 5.10. Estimates of σ_3 have not been included in this Table for either experiment since they are highly dependent on the smoothing process.

The growth curve for experiment I shows a higher degree of synchrony in the second generation than is found in experiment J. This allowed the estimation of σ_2 by the normal procedure and we see from Table 5.10 that a significant negative correlation exists between the mother and daughter cell generation times. However, a considerable shortening of the second generation is evident in this experiment. The control cultures in both of these experiments exhibit doubling times in the range 80-83 minutes. While the results of experiment J are in accord with this rate of growth, the significantly shorter interval for the second generation growth in experiment I suggests some perturbation to the growth pattern of these cells. Bearing this criticism in mind, the results for this experiment may be of doubtful value.

Fig. 5.13 shows the Gaussian fit to the data for both experiments at a cut-off wavelength of 9 data point spacings. The coefficient of variation of the generation time distribution has been estimated in both cases using σ_1 and τ_1, rather than $\tau_2 - \tau_1$. It is noteworthy, in fact, that no lag is discernible in the asynchronous controls nor apparent in the synchronous cultures; τ_1 has the value expected in both experiments, *viz*, about 80 minutes.

Further experimentation will be necessary to assess quantitatively the nature of the mother-daughter correlation in generation times for these succinate-grown cells. However, it can be stated that synchrony does not persist in these cultures to the same extent as those growing at the faster growth rates we have considered (at 37°C). The indications are that correlations, if any, between mother and daughter cell generation times are smaller in magnitude compared to the distinctly negative values we have found up to now.

(e) Glucose + Methionine + Histidine

Under these rapid growth conditions, difficulty was experienced in maintaining cultures in an unperturbed state. As with most other cultures exposed to sucrose an initial lag occurred in the control culture which was reflected in the results for the synchronous culture (Table 5.10). The doubling time of the control culture is approximately 33 minutes. No slowdown in growth over the latter stages is indicated in this culture although the synchronous culture appears to exhibit a prolonged third generation phase of growth. However the pattern of synchronous growth is not well

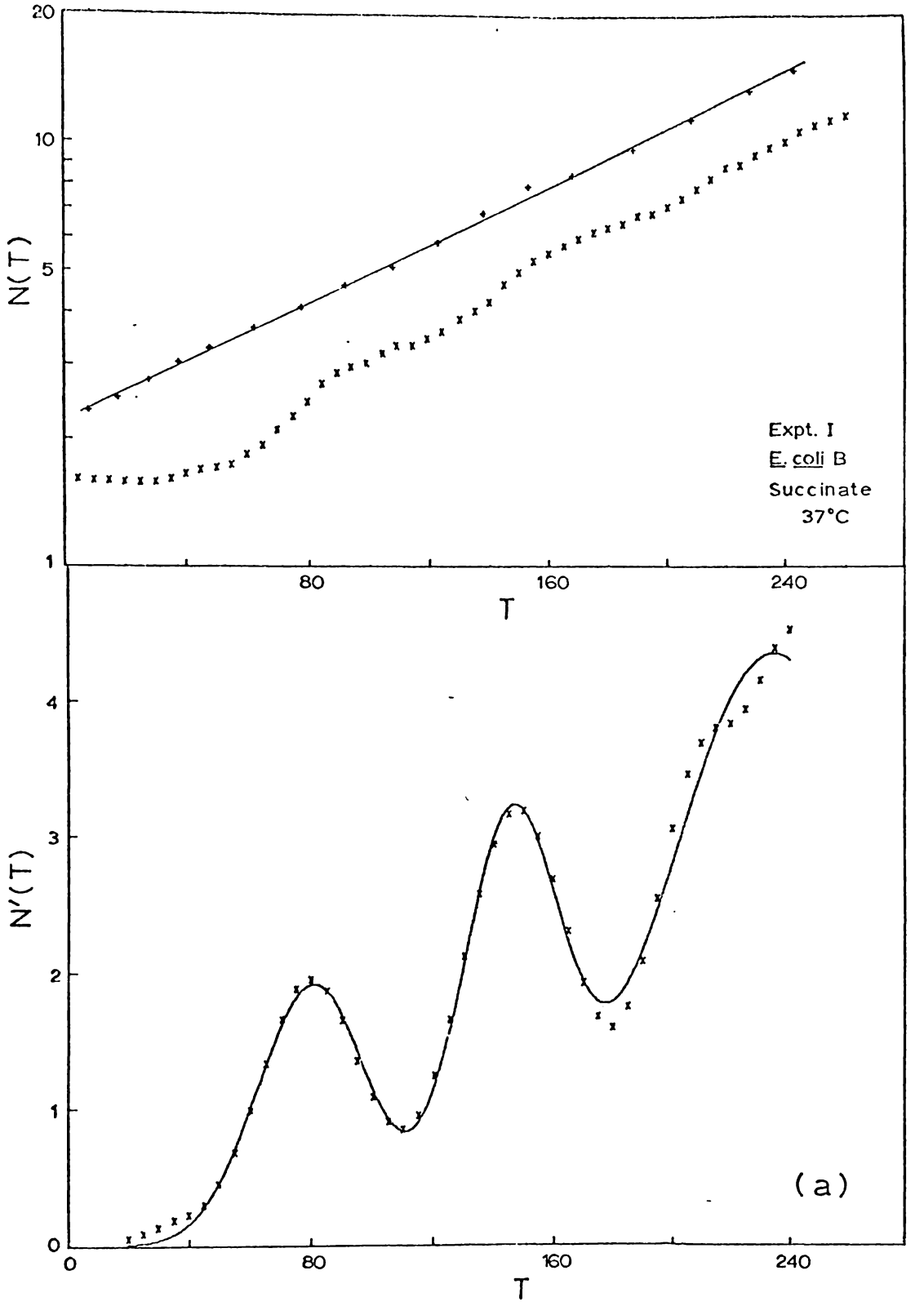
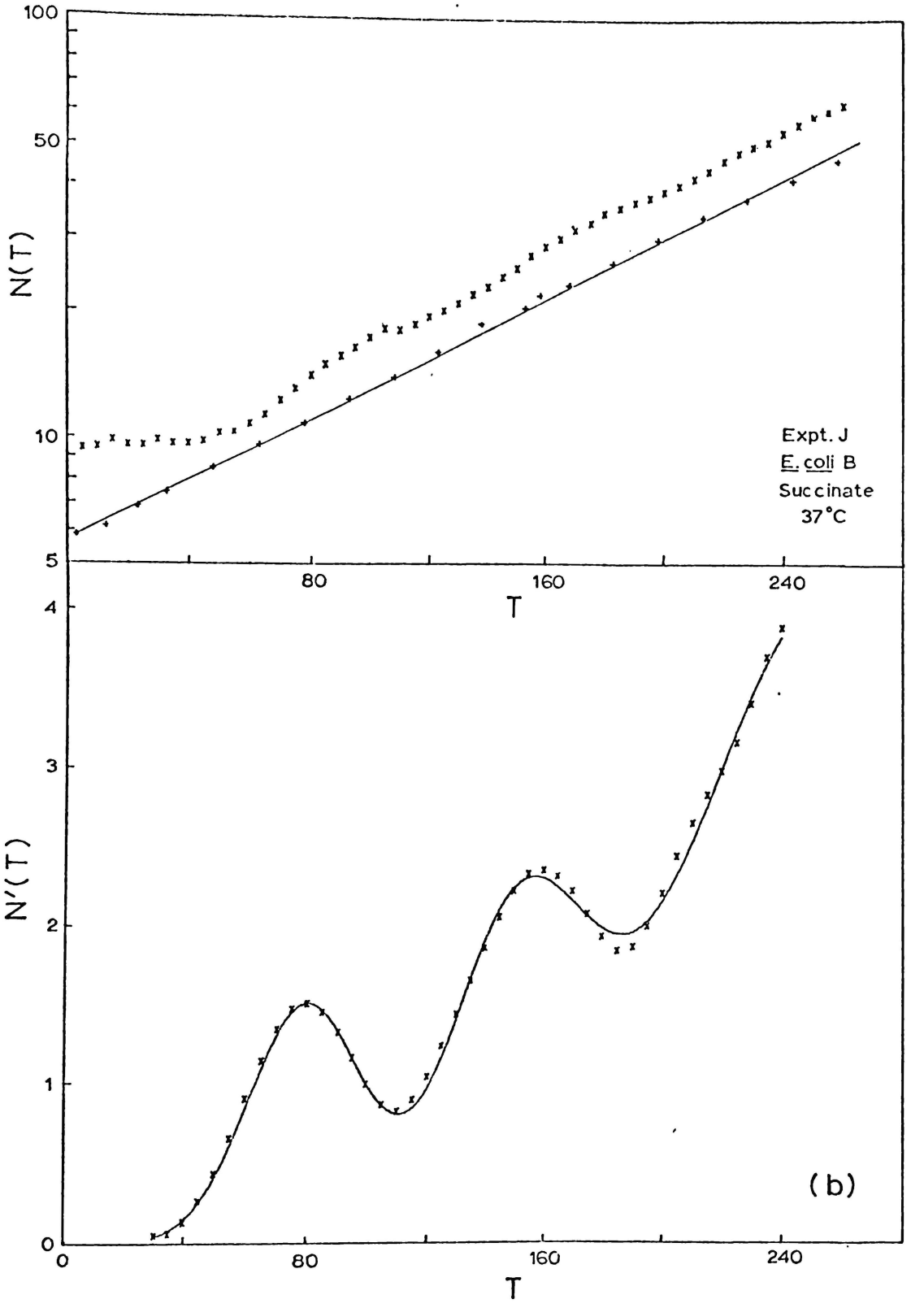


Fig. 5.13 a & b Sum of three Gaussian functions fitted to differentiated cell concentration data for experiments I and J at a digital filtering cut-off wavelength of nine data point spacings. Time (T) is measured in minutes and the ordinates are in arbitrary units.


100
50
10
5
N(T)
80
160
240
T
Expt. J
E. coli B
Succinate
37°C
4
3
2
1
0
N'(T)
0
80
160
240
T
(b)

defined in this region and, as reflected in the curve fitting results, a considerable uncertainty is attached to the estimate of τ_3. Fig. 5.14 shows the fit of a sum of Gaussians to the derivative curve. In contrast to the other experiments we have considered, the fit is not very good around the peak of the generation time distribution. In fact, the data indicate a slight negative skewness in the distribution of generation times. It is difficult, however, to place too much reliance on this observation owing to the disturbed nature of the growth of the culture. The simulation scheme for error estimation places a large uncertainty on the value for $\rho^{(1)}$.

5.6.2 Variable Temperature

Analysis of the synchronous culture data obtained at various temperatures of growth yielded the results presented in Table 5.11. The derivatives calculated for particular digital filtering cut-off wavelengths are shown in Figs. 5.15a to 5.15d for the four experiments, along with the Gaussian functions fitted by least squares.

Control cultures were not produced in experiments L and M. A sum of three Gaussians was fitted to the entire data of these experiments, as shown in Figs. 5.15a and 5.15b. Because the data have been extended only a few points into $F_3(T)$ it is clearly not possible to obtain reliable estimates of τ_3 and σ_3. Values for τ_3 have been mentioned in Table 5.11 but they are subject to considerable uncertainty. The Gaussian function appears to fit the first peak $F_1(T)$ very

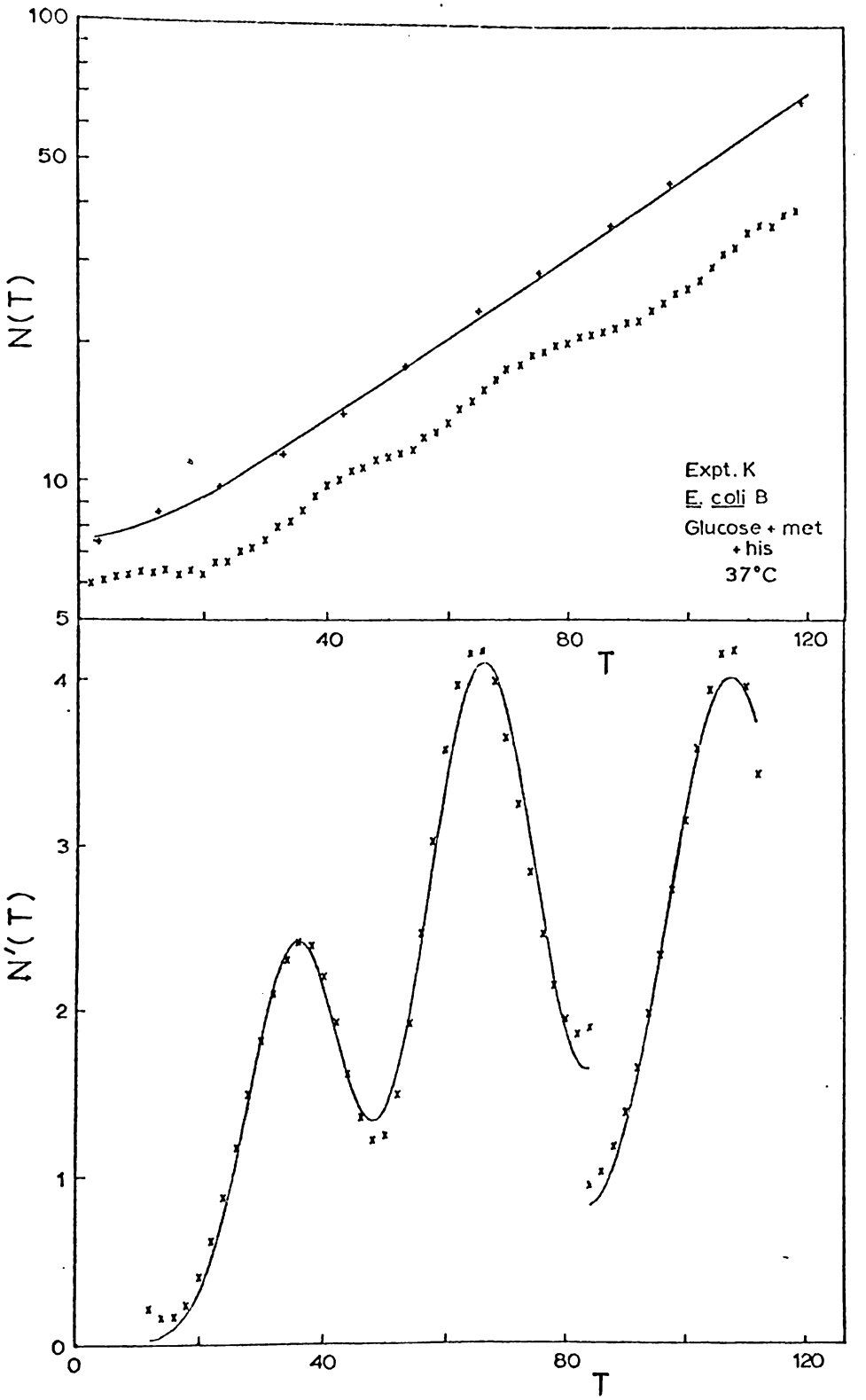


Fig. 5.14 Sum of three Gaussian functions fitted to differentiated cell concentration data after smoothing with a cut-off wavelength of ten data point spacings. Time (T) is measured in minutes and the ordinates are in arbitrary units.

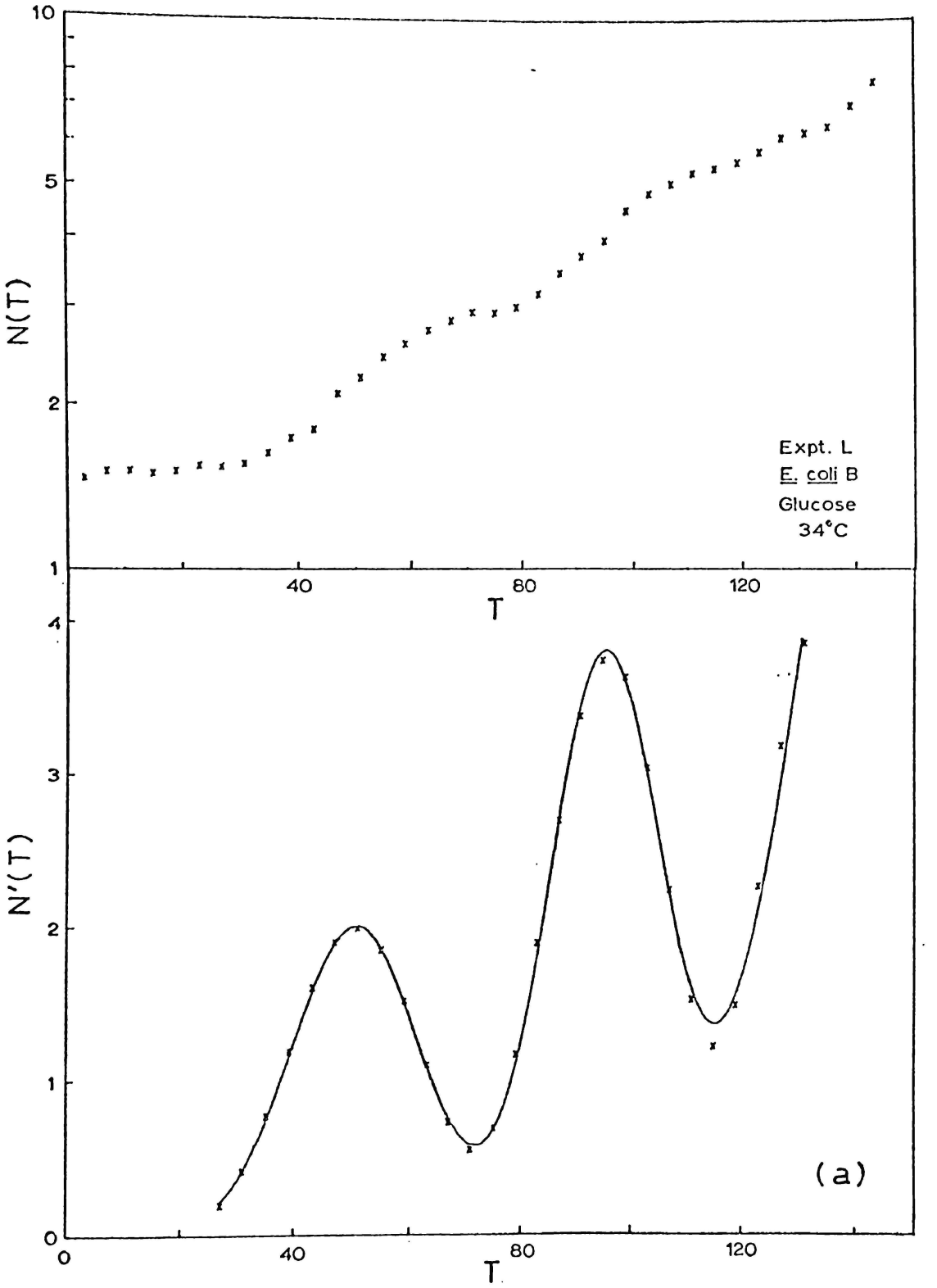
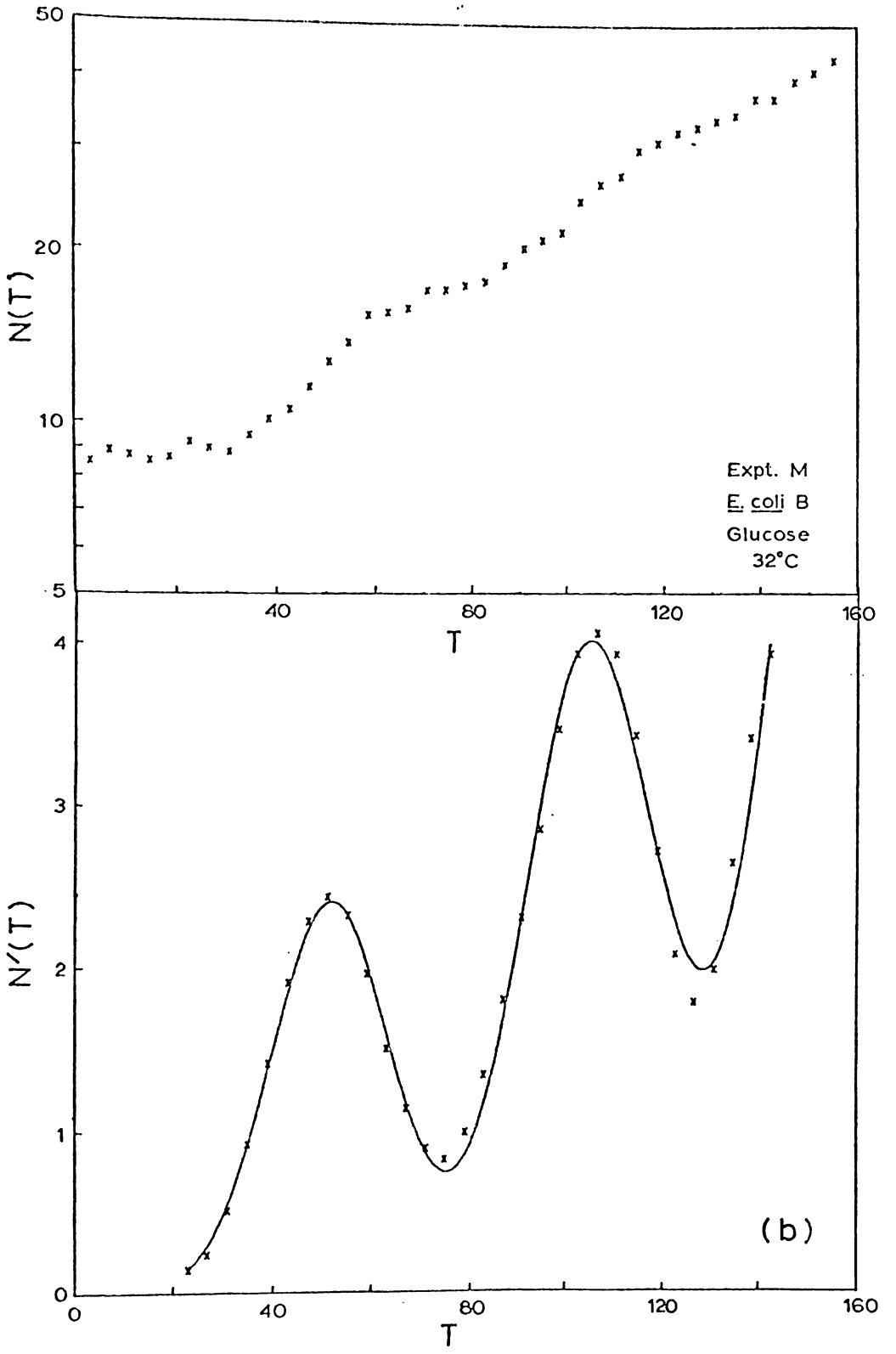
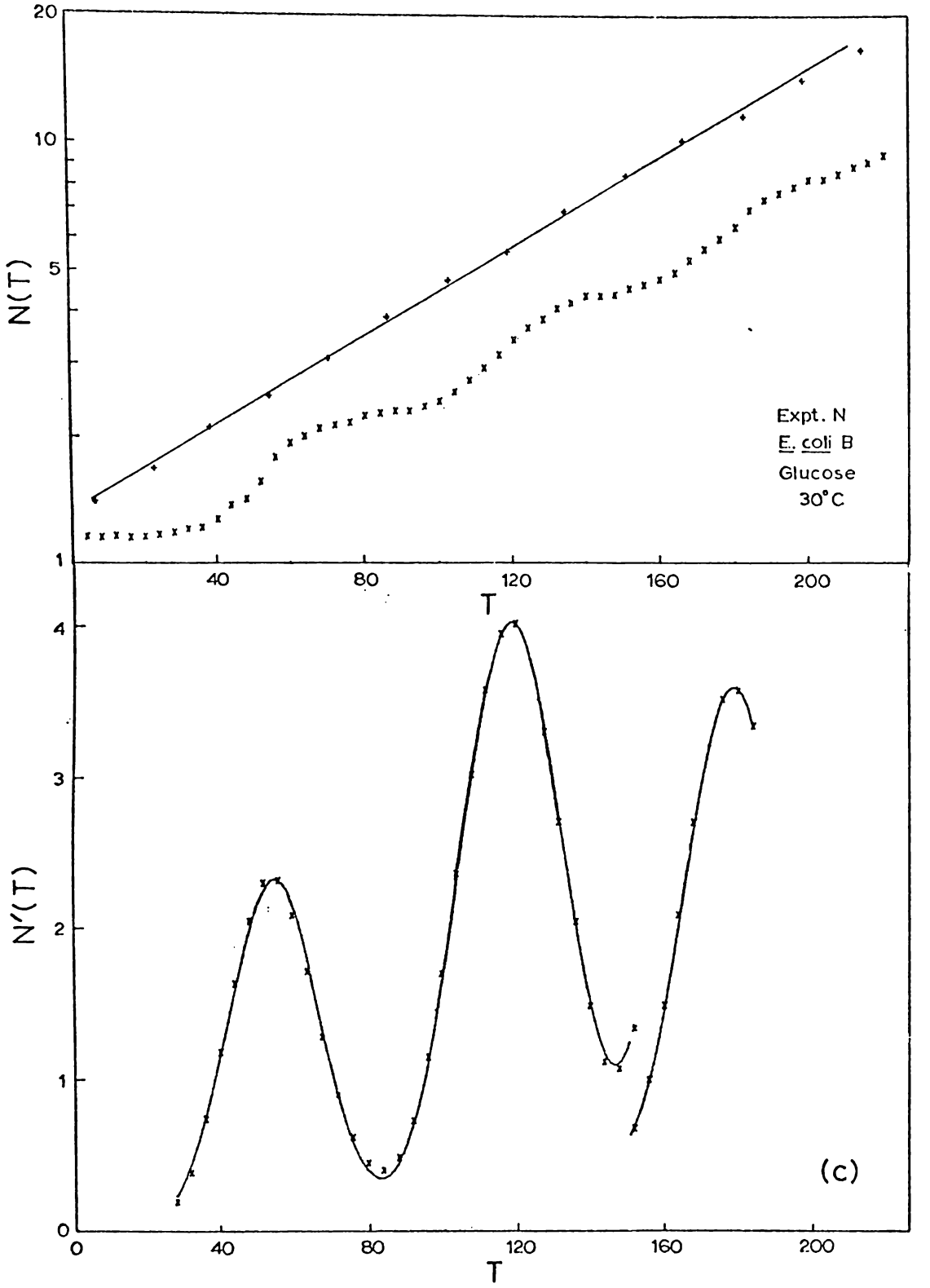
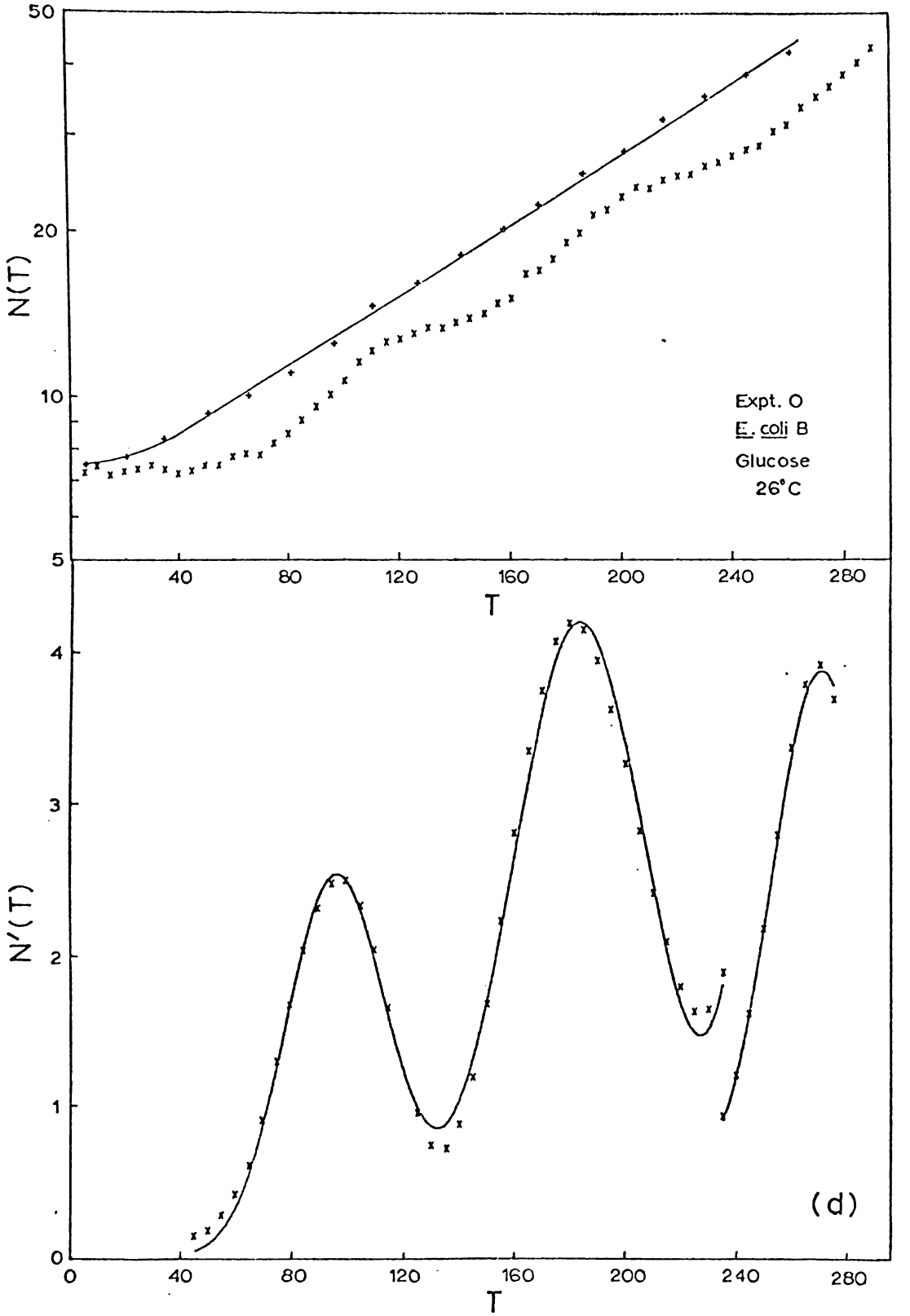


Fig. 5.15a-d Sum of three Gaussians fitted to differentiated cell concentration data for synchronous cultures at various temperatures. The data have been smoothed using digital filtering cut-off wavelengths of (a) eight, (b) eight, (c) nine, and (d) eleven data point spacings. Time (T) is measured in minutes and the ordinates are in arbitrary units.


50
20
10
5
N(T)
Expt. M
E. coli B
Glucose
32°C
40
80
120
160
T
4
3
2
1
0
N'(T)
(b)
0
40
80
120
160
T


20
10
5
1
N(T)
40
80
120
160
200
T
Expt. N
E. coli B
Glucose
30°C
4
3
2
1
0
N'(T)
(c)


50
20
10
5
N(T)
Expt. O
E. coli B
Glucose
26°C
40
80
120
160
200
240
280
T
4
3
2
1
0
N'(T)
(d)
0
40
80
120
160
200
240
280
T

well in these experiments. Values for the coefficient of variation of the generation time distribution and the correlation coefficient $\rho^{(1)}$ are in agreement with the values found earlier for glucose-grown cultures at 37°C.

The asynchronous control of experiment N shows exponential growth, without a lag, with a doubling time of 58 to 59 minutes. With this information and considering the durations of the second and third generation growth, the value derived for τ_1 is too small. This suggests that selection of cells of age greater than zero has occurred. This has been mooted earlier in connection with experiment D and, as in that case, a reduced variability in the generation time distribution results. The coefficient of variation given in Table 5.11 is based on a value of 64 minutes for the true mean of the generation time distribution. A more negative correlation coefficient ($\rho^{(1)}$) than that given, could result if this explanation holds and the true value of σ_1 is slightly larger than measured. The data for experiment N have been taken well into the third generation of growth so that estimates of the parameters of $F_3(T)$ are possible, although not without large error bounds.

The doubling time of *E. coli* B at 26°C as determined from the control culture of experiment O (Fig. 5.15d) is close to 90 minutes. This control shows an initial lag, reflected also in the synchronous culture. The weak synchrony shown by the latter after the first 'step' in the curve causes difficulties in estimating the parameters of $F_2(T)$ and $F_3(T)$. The large uncertainties associated with

TABLE 5.11 Results of Synchronous Culture Experiments with *E. coli* B at Various Temperatures using Glucose-salts Media. (Average of five cut-off wavelengths)

Expt.	Fig.	Temp. °C	Parameters of F_j (in minutes) τ_1	τ_2	τ_3	σ_1	σ_2	σ_3	$\tau_2-\tau_1$ min	$\tau_3-\tau_2$ min	CV	$\rho^{(1)}$
L	5.15a	34	50.4±0.5	95.6±0.6	146±5	11.1±0.6	10.4±0.5	-	45.2±0.8	50±5	0.25±0.02	-0.56±0.09
M	5.15b	32	51.5±0.5	105.4±1.3	161±10	11.9±0.8	14.1±1.2	-	53.9±1.4	56±10	0.22±0.02	-0.30±0.15
N	5.15c	30	54.6±1.0	118.7±1.5	179±15	12.4±0.8	14.3±1.4	14±6	64.1±1.8	60±15	0.19±0.02	-0.34±0.15
O	5.15d	26	96.6±0.8	182.8±3.0	276±20	17.8±1.2	24.4±2.9	-	86.2±3.1	93±20	0.21±0.02	-0.06±0.35

these estimates (see Table 5.11) lead to a considerable lack of precision in $\rho^{(1)}$. While the data suggest that this correlation coefficient is more positive than we have found in experiments at higher growth rates we are not able to show this quantitatively. Certainly, the data are apparently not inconsistent with a value of $\rho^{(1)}$ close to zero.

CHAPTER 6

DISCUSSION

6.1 CONCLUSIONS

Summarised in Table 6.1 are the results for the coefficient of variation of the generation time distribution and the mother-daughter generation time correlation coefficient for *E. coli* B under the various growth conditions considered in this work.

TABLE 6.1 Coefficient of Variation (CV) of the Generation Time Distribution and Mother-Daughter Generation Time Correlation Coefficient ($\rho^{(1)}$) for *E. Coli* B Under Various Growth Conditions

Experiment	Temperature °C	Carbon Source	Approximate Mean Generation Time (min)	CV	$\rho^{(1)}$
A	37	glucose	40	0.24±0.02	-0.52±0.14
B	37	glucose	40	0.27±0.03	-0.65±0.11
C	37	glucose	41	0.22±0.02	-0.39±0.13
D	37	glucose	39	0.19±0.01	-0.39±0.13
E	37	glucose	41	0.21±0.02	-0.35±0.13
F	37	sucrose	47	0.20±0.01	-0.44±0.09
G	37	sucrose	46	0.24±0.02	-0.35±0.09
H	37	glycerol	47	0.23±0.01	-0.49±0.10
I	37	succinate	80	0.23±0.02	-0.53±0.16
J	37	succinate	79	0.24±0.02	(0)
K	37	glucose/ met/his	31	0.25±0.03	-0.18±0.28
L	34	glucose	45	0.25±0.02	-0.56±0.09
M	32	glucose	54	0.22±0.02	-0.30±0.15
N	30	glucose	64	0.19±0.02	-0.34±0.15
O	26	glucose	86	0.21±0.02	-0.06±0.35

These results together with observation of the form of the generation time distributions for the experiments analyzed in the preceding chapter lead to the following important conclusions.

(1) Distribution of Generation Times

The evidence strongly supports a distribution of generation times of normal form. Over the range of growth rates covered (doubling times from 30 to 90 minutes) with growth temperatures in the range 26 to 37°C the normal curve provides a very satisfactory fit to the generation time distribution data, except in one instance. A small tendency towards negative skewness is observed in the generation time distribution for the highest growth rate considered (Fig. 5.14). The Weibull distribution discussed in Section 5.4 would perhaps provide a better fit to this data. However, too much reliance cannot be placed on the results of this experiment (experiment K). We have discussed earlier the difficulty that was experienced in maintaining steady-state conditions in cultures growing at this growth rate. The frequent sampling of the synchronous culture for this experiment (at two minute intervals) may have upset the aeration to the extent of disturbing the normal pattern of growth of the cells. In all other experiments, the normal distribution is a good approximation to the generation time distribution data. Because the data do not have an infinite range they cannot have a precisely normal form. However, the non-truncated normal distribution is an adequate approximation since negative generation times will occur with negligible

frequency in the distributions considered. It is quite likely, of course, that some minimum interdivision time exists at each growth rate. We are interested, however, in representing the main features of the generation time distribution and small adjustments can easily be made, such as truncating the fitted distribution at small times.

(2) Coefficient of Variation of the Generation Time Distribution

For all growth rates and ambient conditions the coefficient of variation of the generation time distribution for *E. coli* B is constant, within experimental error. For any particular experiment the error in the value of this coefficient was usually close to 0.02 and it may be stated that

$$CV = 0.22 \pm 0.02$$

is a suitable estimate of the coefficient of variation covering the results of all experiments.

(3) Mother-Daughter Generation Time Correlation Coefficient

With the exception of the extremes of slow growth (doubling times of 80 to 90 minutes) and rapid growth (doubling time of 30 minutes) the mother-daughter generation time correlation coefficient ($\rho^{(1)}$) is essentially constant and significantly negative at all other growth rates studied. Within the range of doubling times 40 minutes to 64 minutes $\rho^{(1)}$ varied between -0.65 ± 0.11 and -0.30 ± 0.15 with no discernible patterns in this variation. The weighted mean value of this correlation coefficient for the glucose-grown

cultures at 37°C (doubling times of approximately 40 minutes) is -0.47 ± 0.06 and, within experimental uncertainties, the estimates for the other growth conditions are consistent with this value. In other words, within the range of doubling times stated, the uncertainties associated with the values of $\rho^{(1)}$ allow for the possibility that this coefficient is constant or nearly so. A marked change occurs in the patterns of growth of the synchronous cultures in which succinate is the carbon source. These cultures (experiments I and J) exhibit doubling times close to 80 minutes at 37°C. In experiment J, synchrony rapidly weakens after the first generation of growth (Fig. 4.12b). Beyond the second generation the increase in cell numbers is essentially exponential. In such a case the method of analysis presented in the previous chapter is rather unsatisfactory. Considerable overlap of the functions $F_2(T)$ and $F_3(T)$ occurs and the smoothing and differentiation operations are very sensitive to small fluctuations in the data points in this region of the curve. As discussed in Chapter 5, a reasonable estimate of the mother-daughter generation time correlation coefficient is not possible in this case using the curve-fitting procedure. The rapid loss of synchrony in this culture suggests that $\rho^{(1)}$ has a value close to zero and corroboration of this is provided in Fig. 6.1. In this figure we have plotted the synchronous culture data of experiment J along with two growth curves obtained by integrating the sum of four Gaussian functions having peaks at multiples of 80 minutes. Both curves emanate from the same generation time distribution so that they follow the data closely over the first doubling. The solid curve represents a culture in

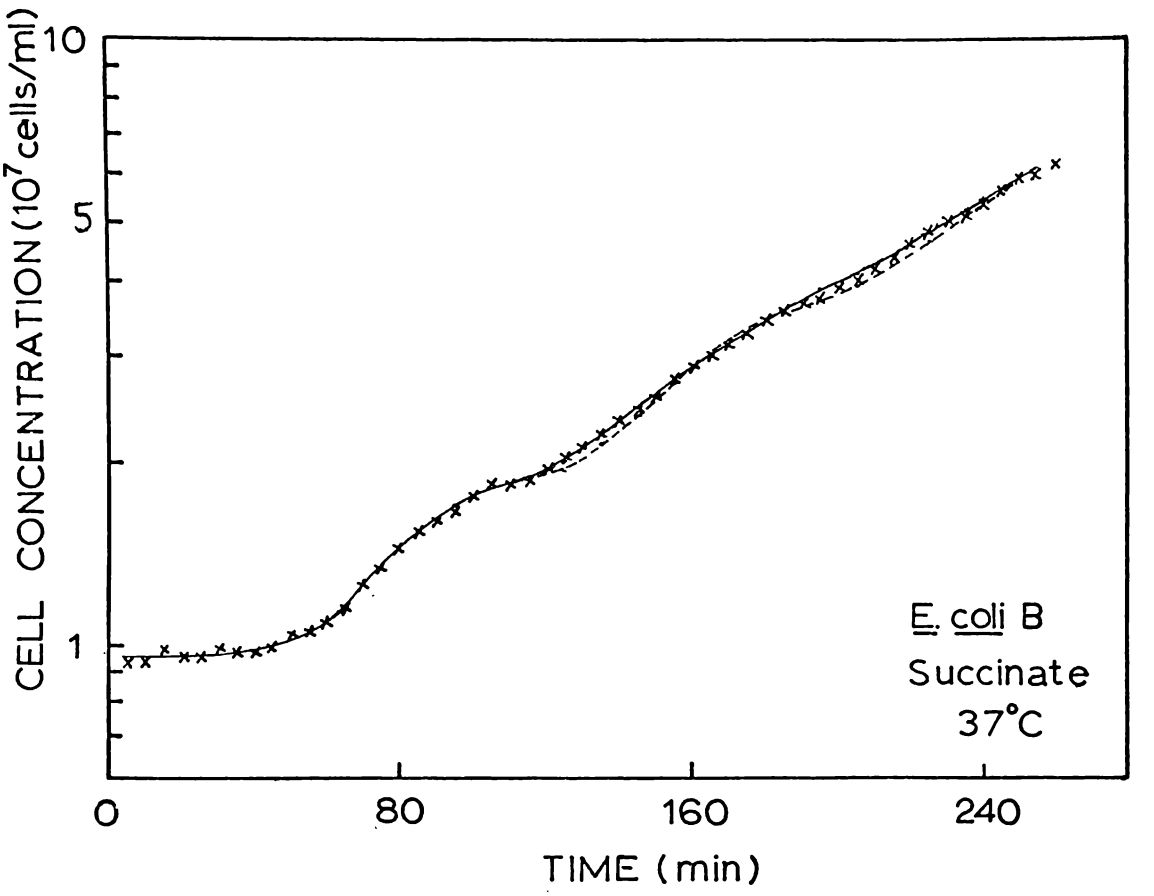


Fig. 6.1 The data of experiment J and idealized concentration curves for mother-daughter generation time correlation coefficients having the values zero (solid line) and -0.4 (dashed line).

which no dependence exists between the generation times of mother cells and their progeny. The dashed line, on the other hand, pertains to a culture in which the correlation coefficient between the generation times of mothers and daughters has a value -0.4. The correlation extending over two generations was assumed to arise purely from the presence of a non-zero $\rho^{(1)}$ so that $\rho^{(2)} = 0.16$. During the growth of the second generation cells the solid curve evidently follows the data more closely and a value for $\rho^{(1)}$ close to zero is a reasonable assumption. It is interesting to compare the curves of Fig. 6.1 with similar curves obtained for the glucose-grown synchronous culture of experiment A, shown in Fig. 6.2. The dashed line of Fig. 6.2 illustrates the case where parent-progeny generation times are independent, and is clearly at variance with the data. The solid line through the data points results when $\rho^{(1)}$ takes the value -0.5. The data of experiment I, also in succinate medium, indicate a less pronounced weakening of synchrony after the first generation (Fig. 4.12a) but the distortion in the pattern of growth of the synchronous culture, discussed previously, means that reliable conclusions cannot be drawn. The disturbed growth pattern consists mainly in a shortened phase of second generation growth and the asynchronous control culture similarly indicates an acceleration of growth from 110 to 150 minutes. By comparison with experiment J the results of experiment O (glucose medium at 26°C), in which the mean generation time is about 90 minutes, indicates that a greater degree of synchrony persists for the three generations of growth shown in Fig. 4.17. It is expected then that a negative mother-daughter generation time

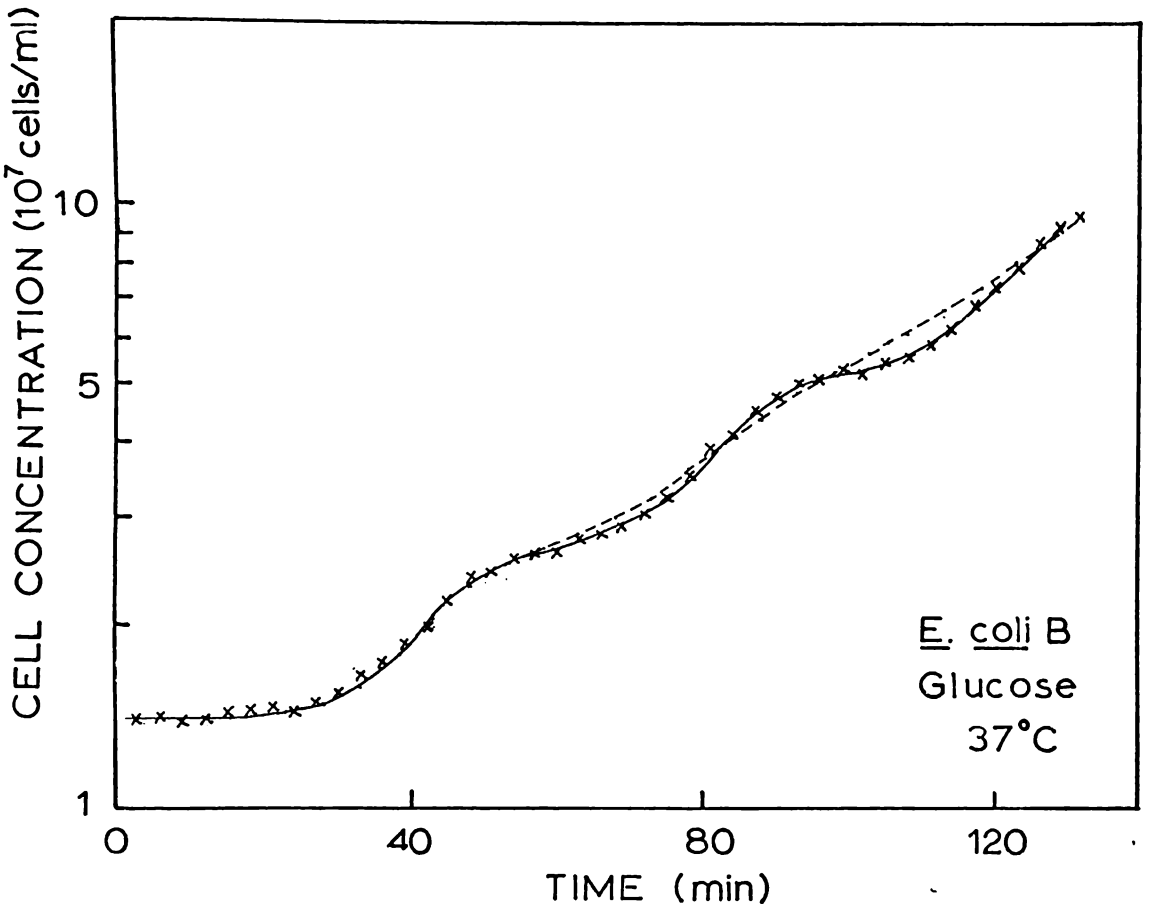


Fig. 6.2 The data of experiment A and concentration curves derived for values of the mother-daughter generation time correlation coefficients of zero (dashed line) and -0.5 (solid line).

correlation coefficient is appropriate for this case, consistent with the result of the analysis of the data. Likewise, the data for the rapidly growing culture (experiment K) suggest a negative value for this correlation coefficient although it must be borne in mind that the difficulty of maintaining this culture in a steady-state may upset the relationship between successive generations of cells.

6.2 COMPARISONS WITH PREDICTIONS OF THEORETICAL MODELS FOR CELL DIVISION REGULATION

Several models for the control of cell division have been proposed in the literature which make predictions concerning the form of the generation time distribution and the extent of association between the generation times of related cells. The results of the work presented here must be explained by any such models which purport to represent the mechanism by which a cell progresses through its division cycle. It is of interest to consider the various theoretical hypotheses for cell division regulation, outlined in Chapter 2 of this thesis, in the light of the conclusions of this work.

The hypotheses of Rahn (1932) and Kendall (1948) can be disposed of briefly, since these models do not allow for association between the generation times of related cells. The generation time in these models arises essentially probabilistically; all cells are initially identical and variability results from the stochastic nature of the individual steps that must be completed before division can occur. This probabilistic view of the generation time

characterizes models based solely on age and time as the fundamental determinants of cell growth and division; the probability that a cell will divide at some time τ is independent of its history. The experiments reported in this thesis show a very distinct correlation between the generation times of mother cells and their daughters over a wide range of growth conditions.

Further, this correlation is negative. Indeed, the existence of a positive correlation between the generation times of mother cells and their daughters would mean, without some compensating tendency operating over longer times, that a stable generation time distribution would never be observed. This point is discussed in greater detail later. A number of models, which we discuss now, can admit correlations between the generation times of related cells. Not all of these models, however, can allow complete symmetry in the distribution of generation times. We deal with this aspect of the generation time distribution before discussing explicitly the correlations predicted by certain models.

All the results obtained in this work indicate that positively skewed distributions, such as the lognormal, Pearson Type III, or reciprocal normal, will not provide a fit to the generation time data as satisfactory as that given by a normal curve. It is clear then that the model proposed by Kubitschek (1971b) in which a reciprocal normal distribution of generation times is predicted, is untenable. The hypothesis of Kendall (1948) leading to a Pearson Type III distribution

of generation times is also discounted on this basis. Through the central limit theorem we may expect a normal distribution to arise when a large number of sources of variation enter together to give rise to the final distribution. Koch (1966c) has shown how normal and lognormal distributions may be generated, depending on how the large number of individual errors combine to produce the distribution. Addition of errors gives rise to the normal distribution, and multiplication of proportionate errors the lognormal distribution. It was mentioned by Burns (1962) that random variations causing generation times to deviate from the mean would lead to a normal distribution of generation times.

A symmetrical generation time distribution is consistent with the hypothesis of Koch & Schaechter (1962) under which cell size regulates division. Under assumptions which are basically those of Koch & Schaechter, we have derived earlier an expression for the distribution of generation times (equation 2.33) by means of the mass distribution model of Eakman *et al.* (1966). This derivation assumed equal size daughters at division and a distribution of division masses having normal form. We have fitted this distribution to a set of experimental data for the generation time distribution. The results of experiment A indicate a generation time distribution of normal form with mean, $\bar{\tau}$, close to 40 minutes and variance equal to 91 (minutes)^2. Assuming this distribution is precisely Gaussian with these parameters, we have fitted, by the nonlinear least squares procedure, the

distribution $f(\tau)$ of equation (2.33). The result is illustrated in Fig. 6.3. The Gaussian distribution was represented by points at two minute intervals, as shown in the figure, and the function $f(\tau)$ was fitted at these points. A smooth curve has been drawn through the points representing the fitted values. A very close fit, nearly indistinguishable from the normal distribution, is obtained for the parameter values

$$k = 0.01733 ,$$

$$\frac{m_c}{\varepsilon} = 6.05$$

where, it will be remembered, k is the mass growth rate constant for the whole population and is taken to be the same as that for an individual cell, m_c is the mean division mass, while $\varepsilon/\sqrt{2}$ is the standard deviation of the division mass distribution. As we expect, from the analysis leading to equation (2.31),

$$\frac{\ln 2}{40} = 0.01733 ,$$

i.e.,

$$k = \frac{\ln 2}{\bar{\tau}} .$$

The coefficient of variation for the division mass distribution is $\varepsilon/\sqrt{2}m_c$ which, in this example, has the approximate value 0.12. As expected this coefficient is close to one-half of the coefficient of variation for the corresponding generation time distribution (0.24 for experiment A). This relationship between the coefficients of variation was shown by Powell (1964) to hold, under certain conditions which are

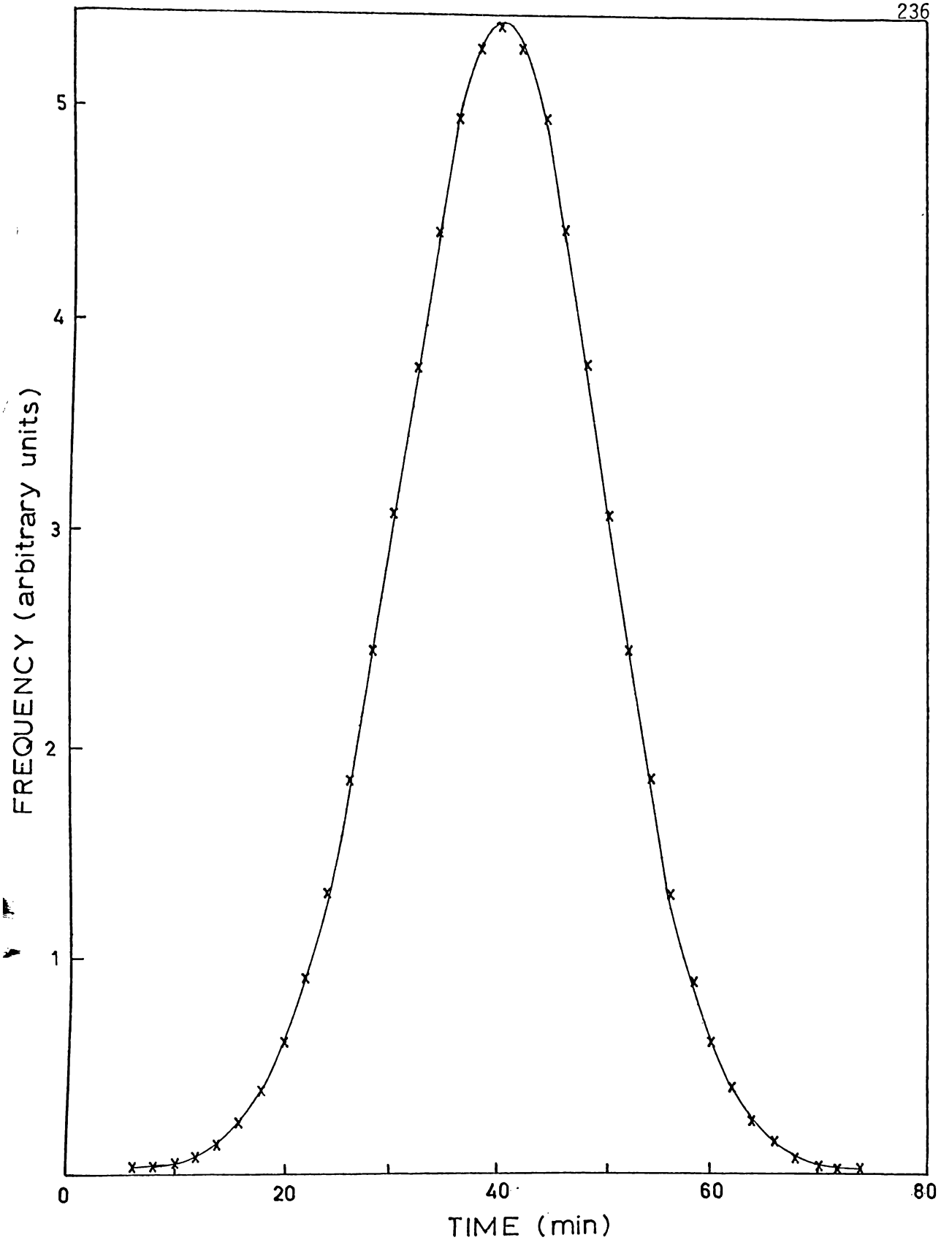


Fig. 6.3 Generation time distribution from the mass distribution model (solid curve) fitted to a Gaussian distribution (x) representing the generation time distribution of experiment A.

obeyed here, when fission is symmetrical.

Koch & Schaechter's (1962) model leads to a mother-daughter generation time correlation coefficient of -0.5 for precisely even division. The symmetry of the distributions of generation time obtained in the work here suggests that such even division operates, provided the other assumptions of the Koch & Schaechter hypothesis hold. Inequalities in the division process will cause a reduction in the magnitude of the correlation coefficient, $\rho^{(1)}$. Koch & Schaechter (1962) show that, approximately,

$$\rho^{(1)} = - \frac{q^2}{2q^2 + q_h^2} \qquad (6.1)$$

where q is the coefficient of variation of the division mass distribution and q_h is the coefficient of variation of the distribution of p where

$$p = \frac{\text{volume of daughter cell at division}}{\text{volume of parent cell at division}} .$$

Expression (6.1) is derived on the assumption that the dispersion of the division mass distribution is the same for each successive generation. It is clear $\rho^{(1)}$ can vary from -0.5 to zero depending on the magnitude of q_h relative to q. Marr *et al.* (1966) determined the distribution of p for *E. coli* strain ML30, the coefficient of variation being quite small and close to 4%. Taking $q_h = 0.04$ and assuming a coefficient of variation for the division mass distribution of 0.12 we find from equation (6.1) that

$$\rho^{(1)} = -0.47 ,$$

in agreement with our estimation of this coefficient for glucose-grown cultures of *E. coli* B at 37°C. Asymmetrical division into daughter cells introduces, however, positive skewness into the generation time distribution. Powell (1964) has rigorously shown the validity of this assertion. While the results reported in this thesis indicate a mother-daughter generation time correlation coefficient generally more positive than -0.5 over the range of doubling times 40 to about 60 minutes, no evidence of positive skewness in the generation time distributions is observed. In addition, a significant change in the degree of correlation between mother and daughter cell generation times is apparent in the succinate-grown cultures. The Koch & Schaechter hypothesis would seem unable to explain such a difference in the magnitude of $\rho^{(1)}$ between these fast and slow growing cultures. It is possible that the growth of individual cells is not exponential, as assumed in the Koch & Schaechter model, but follows a more complicated law. Indeed, measurements by Marr *et al.* (1969) show that the rate of growth of an *E. coli* bacterium is not proportional to its size. It was mentioned in Chapter 2 of this thesis that the analysis of the Koch & Schaechter model by these workers indicated, in addition, the incorrectness of the assumption of Koch & Schaechter that the size of an organism at division is independent of its size at inception. It is more likely that cell size plays an <u>indirect</u> role in the regulation of division.

Evidence exists that the initiation of DNA replication

in *E. coli* B/r occurs when the cell reaches a fixed volume (mass) per chromosomal origin in cultures growing with doubling times less than 70 minutes (Donachie, 1968). In slower growing cultures, however, Helmstetter (1974) has presented results which indicate that initiation occurs at smaller volumes per chromosomal origin. Pierucci (1972), on the other hand, was not able to draw definite conclusions about the relationship between cell size and initiation from results on fast and slow growing cultures of *E. coli* B/r at 37°C and lower temperatures. A close coupling exists between DNA replication and division. Rowbury (1972) has reviewed experimental work on the relationships between initiation and termination of DNA replication and cell division. He has suggested that a double control on division rate may exist in most organisms; requirements for division may be met jointly through an initiation mechanism as well as the termination of DNA replication. We have discussed earlier (see Chapter 2) that the Cooper-Helmstetter hypothesis (Cooper & Helmstetter, 1968) relating initiation of DNA synthesis, termination of a round of replication, and subsequent cell division, can adequately predict the growth of synchronous cultures. The analysis of Marr *et al.* (1969) predicts a generation time distribution having very little skewness in order to obtain agreement with a synchronous culture growth curve for *E. coli* produced by the membrane selection technique. While the precise mechanism of control of initiation is unclear, it is apparent that associations between the generation times of parent and progeny cells may exist, at certain growth rates, because of this intimate

connection between initiation and subsequent cell division and the fact that, at these growth rates, initiation occurs during the previous division cycle. For instance, in cultures growing with a doubling time of 40 minutes at 37°C, an initiation at some time, T, will result in a division 63 minutes later, according to the Cooper-Helmstetter model, and during that time a division will have occurred (at T + 23 minutes, on average). Thus events in one cycle are closely coordinated with events in the next. It is feasible to suggest that some correlation exists in this case between mother and daughter cell generation times. If a tendency towards positive correlation existed, that is, mothers and daughters tend to have essentially the same generation time on the average, then the cells with the shortest generation time would ultimately dominate the population. In this case an invariant generation time distribution would never be observed. Lebowitz & Rubinow (1974) have discussed this point in connection with their theory in which, over a few generations, memory of the generation time tends to be passed on to the descendants, but over long times this memory is diffused so that a generation time distribution is observed. A perfect memory model was presented by Rubinow (1968) who adopted a maturity-time formalism where the concept of 'maturation velocity' of a cell was introduced, this parameter being preserved from one generation to the next so that the generation times of daughter cells were identical to those of their mothers. Such a model seemed to fit the experimental results of Prescott (1959) on animal cells, but no evidence was found in the work reported in this thesis that significant positive correlations exist between mother

and daughter cell generation times. It is reasonable to suggest, particularly if cell size plays a part in determining the time of initiation and the time of division, that a negative correlation could exist between the generation times of mother cells and their daughters in cases where the events of one generation are coordinated with those of another. This argument would hold for all doubling times greater than 31.5 minutes and less than 63 minutes at 37°C, provided the times C (the duration of a round of replication) and D (the time between the end of a round of replication and the following division) are taken to be 41 and 22 minutes respectively. At doubling times greater than 63 minutes (at 37°C), initiation occurs at cell division and thus a linkage between successive division cycles of the type mentioned is absent. Succinate-grown cultures, with doubling times of about 80 minutes at 37°C, would then be expected to exhibit a different pattern of growth compared to the faster growing cultures we have examined. A zero correlation coefficient between mother and daughter generation times is then plausible, consistent with experiment J. At doubling times less than 31.5 minutes, initiation is two division cycles removed from the fission it leads to. In other words, events occurring in a particular cycle are coupled to events occurring in the next cycle but one. These cultures would again be expected to show a somewhat different pattern of growth. It is conceivable that these fast growing cultures, and experiment K in this work, show correlations between the generation times of mothers and daughters and between grandmothers and granddaughters which are significantly different

from the values observed in glucose-grown cultures. We have not been able to show this in the work presented here. According to the results of Pierucci (1972) these arguments would hold also for temperatures less than 37°C. Pierucci found that the periods C and D were equally affected by temperature and, within experimental error, the relative lengthening in C + D, as the temperature was lowered, was the same as that for the doubling times, for all growth media studied. All the experiments reported in this thesis at reduced temperatures were conducted in glucose minimal medium so that the relationships between initiation and division occurring in glucose medium at 37°C would also be expected to hold at lower temperatures. Thus the increases in the times C and D would not be expected to alter the correlations assigned, in the discussion above, to the glucose-grown cultures at 37°C. Our results are in fact consistent with a $\rho^{(1)}$ value in the range -0.4 to -0.5 for all temperatures in glucose medium (noting that a large uncertainty is attached to the result for experiment O at 26°C).

6.3 COMPARISONS WITH OTHER WORK

Synchronous culture data of adequate precision covering a range of growth rates are not available in the literature with which to make comparisons. However, Helmstetter (1969) has published data for glucose-grown *E. coli* B/r at 37°C in which a synchronous culture, produced by the membrane selection technique, was sampled every three minutes. Harvey (1972b) has analyzed these results using the

techniques we have described except that the means and variances of the functions $F_j(T)$ were obtained by straightforward summation. No significant skewness was found in the generation time distribution, which had a coefficient of variation of 0.18. The mother-daughter generation time correlation coefficient was given as

$$\rho^{(1)} = -0.40 \pm 0.08$$

in agreement with our estimates of this parameter under the same conditions of growth. Coefficients of variation for the generation time distributions of *E. coli* have generally been observed to fall in the range 0.18 to 0.23 (Schaechter *et al.*, 1962; Kubitschek, 1962a; Powell & Errington, 1963; Shehata & Marr, 1970). Kubitschek, Freedman, & Silver (1971) have asserted that the shape of the distribution does not appear to change with growth rate, in agreement with the results in this thesis.

6.4 GRANDMOTHER-GRANDDAUGHTER GENERATION TIME CORRELATIONS

Two experiments (D and E) reported in this work were used to estimate the grandmother-granddaughter generation time correlation coefficient, $\rho^{(2)}$. The uncertainties attached to these estimates was so large as to make any quantitative conclusions impossible. The difficulty in estimation arises because the calculation of $\rho^{(2)}$ involves the difference of two nearly equal terms, each having an associated uncertainty of, generally, at least 10%. From equations (5.19), (5.20), and (5.21) the expression for $\rho^{(2)}$ is, in fact,

$$\rho^{(2)} = \frac{\sigma_1^2 + \sigma_3^2 - 2\sigma_2^2}{2\sigma_1^2} ,$$

where we have neglected σ_0^2. The three variances estimated for each of experiments D and E are of comparable magnitude and so lead to difficulties in measuring $\rho^{(2)}$. The values found for this coefficient are consistent with this correlation arising only from the mutual correlation with the daughter cell generation time. It is difficult to see how improved precision can be attained in the results reported here, using present counting techniques. As such, the nature of correlations extending over more than one generation must remain essentially inaccessible using synchronous culture techniques.

6.5 CONCLUDING REMARKS

Synchronization has been assumed, throughout this work, to be initially perfect. In other words, the starting culture consists initially only of newborn cells. If in fact a range of ages is present in this sample the resultant increased dispersion in the functions $F_j(T)$ was shown to be accounted for by an additive variance term σ_0^2 (equations (5.19) to (5.21)). This term represents the age dispersion of the initial sample. The discussion in Chapter 4 where the size distributions of selected fractions from a band of cells in a gradient were measured and assessed indicated that the initial sample for the synchronous culture possessed a size distribution similar to that found for newborn cells in the membrane selection procedure. It is reasonable to suggest

that the age dispersion of this initial sample is suitably narrow and satisfies the requirement of near-perfect synchrony at zero time. Examining equations (5.19) and (5.20) it is clear that in instances where the variances σ_1^2 and σ_2^2 are equal or nearly so the effect of σ_0^2 on the computed correlation coefficient $\rho^{(1)}$ will be small. This effect will become more significant as the difference between σ_1^2 and σ_2^2 increases. Some of the experiments reported here show σ_2^2 to be considerably larger than σ_1^2; however, the experimental uncertainties in the values of σ_2^2, and hence of $\rho^{(1)}$, are generally large in these cases and the effect of σ_0^2 will again tend to be insignificant.

So that sufficiently precise cell numbers could be obtained rapidly it was imperative in this work that an instrument of the Coulter type be used for cell counting. Referring back to Figs. 6.1 and 6.2, we can see graphically the magnitude of the effects we are attempting to discover. At 70 minutes in Fig. 6.2 the difference between the two curves amounts to some 5%. The need for extreme precision in the estimation of cell counts is clear. The Coulter particle counter will not of course differentiate between two cells stuck together and one large cell so that our criterion for cell division is obviously mechanical separation of the two daughter cells. It is expected, however, that cells which have completed division and remain attached will generally become separated during the agitation received while being diluted or under the forces they endure near the Coulter aperture while being counted. In this connection, it

is worth mentioning that in the experiments of Schaechter *et al.* (1962) the coefficient of variation for *E. coli* was as high for the nuclear division as for the cellular division (mechanical separation). Maaløe (1962) pointed out that these results, although subject to much uncertainty, tend to argue against the conclusion of Powell (1958) that division, in the sense of mechanical separation, is less rigorously controlled than physiological separation.

Several of the experiments in this work exhibited a short initial lag in growth. Control cultures followed simultaneously showed the same lag suggesting that its cause was not confined to the youngest cells of the culture. This being the case it is unlikely that our main conclusions will be affected by their presence. However, they are an unsatisfactory feature of certain experiments, in particular those in which sucrose gradients have been used. Sucrose has been generally employed as a gradient material in this work and other work because it gives satisfactory resolution and is applicable to a wide variety of cells. One criticism that has been raised (Rowbury, 1972) is that plasmolysis may be induced in certain cells by the sucrose and the relation between cell division and DNA synthesis perturbed. It would be of interest then to check the results obtained here using a different gradient material, Ficoll for instance. While this material has been used for the succinate-grown cultures it has the disadvantage of reduced resolution and the fact that aggregation may occur with some strains of *E. coli* (Koch & Blumberg, 1976).

Much of the attraction of the density gradient centrifugation technique lies in the fact that it is applicable to a wide range of cell types. The other standard selection procedures for obtaining synchronous cultures have so far been limited to very few strains of bacteria. It would be of great interest to obtain data, of comparable precision to that presented in this work, for other strains of *E. coli*, other species of bacteria, and other cell types. Armed with these data the possibility presents itself of modelling the cell division process in a variety of organisms and observing similarities or differences between diverse species. Chatterjee, Taber & Young (1971) succeeded in synchronizing *Bacillus subtilis* and *Staphylococcus aureus* using the sucrose gradient method. It is interesting to note that several precautions were necessary during their experiments in order that successful synchrony was obtained. For instance, it was important to centrifuge the parent cultures at a low speed and, in the case of *S. aureus*, cold gradients (2-4°C) were necessary to obtain results. Although the reasons for these small modifications are not clear, perseverance with the gradient centrifugation technique would seem to guarantee results for a range of cell types. Microscopic observations by Powell (1955, 1956a) indicate coefficients of variation of the generation time distributions of various species of bacteria ranging from less than 15% to about 50%. *Bacillus* species tended to have rather broad generation time distributions (coefficients of variation greater than 30%) although the synchronous culture growth curve for *B. subtilis* found by Chatterjee *et al.* (1971) indicates a considerably narrower

distribution. A great deal of experimental work is required to establish with precision the characteristics of the generation time distributions for various species of bacteria and other cells and to determine the extent of correlation between the generation times of related cells. With the availability of very precise data for a range of organisms some progress may perhaps be made toward the establishment of a unifying theory for the control of cell division.

The objectives outlined in the Introduction to this thesis consisted principally in resolving the conflict regarding the form of the generation time distribution for *E. coli* and the magnitude and size of the correlation between the generation times of mother and daughter cells. We have successfully established that the generation times of *E. coli* B under a wide variety of growth conditions are distributed in a way which closely resembles a Gaussian distribution. We can also state categorically that, over a limited range of growth rates and ambient conditions, the mother-daughter generation time correlation coefficient is significantly negative for this species of bacteria. The data presented for all growth conditions exhibit clear features which must be explained by theoretical models proposed to account for the variability in cell generation times and the mechanism by which a cell controls its own division.

APPENDICES

APPENDIX A

SOLUTIONS FOR THE MODELS OF RAHN AND KENDALL

A.1 RAHN'S HYPOTHESIS

The differential equations for this model, derived in Section 2.2, are

$$\frac{dP_x(\tau)}{d\tau} = -\beta(g-x)P_x(\tau) + \beta(g-x+1)P_{x-1}(\tau) \qquad (A.1)$$

$$x = 1,2,\ldots,g$$

$$\frac{dP_o(\tau)}{d\tau} = -\beta g P_o(\tau) . \qquad (A.2)$$

These are to be solved subject to the initial conditions

$$P_x(0) = \begin{cases} 1 & x = 0 \\ 0 & x = 1,2,\ldots,g \end{cases} \qquad (A.3)$$

Straightforward integration of equation (A.2) yields

$$P_o(\tau) = e^{-\beta g \tau}$$

satisfying the initial condition (A.3). We now seek the solution of equation (A.1) for $x = 1$. This can be obtained using the Laplace transformation. We denote by $\overline{P_x}(s)$ the Laplace transform of $P_x(\tau)$. Transforming both sides of equation (A.1) with $x = 1$, we obtain

$$s\overline{P_1}(s) = -\beta(g-1)\overline{P_1}(s) + \beta g \overline{P_o}(s)$$

where we have used the initial condition $P_1(0) = 0$.

Rearranging, and noting that

$$\overline{P_o}(s) = \frac{1}{s + \beta g},$$

we find

$$\overline{P_1}(s) = \frac{\beta g}{(s + \beta g)\{s + \beta(g - 1)\}}.$$

This has the well-known inverse*

$$P_1(\tau) = g\{e^{-\beta(g-1)\tau} - e^{-\beta g\tau}\}$$

$$= g\, e^{-\beta(g-1)\tau}\,(1 - e^{-\beta\tau}).$$

Similarly, for $x = 2$, we obtain after a transformation of the equation (A.1)

$$\overline{P_2}(s) = \frac{\beta g\ \beta(g-1)}{(s + \beta g)\{s + \beta(g-1)\}\{s + \beta(g-2)\}}$$

which has inverse

$$P_2(\tau) = \tfrac{1}{2} g(g-1)\{e^{-\beta g\tau} - 2e^{-\beta(g-1)\tau} + e^{-\beta(g-2)\tau}\}$$

$$= \tfrac{1}{2} g(g-1)\, e^{-\beta(g-2)\tau}\,(1 - e^{-\beta\tau})^2.$$

Employing the method of proof by induction, we now assume that the solution for $x = m$ is given by

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Tables of Laplace transforms may be found in, for example, Abramowitz & Stegun (1965).

$$P_m(\tau) = \binom{g}{m} e^{-\beta(g-m)\tau}(1-e^{-\beta\tau})^m$$

where $$\binom{g}{m} = \frac{g!}{m!\,(g-m)!} .$$

We wish to find the solution for $x = m+1$. Equation (A.1) now reads

$$\frac{dP_{m+1}(\tau)}{d\tau} = -\beta(g-m-1)\,P_{m+1}(\tau) + \beta(g-m)\,P_m(\tau) .$$

Applying the Laplace transformation to both sides yields

$$s\bar{P}_{m+1}(s) = -\beta(g-m-1)\,\bar{P}_{m+1}(s) + \beta(g-m)\,\bar{P}_m(s) .$$

Therefore,

$$\bar{P}_{m+1}(s) = \frac{\beta(g-m)}{s+\beta(g-m-1)}\,\bar{P}_m(s) .$$

By the convolution theorem for Laplace transforms we can write immediately

$$P_{m+1}(\tau) = \int_0^\tau \beta(g-m)\,e^{-\beta(g-m-1)(\tau-\eta)}\binom{g}{m} e^{-\beta(g-m)\eta}(1-e^{-\beta\eta})^m\,d\eta$$

$$= \binom{g}{m}\beta(g-m)\,e^{-\beta(g-m-1)\tau}\int_0^\tau e^{-\beta\eta}(1-e^{-\beta\eta})^m\,d\eta .$$

A simple integration gives

$$P_{m+1}(\tau) = \binom{g}{m}\frac{g-m}{m+1}\,e^{-\beta(g-m-1)\tau}(1-e^{-\beta\tau})^{m+1}$$

$$= \binom{g}{m+1} e^{-\beta\{g-(m+1)\}\tau}(1-e^{-\beta\tau})^{m+1} .$$

Hence, if the solution is true for $x = m$ it is true for $x = m+1$, and so for all x, since it holds for $x = 0$.

For $x = g$ we therefore have

$$P_g(\tau) = (1 - e^{-\beta\tau})^g .$$

A.2 Kendall's Hypothesis

We derive here the solution to the Poisson process represented by the system:

$$\frac{dP_x(\tau)}{d\tau} = -\beta P_x(\tau) + \beta P_{x-1}(\tau) \qquad (A.4)$$

$$x = 1,2,\ldots,g$$

$$\frac{dP_o(\ )}{d\tau} = -\beta P_o(\tau) \qquad (A.5)$$

with the initial conditions

$$P_x(0) = \begin{cases} 0 & x = 0 \\ 1 & x = 1,2,\ldots,g \end{cases} . \qquad (A.6)$$

We again let $\bar{P}_x(s)$ denote the Laplace transform of $P_x(\tau)$. Application of the Laplace transformation to equation (A.5) yields, under the initial condition,

$$s\bar{P}_o(s) - 1 = -\beta\bar{P}_o(s)$$

or,

$$\bar{P}_o(s) = \frac{1}{s+\beta} . \qquad (A.7)$$

Inversion gives

$$P_o(\tau) = e^{-\beta\tau} .$$

Now, transforming equation (A.4), we have

$$s\,\bar{P}_x(s) = -\beta\,\bar{P}_x(s) + \beta\bar{P}_{x-1}(s)$$

or,

$$\bar{P}_x(s) = \frac{1}{s+\beta}\,\bar{P}_{x-1}(s) .$$

From equation (A.7) we obtain

$$\bar{P}_1(s) = \frac{\beta}{(s+\beta)^2} \quad , \quad \bar{P}_2(s) = \frac{\beta^2}{(s+\beta)^3}$$

and, in general,

$$\bar{P}_x(s) = \frac{\beta^x}{(s+\beta)^{x+1}} .$$

Inverting this last expression we obtain the Poisson distribution

$$P_x(\tau) = \frac{(\beta\tau)^x}{x!}\, e^{-\beta\tau} . \qquad \text{(A.8)}$$

Kendall's general case

The general system of equations (2.19), *viz.*,

$$\frac{dP_o(\tau)}{d\tau} = -\beta_1 P_o(\tau) \qquad \text{(A.9)}$$

$$\frac{dP_x(\tau)}{d\tau} = -\beta_{x+1} P_x(\tau) + \beta_x P_{x-1}(\tau) \qquad \text{(A.10)}$$

with the initial conditions

$$P_x(0) = \begin{cases} 1 & x = 0 \\ 0 & x = 1,2,\ldots,g \end{cases} \qquad (A.11)$$

cannot be solved in such a compact form as (A.8) above. The Laplace transform approach can again be used to obtain, successively, explicit expressions for the $P_x(\tau)$.

We are interested in the solution for the special case where $\beta_1 = \beta$, $\beta_2 = 2\beta$, ..., $\beta_g = g\beta$. The system of equations we wish to solve is then

$$\frac{dP_o(\tau)}{d\tau} = -\beta\, P_o(\tau)$$

$$\frac{dP_x(\tau)}{d\tau} = -(x+1)\,\beta\, P_x(\tau) + x\beta P_{x-1}(\tau)$$

$$x = 1,2,..,g-1$$

$$\frac{dPg(\tau)}{d\tau} = g\,\beta\, P_{g-1}(\tau)$$

subject to the initial conditions

$$P_x(0) = \begin{cases} 1 & x = 0 \\ 0 & x = 1,2,..,g. \end{cases}$$

Following the Laplace transform method used for the Poisson process we easily find

$$P_o(\tau) = e^{-\beta\tau}$$

$$P_1(\tau) = e^{-\beta\tau}\ (1 - e^{-\beta\tau})$$

$$P_2(\tau) = e^{-\beta\tau}\ (1 - e^{-\beta\tau})^2$$

and

$$\overline{P}_x(s) = \frac{x\beta}{s + (x+1)\beta}\ \overline{P}_{x-1}(s) \quad , \quad x = 1,2,\ldots,g-1 \ .$$

We can prove inductively, in analogous fashion to the proof given in Appendix A.1, that

$$P_x(\tau) = e^{-\beta\tau}\ (1 - e^{-\beta\tau})^x \ , \qquad x = 1,2,\ldots,g-1 \ .$$

It follows that the expected rate at which the g^{th} event occurs

$$\frac{dP_g(\tau)}{d\tau} = g\beta\, P_{g-1}(\tau)$$

$$= g\beta\, e^{-\beta\tau}\ (1 - e^{-\beta\tau})^{g-1} \ .$$

APPENDIX B

THE DISTRIBUTION OF GENERATION TIMES FROM THE MASS DISTRIBUTION MODEL

We derive in this appendix the general equation (2.24) for the distribution of generation times in terms of the distribution of division mass using probabilistic arguments due to Eakman (1966).

We define the following propositions:

A_i : the i^{th} cell has age τ_i to $\tau_i + d\tau_i$ when it divides;

B_i : the i^{th} cell has mass m_{oi} to $m_{oi} + dm_{oi}$ initially;

H : growth rates are dependent only on cell mass (or age) and not on absolute time;

H' : the parent cell had mass m' to m' + dm' when it divided to form the cell under consideration.

For the conditional probability of A_i given that H is true we can write

$$P\{A_i|H\} = f(\tau_i)\, d\tau_i \,, \qquad \text{(B.1)}$$

i.e., the probability that a cell will divide in the age range τ_i to $\tau_i + d\tau_i$ if growth rates are dependent only on cell mass (or age). By our previous definitions, which we reproduce again here,

$p(m, m')\, dm$ = probability that a daughter cell formed from a mother cell of mass m' has a mass between m and $m + dm$

$h(m)\, dm$ = probability that a cell will divide in the mass range m to $m + dm$

we have

$$P\{B_i | H'H\} = p(m_{oi}, m')\, dm_{oi} \tag{B.2}$$

$$P\{H' | H\} = h(m')\, dm' . \tag{B.3}$$

To find the probability of A_i and B_i given that H' and H are true we invoke the well-known multiplicative law of conditional probabilities*

$$P\{A_i B_i | H' H\} = P\{A_i | B_i H' H\}\, P\{B_i | H' H\} . \tag{B.4}$$

The probability that a cell will have a generation time τ_i to $\tau_i + d\tau_i$ given that it had mass m_{oi} initially is just

$$P\{A_i | B_i H' H\} = h\{m(m_{oi}, \tau_i)\}\, r\{m(m_{oi}, \tau_i)\}\, d\tau_i \tag{B.5}$$

since we know that

$$\frac{dm}{d\tau_i} = r\{m(m_{oi}, \tau_i)\} .$$

Thus, substitution of equations (B.2) and (B.5) into (B.4) gives

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*See, for example, Feller (1957).

$$P\{A_iB_i|H'H\} = h\{m(m_{oi}, \tau_i)\}\, r\{m(m_{oi},\tau_i)\}\, p(m_{oi}, m')\, dm_{oi}\, d\tau_i . \tag{B.6}$$

This is the probability that the i^{th} cell has generation time in the range τ_i to $\tau_i + d\tau_i$ and initial mass in the range m_{oi} to $m_{oi} + dm_{oi}$ given that its parent had mass m' at fission.

If A and B_j are propositions conditional on H and all the B_j, $j = 1,2,\ldots,n$, are mutually exclusive and exhaustive propositions, the simple formula

$$P\{A|H\} = \sum_{j=1}^{n} P\{AB_j|H\}$$

holds. This is clear since any event A can only occur in conjunction with some B_j and, since the AB_j are mutually exclusive, their probabilities add. In the case of a continuous distribution the summation is replaced by an integration. Application of this formula to equation (B.6) yields

$$P\{A_i|H'H\} = d\tau_i \int_o^{m'} h\{m(m_{oi},\tau_i)\}\, r\{m(m_{oi},\tau_i)\}\, p(m_{oi},m')\, dm_{oi} \tag{B.7}$$

for the probability that the i^{th} cell will have a generation time between τ_i and $\tau_i + d\tau_i$ given that the parent cell had mass m' at fission. Clearly, an upper limit on the m_{oi} is m'.

Further, we have that

$$P\{A_i H'|H\} = P\{A_i|H'H\}\, P\{H'|H\} .$$

Using equation (B.3) we find, therefore,

$$P\{A_i H' | H\} = d\tau_i \, h(m') \, dm' \int_o^{m'} h\{m(m_{oi}, \tau_i)\} \, r\{m(m_{oi}, \tau_i)\} \, p(m_{oi}, m') \, dm_{oi} .$$

Integration over all possible masses of the parent cell provides

$$P\{A_i | H\} \equiv f(\tau_i) \, d\tau_i$$

$$= d\tau_i \int_o^{\infty} h(m') \, dm' \int_o^{m'} h\{m(m_{oi}, \tau_i)\} \, r\{m(m_{oi}, \tau_i)\} \, p(m_{oi}, m') \, dm_{oi}$$

the required relation for the distribution of generation times.

APPENDIX C

COMPUTER PROGRAMS

C.1 'SMOOTHING, DIFFERENTIATION, AND LEAST SQUARES CURVE FITTING

Extensive use is made in this thesis of a least squares fitting routine designated RLQF. It enables a continuous and differentiable function with M arbitrary parameters, which are to be determined, to be fitted to a set of data points. Nonlinear cases, in which the parameters appear nonlinearly in the function, are handled by a linearization procedure so that the usual linear least squares fit can be applied. In Section C.1.1 a complete FORTRAN listing of this least squares routine is given, together with the calling program. This program is used to accomplish the smoothing and differentiation of the input data followed by unweighted least squares fitting of a sum of three Gaussian functions to the differentiated data.

To call the subroutine RLQF the following statements are needed:

```
EXTERNAL AUX

CALL RLQF (X, Y, YF, W, E1, E2, P, WZ, N, M, NI, ND, EP, AUX,
          MD)
```

The arguments of the CALL statement have the following meanings:

X = an array containing N independent variables.

Y = an array containing N dependent variables.

YF = an array containing N fitted values of the dependent variables (these are calculated by the subroutine).

W = an array containing N values of the weights associated with the dependent variables.

E1 = an array containing the root mean square statistical error of estimate for each of the M parameters P_i.

E2 = an array containing the root mean square total error of estimate for each of the M parameters P_i.

P = an array containing the initial estimates of the M parameters (the subroutine will change this array to contain the final estimates of these parameters).

WZ = 0., if all the weights are to be assumed equal to 1, in which case the weights W_i should not be provided.

= 1., if the weights are to be provided for each point by the user.

N = number of data points.

M = number of parameters in the function to which the data is to be fitted. (M must be less than 10).

NI = maximum number of iterations.

ND (set by the subroutine)

= 1 if the system of equations was solved successfully,

= 0 if the system of equations was not solved successfully.

EP = value of ε such that if

$$\left|\frac{\Delta P_i{}^j}{P_i{}^j}\right| < \varepsilon \ , \quad i = 1, \ldots, M, \text{ the}$$

computation will stop at this, the j^{th} iteration.

ΔP_i^j is the calculated change in the i^{th} parameter on the j^{th} iteration ($j < NI$).

AUX = the name of the function to be fitted. This name must be declared EXTERNAL in the routine in which the call to RLQF appears.

MD = an array containing '0's' or '1's'. When a zero is in MD(i) it indicates that the i^{th} parameter P_i is to be held constant. A '1' in MD(i) indicates that P_i will be allowed to vary. The array MD is destroyed inside the subroutine and so must be renewed each time RLQF is called.

The intermediate and final estimates of the parameters P_i are printed within the subroutine itself and the final estimates of the P_i's are available in the array P after returning from the subroutine. Except as indicated above, none of the quantities that appear in the argument list of the subroutine are modified by the subroutine.

Subroutine SOLMT is called by RLQF during execution of the least squares fitting.

The auxiliary subroutine AUX defines the function to be fitted. This subroutine must begin:

```
SUBROUTINE AUX (F, P, D, X, L)
DIMENSION  P (10), D (10)
```

where,

F = value of the function.

P = an array as indicated above.

D = an array containing the partial derivatives of the function with respect to each parameter, *i.e.*,

$$D(I) = \frac{\partial f}{\partial P_i}$$

where f is the function which is being fitted to the data.

X = current value of the independent variable (this value is provided by the subroutine RLQF).

L = if the current value of X is X_1, then L contains the value 1 in case it is needed by the subroutine AUX.

This subroutine should return control to RLQF with AUX set equal to the value of the function.

In the program given in Section C.1.1, AUX represents the sum of three Gaussian functions, each with a normalisation parameter, so that nine parameters are varied during the fitting procedure. RLQF is called several times with some of these parameters held constant, before all nine parameters are varied simultaneously in the final execution.

The input experimental data is represented by the array B and

N4 = number of experimental data points,

NMIN = data point spacing.

Smoothing of this data is achieved by the digital filtering procedure discussed in Chapter 5 of this thesis. XK represents the digital filtering cut-off wavelength in units of data point spacings. The smoothed data values are held in the array C. A six-point numerical differentiation formula applied to the smoothed data produces the derivative values held in the array D. E is an array, which is not used further, containing the approximate derivative values obtained

by simple differencing of the smoothed data. The function AUX is fitted to the differentiated data between the bounds (cut-off points) S1 and S2. For example, S1 = 6, S2 = 41 for fitting between the 6th and 41st data points. Clearly, the first three and last three points of the array D will be zero since a six-point formula has been used to obtain this array. This array should not be confused with that defined in the subroutine AUX. In the main program the number of data points to be fitted is represented by NN4 and the arrays of independent and dependent variables by JX and G, respectively. These arguments correspond to N, X and Y, respectively, in the subroutine RLQF.

The initial estimates of the parameters are held in the array PP. The complete program may be repeatedly cycled for different cut-off wavelengths; at the end of each cycle the array P of final estimates is printed and then reset to the array PP of initial estimates.

A sample print-out of the application of this program to the data of experiment A is provided in Section C.1.2.

Several other functions were fitted to the data of experiment A. FORTRAN listings of the 'AUX' subroutines used in these cases are provided in Section C.1.3. The subroutine for the mass distribution model is given also. This function was fitted to a single Gaussian representing the generation time distribution for experiment A.

C.1.1 Program Listing for Smoothing and Differentiation of Data Followed by Least Squares Fitting of a Sum of Gaussians

```
C  PROGRAM FITS FUNCTION 'AUX' TO DATA AFTER SMOOTHING AND
C        NUMERICAL DIFFERENTIATION

      DIMENSION B(100),BB(400),E(100),D(100),S(50),C(100)
      DIMENSION P(10),E1(10),E2(10),YF(100),W(10),MD(10),JZ(100),G(100)
      DIMENSION JX(100),PP(10)
      EXTERNAL AUX
      INTEGER S1,S2
200   READ(5,160)N4,NMIN

160   FORMAT(2I5)
      READ(5,170)(B(I),I=1,N4)
      READ(5,160)M,NI
      READ(5,170)(PP(I),I=1,M)
170   FORMAT(F12.6)
      DO20I=1,M
20    P(I)=PP(I)
      DO1I=1,N4
      BB(I+N4)=B(I)
      BB(N4+1-I)=B(I)
1     BB(2*N4+I)=B(N4+1-I)
131   PI=3.14159265
      V=0.5
      READ(5,170,END=99) XK
      WRITE(6,115)XK

115   FORMAT(' CUTOFF WAVELENGTH =',F5.2,'DATA POINTS')
      K=XK*2.5
      DO3I=1,K
      X=5*I*PI/K
      S(I)=SIN(X)*(1-X/(5*PI))/X
3     V=V+S(I)
      DO4I=1,K
4     S(I)=S(I)/(2*V)
      N41=N4+1
      N5=2*N4+1
      DO5J=N41,N5
      SU=BB(J)/(2*V)
      DO6I=1,K
6     SU=SU+S(I)*(BB(J+I)+BB(J-I))
      C(J-N4)=SU
      IF(J.NE.N41) GOTO 12
      E(J-N4)=SU
      GOTO 5
12    E(J-N4)=(SU-C(J-N41))/NMIN
5     CONTINUE
      ST=0.
      JM4=N4-3
      DO7J=4,JM4
      SX=(C(J+3)-C(J-3))/60-3*(C(J+2)-C(J-2))/20
7     D(J)=(SX+0.75*(C(J+1)-C(J-1)))/NMIN
      DO9J=1,N4
      JZ(J)=NMIN*(J-1)
9     WRITE(6,10) JZ(J),B(J),C(J),D(J),E(J)
10    FORMAT(I5,2F10.3,2F11.4)
```

```
350   READ(5,142)S1,S2
      WRITE(6,137)S1,S2
137   FORMAT(' CUTOFF POINTS ARE',2I6)
142   FORMAT(2I12)
      DO340J=S1,S2
      JX(J+1-S1)=JZ(J)
340   G(J+1-S1)=D(J)
      EP=0.0001
      WZ=0.
      NN4=S2-S1+1
      DO15I=1,3
15    MD(I)=0
      MD(4)=1
      MD(5)=1
      DO25I=6,9
25    MD(I)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      MD(1)=1
      MD(2)=1
      MD(3)=0
      MD(4)=1
      MD(5)=1
      DO26I=6,9
26    MD(I)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      MD(1)=1
      MD(2)=1
      MD(3)=0
      MD(4)=1
      MD(5)=1
      MD(6)=0
      MD(7)=1
      MD(8)=1
      MD(9)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      MD(1)=1
      MD(2)=1
      MD(3)=0
      DO27I=4,8
27    MD(I)=1
      MD(9)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      DO22I=1,8
22    MD(I)=1
      MD(9)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      DO29K=1,9
      IF(P(K).LE.0)GOTO 30
29    CONTINUE
      DO14I=1,9
14    MD(I)=1
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
30    DO19I=1,M
19    P(I)=PP(I)
      GOTO 131
99     CALL EXIT
      END
```

```
      SUBROUTINE RLQF(X,Y,YF,W,E1,E2,P,WZ,N,M,NI,ND,EP,AUX,MD)
      DIMENSION X(1),Y(1),YF(1),W(1)
      DIMENSION C(55),V(10),CU(10,10),VV(10,1),U(10)
      DIMENSION MD(10),P(10),E1(10),E2(10)
      DIMENSION D(10)
      EQUIVALENCE (V(1),VV(1,1))
      COMMON /RLQF/ S
      ND=1
      WRITE(6,2)
2     FORMAT(/53H INTERMEDIATE ESTIMATES OF PARAMETERS, SUM OF SQUARES)
      LM=0
      DO 4 IM=1,M
      IF(MD(IM).EQ.0) GOTO 4
      LM=LM+1
      MD(LM)=IM
4     CONTINUE
      SXX=1.E20
      NT=0
      IV=0
5     IJ=0
      NT=NT+1
      DO 10 I=1,LM
      V(I)=0.
      DO 10 J=1,I
      IJ=IJ+1
10    C(IJ)=0.
      XX=0.
      TT=0.
      DO 40 L=1,N
      IF(WZ)15,25,15
15    WT=W(L)
      IF(WT)28,40,28
      GOTO 28
25    WT=1.
28    CALL AUX(F,P,D,X(L),L)

      A=F-Y(L)
      XX=XX+A*A
      IJ=0
      DO 40 I=1,LM
      K=MD(I)
      DO 30 J=I,LM
      KJ=MD(J)
      IJ=IJ+1
30    C(IJ)=C(IJ)+WT*D(K)*D(KJ)
      V(I)=V(I)-WT*A*D(K)
40    CONTINUE
      WRITE(6,55) (P(I),I=1,M),XX
55    FORMAT(1X,8E15.5)
      IF(IV.EQ.1) GOTO 78
      IF(NT.GT.NI) GOTO 125
      IF(XX.GT.SXX) GOTO 71
      SXX=XX
      CALL SOLMT(C,VV,1,IJ,LM,KEY)
      IF(KEY.EQ.1) GOTO 120
      PMAX=ABS(P(MD(1)))
      DO 70 I=2,LM
      IF(MD(I).EQ.0) GOTO 70
      PK=ABS(P(MD(I)))
      IF(PK.GT.PMAX) PMAX=PK
70    CONTINUE
```

```
      PMAX=PMAX*1.E-8
      GOTO 73
71    DO 72 I=1,LM
      K=MD(I)
      P(K)=P(K)-U(I)
72    V(I)=U(I)*.3
73    DO 75 I=1,LM
      K=MD(I)
      P(K)=P(K)+V(I)
C  RETURN IF NEGATIVE PARAMETER IN GAUSSIAN FUNCTION
      IF(P(K).LE.0) GOTO 127
      U(I)=V(I)
      IF(ABS(P(K)).LT.PMAX) GOTO 75
      TC=ABS(V(I)/P(I))
      IF(TC.LE.TT) GOTO 75
      TT=TC
75    CONTINUE
      IF(EP.GT.TT) IV=1
      GOTO 5
78    DO 80 I=1,LM
      DO 80 J=1,LM
80    CU(I,J)=0.
      DO 82 I=1,LM
82    CU(I,I)=1.
      CALL SOLMT(C,CU,M,IJ,LM,KEY)
      IF(KEY.EQ.1) GOTO 120
      DO 85 I=1,LM
      K=MD(I)
      DO 85 J=1,LM
85    P(K)=P(K)+CU(I,J)*V(J)
      DO 91 I=1,M
91    E1(I)=0.
      DO 95 I=1,LM
      K=MD(I)
95    E1(K)=SQRT(CU(I,I))
      WRITE(6,96) NT
96    FORMAT(/30H FINAL ESTIMATES OF PARAMETERS35X,11HITERATIONS=,I5)
      WRITE(6,55) (P(I),I=1,M)
      SS=0.
      S=0.
      IF(MD(9).EQ.0) GOTO 206
      WRITE(6,860)
860   FORMAT('0FITTED VALUES')
206   CONTINUE
      DO105L=1,N
      IF(WZ.EQ.0.) GOTO100
      WT=W(L)
      GOTO 102
100   WT=1.
      INT=1
102   CALL AUX(F,P,D,X(L),L)

      IF(MD(9).EQ.0) GOTO 207
      WRITE(6,855)F
855   FORMAT(F12.6)
207   CONTINUE
      INT=0
      YF(L)=F
      XX=(Y(L)-F)**2
      S=XX*WT+S
      SS=XX+SS
105   CONTINUE
```

```
      PP=N-M
      FI=SQRT(S/PP)
      DO 115 I=1,M
115   E2(I)=FI*E1(I)
      WRITE(6,117) SS,S
117   FORMAT('0SUM OF SQUARES',E15.5,' WEIGHTED S S',E15.5)
      WRITE(6,118)
118   FORMAT('0RMS STATISTICAL ERRORS')
      WRITE(6,55) (E1(I),I=1,M)
      WRITE(6,119)
119   FORMAT('0RMS TOTAL ERRORS')
      WRITE(6,55) (E2(I),I=1,M)
      RETURN
120   WRITE(6,121)
121   FORMAT(22H LINEAR EQUATIONS FAIL)
      ND=0
      RETURN
125   WRITE(6,126)
126   FORMAT('0NO CONVERGENCE')
      RETURN
127   WRITE(6,866)
866   FORMAT('0NEGATIVE PARAMETER')
      RETURN
      END



      SUBROUTINE  SOLMT(A,B,L,M,N,KEY)
      DIMENSION A(1),B(10,1)
      IF(A(1).EQ.0.) GOTO 150
      IF(M.EQ.1) GOTO 160
      A(1)=1./SQRT(A(1))
      DO 10 I=2,N
   10 A(I)=A(I)*A(1)
      INC=N
      I1=1
      IN=N
      NM1=N-1
   20 INC=INC-1
      I1=IN+1
      IN=IN+INC
      NS=N-INC
      X=0.
      ISUB=I1
      DO 30 I=INC,NM1
      ISUB=ISUB-I
   30 X=X+A(ISUB)**2
      IF(A(I1).LT.X) GOTO 150
      A(I1)=SQRT(A(I1)-X)
      IF(A(I1).EQ.0.) GOTO 150
      A(I1)=1./A(I1)
      IF(INC.EQ.1) GOTO 90
      I11=I1+1
      L11=I1-INC
      DO 50 I=I11,IN
      X=0.
      L1=L11
      L2=I-INC
```

```
      DO 40 J=1,NS
      X=X+A(L1)*A(L2)
      L1=L1-INC-J
   40 L2=L2-INC-J
   50 A(I)=A(I1)*(A(I)-X)
      GOTO 20
   90 DO 130 K=I,L
      B(1,K)=B(1,K)*A(1)
      DO 110 I=2,N
      JM=I-1
      ISUB=I
      INC=N
      X=0.
      DO 100 J=1,JM
      X=A(ISUB)*B(J,K)+X
      INC=INC-1
  100 ISUB=ISUB+INC
  110 B(I,K)=A(ISUB)*(B(I,K)-X)
      B(N,K)=B(N,K)*A(M)
      INC=-1
      J1=M+1
      DO 125 I=2,N
      INC=INC+1
      JM=J1-2
      J1=JM-INC
      JSUB=N-INC-1
      II=JSUB
      X=0.
      DO 120 J=J1,JM
      JSUB=JSUB+1
  120 X=X+A(J)*B(JSUB,K)
  125 B(II,K)=A(J1-1)*(B(II,K)-X)
  130 CONTINUE
      KEY=0
      RETURN
  150 KEY=1
      RETURN
  160 B(1,1)=B(1,1)/A(1)
      KEY=0
      RETURN
      END
```

```
      SUBROUTINE AUX(F,P,D,X,L)
      DIMENSION P(10),D(10)
      PI=3.14159265
      F1=1./SQRT(2.*PI)
      F11=(EXP(-(X-P(1))**2/(2.*P(4))))/SQRT(P(4))
      F12=(EXP(-(X-P(2))**2/(2.*P(5))))/SQRT(P(5))
      F13=(EXP(-(X-P(3))**2/(2.*P(6))))/SQRT(P(6))
      F=F1*(P(7)*F11+P(8)*F12+P(9)*F13)
      IF(INT.EQ.1) GOTO 801
      D(7)=F1*F11
      D(8)=F1*F12
      D(9)=F1*F13
      DO 800 I=1,3
      J=I+3
      D(I)=P(I+6)*EXP(-(X-P(I))**2/(2.*P(J)))
      D(J)=(D(I)/(2.*P(J)*SQRT(2.*PI*P(J))))*((X-P(I))**2/P(J)-1.)
800   D(I)=D(I)*(X-P(I))/(P(J)*SQRT(2.*PI*P(J)))
801   RETURN
      END
```

C.1.2 Sample Results of Analysis of Experiment A

The results of an application of the least squares fitting routine to the data of experiment A are presented here. In this example, smoothing was performed at a digital filtering cut-off wavelength of eight data point spacings and the sum of three Gaussian functions was fitted to the data between the 6th and 41st points. After the final estimates for simultaneous variation of all nine parameters are printed out, the values of the function are given for this best fit. For ease of handling, the data record begins at time zero which does not correspond to the zero of the experiment. This simple time translation is easily corrected for by adjusting the computed means. Examination of the subroutine AUX reveals that the parameters P(1), P(2), P(3) represent the means, and P(4), P(5), P(6) the variances of the Gaussian functions. It should be noted that the powers of ten associated with the input data points (representing cell concentrations) are suppressed; this involves no loss of information.

CUTOFF WAVELENGTH = 8.00DATA POINTS

0	1.390	1.382	0.0000	1.3823
3	1.400	1.386	0.0000	0.0012
6	1.370	1.393	0.0000	0.0023
9	1.380	1.403	0.0036	0.0032
12	1.430	1.414	0.0036	0.0037
15	1.440	1.424	0.0035	0.0035
18	1.440	1.436	0.0044	0.0038
21	1.420	1.453	0.0079	0.0059
24	1.490	1.486	0.0139	0.0107
27	1.540	1.539	0.0222	0.0179
30	1.630	1.620	0.0320	0.0271
33	1.730	1.731	0.0418	0.0370
36	1.860	1.869	0.0496	0.0459
39	1.990	2.025	0.0538	0.0520
42	2.200	2.187	0.0530	0.0539
45	2.400	2.338	0.0467	0.0503
48	2.430	2.464	0.0376	0.0421
51	2.570	2.563	0.0282	0.0330
54	2.620	2.635	0.0203	0.0240
57	2.660	2.689	0.0169	0.0182
60	2.780	2.742	0.0188	0.0174
63	2.810	2.809	0.0271	0.0223
66	2.900	2.911	0.0419	0.0340
69	3.050	3.065	0.0611	0.0513
72	3.260	3.278	0.0812	0.0713
75	3.560	3.548	0.0978	0.0900
78	3.870	3.857	0.1068	0.1030
81	4.160	4.180	0.1065	0.1074
84	4.510	4.487	0.0967	0.1024
87	4.760	4.753	0.0796	0.0885
90	4.980	4.962	0.0597	0.0697
93	5.110	5.113	0.0422	0.0505
96	5.300	5.222	0.0310	0.0361
99	5.170	5.311	0.0314	0.0298
102	5.440	5.423	0.0449	0.0374
105	5.610	5.592	0.0697	0.0562
108	5.870	5.852	0.1053	0.0868
111	6.230	6.230	0.1469	0.1258
114	6.760	6.731	0.1860	0.1671
117	7.280	7.333	0.2127	0.2008
120	7.940	7.984	0.2163	0.2169
123	8.570	8.600	0.0000	0.2054
126	9.090	9.086	0.0000	0.1619
129	9.460	9.355	0.0000	0.0898

CUTOFF POINTS ARE 6 41

INTERMEDIATE ESTIMATES OF PARAMETERS, SUM OF SQUARES

.36000E+02	.78000E+02	.12000E+03	.60000E+02	.80000E+02	.12000E+03	.13000E+01	.26000E+01
.52000E+01	.53206E-02						
.36000E+02	.78000E+02	.12000E+03	.90791E+02	.90074E+02	.12000E+03	.13000E+01	.26000E+01
.52000E+01	.40950E-02						
.36000E+02	.78000E+02	.12000E+03	.10273E+03	.90572E+02	.12000E+03	.13000E+01	.26000E+01
.52000E+01	.40438E-02						
.36000E+02	.78000E+02	.12000E+03	.10292E+03	.90603E+02	.12000E+03	.13000E+01	.26000E+01
.52000E+01	.40438E-02						
.36000E+02	.78000E+02	.12000E+03	.10291E+03	.90605E+02	.12000E+03	.13000E+01	.26000E+01
.52000E+01	.40438E-02						
.36000E+02	.78000E+02	.12000E+03	.10291E+03	.90605E+02	.12000E+03	.13000E+01	.26000E+01
.52000E+01	.40438E-02						

FINAL ESTIMATES OF PARAMETERS ITERATIONS= 6

.36000E+02	.78000E+02	.12000E+03	.10291E+03	.90605E+02	.12000E+03	.13000E+01	.26000E+01
.52000E+01							

```
SUM OF SQUARES      .40438E-02 WEIGHTED S S      .40438E-02

RMS STATISTICAL ERRORS
     0.              0.              0.              .19369E+04      .81472E+03      0.              0.              0.
     0.

RMS TOTAL ERRORS
     0.              0.              0.              .23704E+02      .99706E+01      0.              0.              0.
     0.

INTERMEDIATE ESTIMATES OF PARAMETERS, SUM OF SQUARES
     .36000E+02      .78000E+02      .12000E+03      .10291E+03      .90605E+02      .12000E+03      .13000E+01      .26000E+01
     .52000E+01      .40438E-02
     .39886E+02      .78995E+02      .12000E+03      .10697E+03      .87456E+02      .12000E+03      .13000E+01      .26000E+01
     .52000E+01      .27383E-02
     .39647E+02      .78936E+02      .12000E+03      .88901E+02      .86266E+02      .12000E+03      .13000E+01      .26000E+01
     .52000E+01      .26531E-02
     .39709E+02      .78954E+02      .12000E+03      .91018E+02      .86258E+02      .12000E+03      .13000E+01      .26000E+01
     .52000E+01      .26511E-02
     .39707E+02      .78954E+02      .12000E+03      .90998E+02      .86248E+02      .12000E+03      .13000E+01      .26000E+01
     .52000E+01      .26511E-02
     .39707E+02      .78954E+02      .12000E+03      .91000E+02      .86248E+02.     .12000E+03      .13000E+01      .26000E+01
     .52000E+01      .26511E-02

FINAL ESTIMATES OF PARAMETERS                                                   ITERATIONS=  6
     .39707E+02      .78954E+02      .12000E+03      .91000E+02      .86248E+02      .12000E+03      .13000E+01      .26000E+01
     .52000E+01

SUM OF SQUARES      .26511E-02 WEIGHTED S S      .26511E-02

RMS STATISTICAL ERRORS
     .10595E+03      .50890E+02      0.              .16562E+04      .77265E+03      0.              0.              0.
     0.

RMS TOTAL ERRORS
     .10498E+01      .50427E+00      0.              .16411E+02      .76562E+01      0.              0.              0.
     0.

INTERMEDIATE ESTIMATES OF PARAMETERS, SUM OF SQUARES
     .39707E+02      .78954E+02      .12000E+03      .91000E+02      .86248E+02      .12000E+03      .13000E+01      .26000E+01
     .52000E+01      .26511E-02
     .39869E+02      .78979E+02      .12000E+03      .94806E+02      .80120E+02      .12000E+03      .13101E+01      .24703E+01
     .52000E+01      .25479E-02
     .39862E+02      .78998E+02      .12000E+03      .94799E+02      .80595E+02      .12000E+03      .13094E+01      .24749E+01
     .52000E+01      .25476E-02
     .39863E+02      .78996E+02      .12000E+03      .94829E+02      .80531E+02      .12000E+03      .13096E+01      .24744E+01
     .52000E+01      .25476E-02
     .39863E+02      .78996E+02      .12000E+03      .94827E+02      .80538E+02      .12000E+03      .13096E+01      .24745E+01
     .52000E+01      .25476E-02

FINAL ESTIMATES OF PARAMETERS                                                   ITERATIONS=  5
     .39863E+02      .78996E+02      .12000E+03      .94827E+02      .80537E+02      .12000E+03      .13096E+01      .24745E+01
     .52000E+01

SUM OF SQUARES      .25476E-02 WEIGHTED S S      .25476E-02

RMS STATISTICAL ERRORS
     .10963E+03      .51418E+02      0.              .22320E+04      .95672E+03      0.              .12990E+02      .12466E+02
     0.

RMS TOTAL ERRORS
     .10649E+01      .49946E+00      0.              .21680E+02      .92932E+01      0.              .12618E+00      .12109E+00
     0.
```

```
INTERMEDIATE ESTIMATES OF PARAMETERS, SUM OF SQUARES
     .39963E+02     .78996E+02     .12000E+03     .94827E+02     .80537E+02     .12000E+03     .13096E+01     .24745E+01
     .52000E+01     .25476E-02
     .39681E+02     .79451E+02     .12000E+03     .89448E+02     .92951E+02     .81208E+02     .12834E+01     .26230E+01
     .52000E+01     .48202E-03
     .39697E+02     .79374E+02     .12000E+03     .89897E+02     .92141E+02     .89170E+02     .12850E+01     .26018E+01
     .52000E+01     .18628E-03
     .39697E+02     .79369E+02     .12000E+03     .89926E+02     .92031E+02     .89375E+02     .12852E+01     .26004E+01
     .52000E+01     .18613E-03
     .39697E+02     .79369E+02     .12000E+03     .89927E+02     .92028E+02     .89367E+02     .12852E+01     .26004E+01
     .52000E+01     .18613E-03

FINAL ESTIMATES OF PARAMETERS                                  ITERATIONS=   5
     .39697E+02     .79369E+02     .12000E+03     .89927E+02     .92028E+02     .89367E+02     .12852E+01     .26004E+01
     .52000E+01

SUM OF SQUARES      .18613E-03 WEIGHTED S S      .18613E-03

RMS STATISTICAL ERRORS
     .10771E+03     .54571E+02     0.             .21329E+04     .11091E+04     .52521E+03     .12866E+02     .13142E+02
     0.

RMS TOTAL ERRORS
     .28281E+00     .14328E+00     0.             .56000E+01     .29119E+01     .13790E+01     .33780E-01     .34504E-01
     0.

INTERMEDIATE ESTIMATES OF PARAMETERS, SUM OF SQUARES
     .39697E+02     .79369E+02     .12000E+03     .89927E+02     .92028E+02     .89367E+02     .12852E+01     .26004E+01
     .52000E+01     .18613E-03
     .39752E+02     .79288E+02     .11952E+03     .91461E+02     .88455E+02     .89788E+02     .12931E+01     .25677E+01
     .52000E+01     .23831E-04
     .39748E+02     .79293E+02     .11952E+03     .91401E+02     .88661E+02     .89467E+02     .12927E+01     .25694E+01
     .52000E+01     .23441E-04
     .39748E+02     .79292E+02     .11952E+03     .91405E+02     .88651E+02     .89471E+02     .12927E+01     .25693E+01
     .52000E+01     .23440E-04
     .39748E+02     .79292E+02     .11952E+03     .91405E+02     .88652E+02     .89471E+02     .12927E+01     .25693E+01
     .52000E+01     .23440E-04

FINAL ESTIMATES OF PARAMETERS                                  ITERATIONS=   5
     .39748E+02     .79292E+02     .11952E+03     .91405E+02     .88652E+02     .89471E+02     .12927E+01     .25693E+01
     .52000E+01

SUM OF SQUARES      .23440E-04 WEIGHTED S S      .23440E-04

RMS STATISTICAL ERRORS
     .10837E+03     .54023E+02     .37861E+02     .21661E+04     .11003E+04     .51138E+03     .12918E+02     .13236E+02
     0.

RMS TOTAL ERRORS
     .10098E+00     .50337E-01     .35277E-01     .20183E+01     .10252E+01     .47648E+00     .12036E-01     .12333E-01
     0.

INTERMEDIATE ESTIMATES OF PARAMETERS, SUM OF SQUARES
     .39748E+02     .79292E+02     .11952E+03     .91405E+02     .88652E+02     .89471E+02     .12927E+01     .25693E+01
     .52000E+01     .23440E-04
     .39726E+02     .79341E+02     .11907E+03     .90779E+02     .90195E+02     .83229E+02     .12895E+01     .25855E+01
     .49733E+01     .10501E-04
     .39728E+02     .79338E+02     .11910E+03     .90813E+02     .90120E+02     .83637E+02     .12897E+01     .25846E+01
     .49889E+01     .10414E-04
     .39728E+02     .79339E+02     .11910E+03     .90812E+02     .90122E+02     .83634E+02     .12897E+01     .25846E+01
     .49889E+01     .10414E-04

FINAL ESTIMATES OF PARAMETERS                                  ITERATIONS=   4
     .39728E+02     .79339E+02     .11910E+03     .90812E+02     .90122E+02     .83634E+02     .12897E+01     .25846E+01
     .49889E+01
```

FITTED VALUES
0.001863
0.004013
0.007828
0.013931
0.022129
0.032067
0.042083
0.050018
0.053847
0.052525
0.046485
0.037509
0.028083
0.020671
0.017262
0.019258
0.027436
0.041685
0.060511
0.080738
0.097905
0.107568
0.107007
0.096419
0.078886
0.059187
0.042268
0.032231
0.032144
0.044147
0.069074
0.105275
0.147382
0.186385
0.211990
0.216600

SUM OF SQUARES .10414E-04 WEIGHTED S S .10414E-04

RMS STATISTICAL ERRORS
.10830E+03 .55748E+02 .11382E+03 .21604E+04 .11904E+04 .16050E+04 .12930E+02 .13938E+02
.55297E+02

RMS TOTAL ERRORS
.67261E-01 .34623E-01 .70690E-01 .13417E+01 .73930E+00 .99682E+00 .80304E-02 .86566E-02
.34343E-01

C.1.3(a) Gamma Distribution

The partial derivatives of this function with respect to the parameters are computed in the additional subroutine GDERIV. These derivatives are obtained numerically by application of the limit formula.

$$D(I) = \lim_{\Delta P \to 0} \frac{f(P + \Delta P) - f(P)}{\Delta P}$$

```
      SUBROUTINE AUX(F,P,D,X,L)
      DOUBLE PRECISION D(10)
      DOUBLE PRECISION F1
      DOUBLE PRECISION GD
      DIMENSION P(10)
      F=0.
      DO 70 I=1,2
      I2=I+2
      I4=I+4
      I6=I+6
      IF(X.GT.P(I6)) GOTO 71
      GO TO 70
71    PP1=P(I2)+1.
      GA=GAMMA(PP1)
      F1=X-P(I6)
      F2=(DEXP(-P(I)*F1))/GA
      F3=F2*(P(I)**P(I2))*(F1**(P(I2)-1.))
      F4=F3*P(I4)
      F5=F4*F1*P(I)
      F=F+F5
      IF(INT.EQ.1) GOTO 70
      D(I)=F4*F1*(PP1-P(I)*F1)
      D(I6)=F4*P(I)*(P(I)*F1-P(I2))
      D(I4)=F3*P(I)*F1
      CALL GDERIV(PP1,GD)
      F1=DABS(F1)
      P(I)=ABS(P(I))
      D(I2)=F5*(DLOG(F1)+ALOG(P(I))-GA/(GD**2))
70    CONTINUE
      RETURN
      END

      SUBROUTINE GDERIV(T,GD)
C  EVALUATE DERIVATIVE OF GAMMA FUNCTION
      DOUBLE PRECISION GD
      IT=100
      H=0.5
      GD=0.
      EPS=0.0000001
      DO 500 I=1,IT
      GDL=GD
      IF((T-H).LE.0.) GOTO 500
      GD=(GAMMA(T+H)-GAMMA(T-H))/(2.*H)
      GM=DABS((GDL-GD)/GD)
      IF(GM.LE.EPS) GOTO 510
500   H=0.5*H
511   WRITE(6,512) IT
512   FORMAT('0NUMERICAL DIFFERENTIATION DOES NOT CONVERGE; ITERATIONS =
     1',I4)
510   RETURN
      END
```

(b) Lognormal Distribution

```
      SUBROUTINE AUX(F,P,D,X,L)
C  SUM OF TWO LOGNORMAL DISTRIBUTIONS
      DIMENSION P(10)
      DOUBLE PRECISION D(10)
      F=0.
      PI=3.14159265
      DO 801 I=1,2
      I2=I+2
      I4=I+4
      I6=I+6
      IF(X.GT.P(I6)) GO TO 805
      F7=0.
      GO TO 800
805   F1=X-P(I6)
      F8=ALOG(F1)
      F2=(P(I2)+P(I)*F8)
      F3=F2**2
      F4=EXP(-0.5*F3)
      F5=F4/(SQRT(2.*PI)*F1)
      F6=P(I)*F5
      F7=P(I4)*F6
800   F=F+F7
      IF(X.LE.P(I6)) GO TO 801
      IF(INT.EQ.1) GO TO 801
      D(I4)=F6
      D(I2)=-F7*F2
      D(I)=P(I4)*F5*(1.-P(I)*F8*F2)
      D(I6)=(F7/F1)*(1.+P(I)*F2)
801   CONTINUE
      RETURN
      END
```

(c) Weibull Distribution

```
      SUBROUTINE AUX(F,P,D,X,L)
C  SUM OF TWO GENERAL WEIBULL DISTRIBUTIONS
      DOUBLE PRECISION D(10)
      DIMENSION P(10)
      F=0.
      DO 801 I=1,2
      I2=I+2
      I4=I+4
      I6=I+6
      IF(P(I2).LE.0.) GOTO 806
      IF(P(I).LE.0.) GOTO 808
      PM1=P(I)-1.
      IF(X.GT.P(I6)) GOTO 805
      F5=0.
      GOTO 800
805   F4=(X-P(I6))/P(I2)
      F1=F4**PM1
      F2=EXP(-F1*F4)
      F3=F1*F2*P(I)/P(I2)
      F5=F3*P(I4)
800   F=F+F5
      IF(X.LE.P(I6)) GOTO 801
      IF(INT.EQ.1) GOTO 801
      D(I4)=F3
      D(I6)=(F5/(F4*P(I2)))*(1.-P(I)+P(I)*F1*F4)
      D(I2)=(F5/P(I2))*(P(I)+F1*F4)
      D(I)=(F5/P(I))*(1.+P(I)*ALOG(F4)*(1.-F1*F4))
801   CONTINUE
      RETURN
      END
```

(d) Gaussian Distribution with Adjustment for Skewness.

```
      SUBROUTINE AUX(F,P,D,X,L)
C  SUM OF TWO GAUSSIANS EACH WITH SKEWNESS ADJUSTMENT
      DOUBLE PRECISION D(10)
      DIMENSION P(10)
      PI=3.14159265
      F1=1./SQRT(2.*PI)
      X3=X**3
      P3Q=SQRT(P(3))
      P4Q=SQRT(P(4))
      FE1=(EXP(-(X-P(1))**2/(2.*P(3))))/P3Q
      FC1=1.-(P(7)/2.)*(X/P3Q-X3/(3.*P3Q*P(3)))
      F11=FE1*FC1
      FE2=(EXP(-(X-P(2))**2/(2.*P(4))))/P4Q
      FC2=1.-(P(8)/2.)*(X/P4Q-X3/(3.*P4Q*P(4)))
      F12=FE2*FC2
      F=F1*(P(5)*F11+P(6)*F12)
      IF(INT.EQ.1) GOTO 801
      D(5)=F1*F11
      D(6)=F1*F12
      D(7)=-P(5)*F1*FE1*0.5*(X/P3Q-X3/(3.*P3Q*P(3)))
      D(8)=-P(6)*F1*FE2*0.5*(X/P4Q-X3/(3.*P4Q*P(4)))
      D(1)=P(5)*D(5)*(X-P(1))/P(3)
      D(2)=P(6)*D(6)*(X-P(2))/P(4)
      D(3)=F1*P(5)*FE1*((FC1/P3Q)*((X-P(1))**2/P(3)-1.)+(1.-X**2/P(3))
     1*P(7)*X/(2.*P(3)))
      D(4)=F1*P(6)*FE2*((FC2/P4Q)*((X-P(2))**2/P(4)-1.)+(1.-X**2/P(4))
     1*P(8)*X/(2.*P(4)))
801   RETURN
      END
```

(e) Mass Distribution Model

```
      SUBROUTINE AUX(F,P,D,X,L)
      REAL MT
      DOUBLE PRECISION D(10)
      DIMENSION P(10)
      PI=3.14159265
      SPI=SQRT(PI)
      MT=X*P(1)
      EM=EXP(-MT)
      EP=EXP(MT)
      Q=4./(4.*EM+EP)
      QP1=1.+Q
      SQP1=SQRT(QP1)
      QM1=Q-1.
      PS=P(2)**2
      F1=EXP(PS*QM1)
      F2=(P(1)*P(2)/(2.*SPI))*Q*SQP1*F1
      F=P(3)*F2
      IF(INT.EQ.1) GOTO 70
      D(3)=F2
      D(2)=F*(1.+2.*P(2)*QM1)
      Q2=4.*EM-EP
      D(1)=(F/P(1))*(1.+MT*Q2+MT*0.5*Q*Q2/QP1+MT*PS*Q*Q2)
70    CONTINUE
      RETURN
      END
```

C.2 SIMULATION OF NOISY DATA

It was explained in Chapter 5 that, to estimate the uncertainties in the parameters derived by the least squares fitting procedure, noisy data was generated imitating the real experimental data and the fitting routine was applied repetitively. For each repetition of the program appropriate noise was impressed on the data points by generating normally distributed random numbers. We present in this section the main program designed to accomplish this simulation together with the subroutines called to generate the data and to generate the random errors. Subroutines RLQF, SOLMT and AUX provided in Section C.1.1 must also be coupled to this program. This program listing is followed by a sample printout of the results of simulating the data of experiment A and carrying out the least squares fitting of a sum of three Gaussians thirty times. For the purposes of this simulation the integration of a sum of four Gaussians generated by subroutine FUNC was performed. The parameters for these Gaussians are listed in the print-out. During each repetition of the program each point of the integrated function is multiplied by a random number from a normal distribution of mean unity and standard deviation 0.015 furnished by subroutine GAUSS. The resultant points are smoothed and differentiated and the results of the first such repetition are reproduced in the print-out. Finally, the arithmetic means of the parameter estimates for the full 30 cycles are given together with their computed standard deviations.

```
C    SIMULATED ERROR PROGRAM - PROGRAM GENERATES SET OF 'PERFECT' DATA,
C        IMPRESSES A RANDOM ERROR ON EACH DATA POINT, PERFORMS A
C        SMOOTHING OPERATION, NUMERICAL DIFFERENTIATION AND A NONLINEAR
C        LEAST SQUARES FIT.  FOR A ZERO ERROR RUN PUT SD=0.0 AND L=1

      DIMENSION B(100),BB(200),E(100),S(50),C(100)
      DIMENSION D(100),G(100)
      DIMENSION P(10),E1(10),E2(10),YF(100),W(10),MD(10)
      DIMENSION JX(100),JZ(100)
      DIMENSION PP(10)
      DIMENSION BR(100)
      DIMENSION PS(40,10),RHO(40)
      DIMENSION PS5(6),ERR(6)
      DIMENSION RHO2(40)
      DIMENSION Q(15),QS(5),QD(5)
      EXTERNAL AUX
      INTEGER S1,S2
C    MQ IS THE NUMBER OF PARAMETERS IN THE DATA GENERATING FUNCTION
      READ(5,150)MQ

      READ(5,160)N4,NMIN
150   FORMAT(I5)
160   FORMAT(2I5)
      READ(5,170) (Q(I),I=1,MQ)
170   FORMAT(F12.6)
      WRITE(6,191)

191   FORMAT(' PARAMETERS ARE: ')
      WRITE(6,190) (Q(I),I=1,MQ)
190   FORMAT(1X,12F10.1)
      DO 2 I=1,4
      I4=I+4
      I8=I+8
      QS(I)=SQRT(Q(I4))
      SQ=SQRT(2.)
2     QD(I)=Q(I8)/2.
      WRITE(6,181)
181   FORMAT(14X,'FUNCTION',10X,'INTEGRAL')
      DO 111 I=1,N4
      JJ=(I-1)*NMIN
      CALL FUNC(F,Q,JJ)
      T=0.
      DO 333 J=1,4
      S2Q=SQ*QS(J)
333   T=T+QD(J)*(ERF(Q(J)/S2Q)+ERF((JJ-Q(J))/S2Q))
      T=T+1.
      BR(I)=T
111   WRITE(6,180) JJ,F,T
180   FORMAT(I5,6X,F12.6,6X,F12.6)
300   CONTINUE
C    INPUT NUMBER OF PARAMETERS IN FITTING FUNCTION, NUMBER OF
C        ITERATIONS AND INITIAL ESTIMATES OF PARAMETERS
      READ(5,160)M,NI
      READ(5,170)(PP(I),I=1,M)
      DO 20 I=1,M
20    P(I)=PP(I)
C    INPUT DIGITAL FILTERING CUTOFF WAVELENGTH IN UNITS OF DATA POINT
C        SPACINGS; FUNCTION IS FITTED TO POINTS BETWEEN BOUNDS S1,S2
133   READ(5,170,END=99) XK
      READ(5,142) S1,S2
142   FORMAT(2I12)
```

```
      AM=1.0
      SD=0.015
      IX=5
      IR=0
      WRITE(6,54)SD
54    FORMAT(//' S.D. =',F10.3)
      WRITE(6,115)XK
115   FORMAT(' CUTOFF WAVELENGTH =',F5.2,'DATA POINTS')
      WRITE(6,137)S1,S2
137   FORMAT(' CUTOFF POINTS ARE',2I6)
C   NONLINEAR LEAST SQUARES FIT IS PERFORMED L TIMES
      L=30
      DO 132 JK=1,L
      IF(JK.GT.1) GOTO 56
      WRITE(6,55)
55    FORMAT(' RAW DATA',2X,'RANDOM ERROR',X,'RESULTANT')
56    DO51I=1,N4
      CALL GAUSS(IX,SD,AM,V)
      B(I)=BR(I)*V
      IF(JK.GT.1) GOTO 51
      WRITE(6,53) BR(I),V,B(I)
53    FORMAT(3F10.3)
51    CONTINUE
C   SMOOTHING OPERATION; TO BYPASS PUT LL=1
      LL=0
      IF(LL.EQ.1) GOTO 888
      DO 1 I=1,N4
      BB(I+N4)=B(I)
      BB(N4+1-I)=B(I)
1     BB(2*N4+I)=B(N4+1-I)
131   PI=3.14159265
      V=0.5
      K=XK*2.5
      DO 3 I=1,K
      X=5*I*PI/K
      S(I)=SIN(X)*(1-X/(5*PI))/X
3     V=V+S(I)
      DO 4 I=1,K
4     S(I)=S(I)/(2*V)
      N41=N4+1
      N5=2*N4+1
      DO 5 J=N41,N5
      SU=BB(J)/(2*V)
      DO 6 I=1,K
6     SU=SU+S(I)*(BB(J+I)+BB(J-I))
      C(J-N4)=SU
      IF(J.NE.N41) GO TO 12
      E(J-N4)=SU
      GO TO 5
12    E(J-N4)=(SU-C(J-N41))/NMIN
5     CONTINUE
      GO TO 889
888   DO 890 I=1,N4
      C(I)=B(I)
890   E(I)=0.
889   ST=0.
      JM4=N4-3
      DO 7 J=4,JM4
      SX=(C(J+3)-C(J-3))/60-3*(C(J+2)-C(J-2))/20
7     D(J)=(SX+0.75*(C(J+1)-C(J-1)))/NMIN
```

```
      DO 9 J=1,N4
      JZ(J)=NMIN*(J-1)
      IF(JK.GT.1) GOTO 9
      WRITE(6,10) JZ(J),B(J),C(J),D(J),E(J)
10    FORMAT(I5,2F10.3,2F10.4)
9     CONTINUE
      DO 340 J=S1,S2
      JX(J+1-S1)=JZ(J)
340   G(J+1-S1)=D(J)
      EP=0.001
      WZ=0.
      NN4=S2-S1+1
      DO 15 I=1,3
15    MD(I)=0
      MD(4)=1
      MD(5)=1
      DO 25 I=6,9
25    MD(I)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      MD(1)=1
      MD(2)=1
      MD(3)=0
      MD(4)=1
      MD(5)=1
      DO 26 I=6,9
26    MD(I)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      DO 28 I=1,6
28    MD(I)=0
      MD(7)=1
      MD(8)=1
      MD(9)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      MD(1)=1
      MD(2)=1
      MD(3)=0
      MD(4)=1
      MD(5)=1
      MD(6)=0
      MD(7)=1
      MD(8)=1
      MD(9)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      MD(1)=1
      MD(2)=1
      MD(3)=0
      DO 27 I=4,8
27    MD(I)=1
      MD(9)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      DO 22 I=1,8
22    MD(I)=1
      MD(9)=0
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      DO 29 K=1,9
      IF(P(K).LE.0)GOTO 30
29    CONTINUE
      DO 14 I=1,9
14    MD(I)=1
      CALL RLQF(JX,G,YF,W,E1,E2,P,WZ,NN4,M,NI,ND,EP,AUX,MD)
      DO 31 K=1,9
      IF(P(K).LE.0.) GOTO 30
31    CONTINUE
```

```
C    CALCULATE PARENT-DAUGHTER GEN. TIME CORRELATION COEFF.
       IR=IR+1
       DO 400 I=1,9
400    PS(IR,I)=P(I)
       RHO(IR)=P(5)/(2.*P(4))-1.
       ALPHA=((P(5)/(2.*(P(4))**2))**2)*E2(4)**2+((1./(2.*P(4)))**2)*E2(5
      1)**2
       ALPHA=SQRT(ALPHA)
C CALCULATE PARENT-GRANDDAUGHTER GEN. TIME CORRELATION COEFF.
       RHO2(IR)=(P(6)-2.*P(5))/(2.*P(4))+0.5
       ALPHA=((0.5*(P(6)-2.*P(5))/P(4))**2)*E2(4)**2
       ALPHA=ALPHA+E2(5)**2+(E2(6)**2)*0.25
       ALPHA=ALPHA/(P(4)**2)
30     DO 19 I=1,M
19     P(I)=PP(I)
132    CONTINUE
C    DETERMINE MEAN VALUES OF PARAMETERS
       IF(L.EQ.1) GOTO 98
       WRITE(6,415) IR
415    FORMAT(//' UNWEIGHTED ARITHMETIC MEANS OF PARAMETERS FOR',I3,' CYC
      1LES ARE:')
       DO 440 I=1,6
       PSS(I)=0.
       DO 411 J=1,IR
411    PSS(I)=PSS(I)+PS(J,I)
       PSS(I)=PSS(I)/IR
       ERR(I)=0.
       DO 412 J=1,IR
412    ERR(I)=ERR(I)+(PS(J,I)-PSS(I))**2
       ERR(I)=ERR(I)/(IR-1)
       ERR(I)=SQRT(ERR(I))
       IF(I.GT.3) GOTO 431
       WRITE(6,430) I,PSS(I),ERR(I)
430    FORMAT('0MEAN OF F',I1,'=',F7.3,5X,'S.D. =',F7.3)
       GOTO 440
431    IM3=I-3
       WRITE(6,432) IM3,PSS(I),ERR(I)
432    FORMAT('0VARIANCE OF F',I1,'=',F10.3,5X,'S.D. =',F10.3)
440    CONTINUE
       RHOS=0.
       RHOSS=0.
       RHO2S=0.
       RHO2E=0.
       DO 420 I=1,IR
       RHOS=RHOS+RHO(I)
420    RHO2S=RHO2S+RHO2(I)
       RHOS=RHOS/IR
       RHO2S=RHO2S/IR
       DO 421 I=1,IR
       RHOSS=RHOSS+(RHO(I)-RHOS)**2
421    RHO2E=RHO2E+(RHO2(I)-RHO2S)**2
       RHOSS=RHOSS/(IR-1)
       RHO2E=RHO2E/(IR-1)
       RHOSS=SQRT(RHOSS)
       RHO2E=SQRT(RHO2E)
       WRITE(6,433) RHOS,RHOSS
433    FORMAT('0AVERAGE RHO(1) =',F8.4,5X,'S.D. =',F7.4)
       WRITE(6,435) RHO2S,RHO2E
435    FORMAT('0AVERAGE RHO(2) =',F8.4,5X,'S.D. =',F7.4)
98     GO TO 133
99     CALL EXIT
       END
```

```
      SUBROUTINE FUNC(F,P,X)
      DIMENSION P(15)
      PI=3.14159265
      F1=1./SQRT(2.*PI)
      F11=(EXP(-(X-P(1))**2/(2.*P(5))))/SQRT(P(5))
      F12=(EXP(-(X-P(2))**2/(2.*P(6))))/SQRT(P(6))
      F13=(EXP(-(X-P(3))**2/(2.*P(7))))/SQRT(P(7))
      F14=(EXP(-(X-P(4))**2/(2.*P(8))))/SQRT(P(8))
      F=F1*(P(9)*F11+P(10)*F12+P(11)*F13+P(12)*F14)
      RETURN
      END



      SUBROUTINE GAUSS(IX,SD,AM,V)
      A=0.0
      DO 50 I=1,12
      Y=RANDOM(IX)
50    A=A+Y
      V=(A-6.0)*SD + AM
      RETURN
      END
```

PARAMETERS ARE:

40.0 80.0 120.0 160.0 90.0 90.0 90.0 120.0 1.0 2.0 4.0 8.0

	FUNCTION	INTEGRAL
0	0.000006	1.000000
3	0.000021	1.000036
6	0.000068	1.000157
9	0.000202	1.000530
12	0.000540	1.001569
15	0.001306	1.004192
18	0.002858	1.010185
21	0.005660	1.022588
24	0.010142	1.045833
27	0.016445	1.085281
30	0.024128	1.145908
33	0.032031	1.230287
36	0.038477	1.336636
39	0.041827	1.456029
42	0.041156	1.583535
45	0.036692	1.701132
48	0.029754	1.801193
51	0.022257	1.879098
54	0.016121	1.936111
57	0.012894	1.978751
60	0.013671	2.017495
63	0.019112	2.065461
66	0.029292	2.136938
69	0.043335	2.245121
72	0.059082	2.398692
75	0.073247	2.598041
78	0.082279	2.833005
81	0.083678	3.084008
84	0.077078	3.326991
87	0.064458	3.540399
90	0.049389	3.711278
93	0.035821	3.838254
96	0.027141	3.931121
99	0.025835	4.008500
102	0.033520	4.095152
105	0.050805	4.219274
108	0.076664	4.408640
111	0.107672	4.684498
114	0.137898	5.053935
117	0.160178	5.503897
120	0.168591	6.001006
123	0.160979	6.499247
126	0.140076	6.953451

S.D. = 0.015
CUTOFF WAVELENGTH = 8.00DATA POINTS
CUTOFF POINTS ARE 4 40

RAW DATA	RANDOM ERROR	RESULTANT
1.000	1.016	1.016
1.000	0.986	0.986
1.000	0.987	0.987
1.001	0.986	0.987
1.002	0.994	0.996
1.004	0.992	0.996
1.010	1.019	1.030
1.023	0.996	1.019
1.046	0.996	1.042
1.085	0.998	1.084
1.146	1.036	1.187
1.230	0.996	1.225
1.337	0.989	1.322
1.458	0.971	1.415
1.584	1.008	1.596
1.701	1.029	1.750
1.801	0.981	1.768
1.879	1.020	1.917
1.936	1.007	1.949
1.979	1.012	2.002
2.017	0.974	1.966
2.065	1.019	2.105
2.137	1.012	2.162
2.245	1.002	2.250
2.399	0.970	2.327
2.598	1.000	2.598
2.833	1.029	2.915
3.084	1.007	3.107
3.327	1.000	3.328
3.540	1.001	3.544
3.711	0.992	3.683
3.838	0.992	3.806
3.931	1.006	3.955
4.006	0.997	3.998
4.095	0.990	4.054
4.219	0.997	4.208
4.409	0.968	4.269
4.684	1.030	4.826
5.054	1.010	5.105
5.504	0.984	5.418
6.001	1.027	6.164
6.499	1.013	6.582
6.953	0.998	6.938

0	1.016	0.999	0.0000	0.9986
3	0.986	0.996	0.0000	-0.0008
6	0.987	0.994	0.0000	-0.0008
9	0.987	0.993	0.0002	-0.0003
12	0.996	0.995	0.0014	0.0008
15	0.996	1.001	0.0028	0.0021
18	1.030	1.012	0.0044	0.0036
21	1.019	1.028	0.0068	0.0055
24	1.042	1.055	0.0108	0.0087
27	1.084	1.095	0.0163	0.0134
30	1.187	1.153	0.0228	0.0195
33	1.225	1.232	0.0299	0.0263
36	1.322	1.332	0.0368	0.0335
39	1.415	1.451	0.0417	0.0395
42	1.596	1.579	0.0428	0.0427
45	1.750	1.703	0.0391	0.0414
48	1.768	1.810	0.0322	0.0357
51	1.917	1.895	0.0237	0.0282
54	1.949	1.953	0.0156	0.0194
57	2.002	1.992	0.0108	0.0129
60	1.966	2.024	0.0116	0.0106
63	2.105	2.067	0.0181	0.0145
66	2.162	2.137	0.0295	0.0233
69	2.250	2.248	0.0446	0.0369
72	2.327	2.406	0.0610	0.0528
75	2.598	2.611	0.0745	0.0682
78	2.915	2.846	0.0812	0.0785
81	3.107	3.091	0.0810	0.0816
84	3.328	3.326	0.0750	0.0784
87	3.544	3.536	0.0641	0.0699
90	3.683	3.708	0.0503	0.0574
93	3.806	3.838	0.0362	0.0432
96	3.955	3.928	0.0244	0.0300
99	3.998	3.992	0.0197	0.0212
102	4.054	4.059	0.0273	0.0223
105	4.208	4.170	0.0491	0.0371
108	4.269	4.367	0.0846	0.0657
111	4.826	4.683	0.1254	0.1054
114	5.105	5.113	0.1595	0.1433
117	5.418	5.624	0.1772	0.1702
120	6.164	6.146	0.0000	0.1741
123	6.582	6.575	0.0000	0.1432
126	6.938	6.820	0.0000	0.0815

UNWEIGHTED ARITHMETIC MEANS OF PARAMETERS FOR 30 CYCLES ARE:

MEAN OF F1= 40.082 S.D. = 0.534

MEAN OF F2= 80.390 S.D. = 0.496

MEAN OF F3=117.376 S.D. = 0.563

VARIANCE OF F1= 93.912 S.D. = 14.228

VARIANCE OF F2= 98.712 S.D. = 14.173

VARIANCE OF F3= 53.304 S.D. = 7.339

AVERAGE RHO(1) = -0.4548 S.D. = 0.1503

AVERAGE RHO(2) = -0.2990 S.D. = 0.2607

REFERENCES

Abbo, F.E., and A.B. Pardee, 1960. Synthesis of macromolecules in synchronously dividing bacteria. *Biochimica et Biophysica Acta 39,* 478-485.

Abramowitz, M., and I.A. Stegun, 1965. *Handbook of Mathematical Functions.* New York: Dover.

Adolph, E.F., and S. Bayne-Jones, 1932. Growth in size of microorganisms measured from motion pictures. II. *Bacillus megaterium. Journal of Cellular and Comparative Physiology 1,* 409-427.

Allen, T., and K. Marshall, 1972. *The Electrical Sensing Zone Method of Particle Size Measurement. (The Coulter Principle).* University of Bradford.

Anderson, E.C., and G.I. Bell, 1971. Cell growth and division. V. Error analysis of the Collins-Richmond equation. *Biophysical Journal 11,* 308-321.

Anderson, E.C., D.F. Peterson, and R.A. Tobey, 1967. Biochemical balance and synchronized cell cultures. *Nature, London 215,* 1083-1084.

Anderson, P.A., and D.E. Pettijohn, 1960. Synchronization of division in *Escherichia coli. Science 131,* 1098.

Bell, G.I., and E.C. Anderson, 1967. Cell growth and division. I. A mathematical model with applications to cell volume distributions in mammalian suspension cultures. *Biophysical Journal 7,* 329-351.

Bostock, C.J., W.D. Donachie, M. Masters, and J.M. Mitchison, 1966. Synthesis of enzymes and DNA in synchronous cultures of *Schizosaccharomyces pombe. Nature, London 210,* 808-810.

Britten, R.J., and R.B. Roberts, 1960. High resolution density gradient sedimentation analysis. *Science 131,* 32-33.

Burdett, I.D.J., and R.G.E. Murray, 1974. Electron microscope study of septum formation in *Escherichia coli* strains B and B/r during synchronous growth. *Journal of Bacteriology 119,* 1039-1056.

Burns, V.W., 1962. Cell division synchronization, a means of amplification of division correlated processes. *Progress in Biophysics and Molecular Biology 12,* 2-23.

Campbell, A., 1957. Synchronization of cell division. *Bacteriological Reviews 21,* 263-272.

Chatterjee, A.N., H. Taber, and F.E. Young, 1971. A rapid method for synchronization of *Staphylococcus aureus* and *Bacillus subtilis. Biochemical and Biophysical Research Communications 44,* 1125-1130.

Clark, D.J., and O. Maaløe, 1967. Deoxyribonucleic acid replication and the division cycle in *Escherichia coli*. *Journal of Molecular Biology 23*, 99-112.

Collins, J.F., and M.H. Richmond, 1962. Rate of growth of *Bacillus cereus* between divisions. *Journal of General Microbiology 28*, 15-33.

Cook, J.R., and B. Cook, 1962. Effect of nutrients on the variation of individual generation times. *Experimental Cell Research 28*, 524-530.

Cook, J.R., and T.W. James, 1964. Age distribution of cells in logarithmically growing cell populations. In *Synchrony in Cell Division and Growth*. Ed. E. Zeuthen, pp. 485-495. New York: Interscience.

Cooper, S., and C.E. Helmstetter, 1968. Chromosome replication and the division cycle of *Escherichia coli* B/r. *Journal of Molecular Biology 31*, 519-540.

Coulter, W.H., 1956. High speed automatic blood cell counter and cell size analyzer. *Proceedings of National Electronics Conference 12*, 1034.

Croxton, F.E., D.J. Cowdon, and S. Klein, 1968. *Applied General Statistics*. London: Pitman.

Cummings, D.J., 1965. Macromolecular synthesis during synchronous growth of *Escherichia coli* B/r. *Biochimica et Biophysica Acta 85*, 341-350.

Cummings, D.J., 1970. Synchronization of *Escherichia coli* K12 by membrane selection. *Biochemical and Biophysical Research Communications 41*, 471-476.

Deering, R.A., 1958. Studies on division inhibition and filament formation of *Escherichia coli* by ultraviolet light. *Journal of Bacteriology 76*, 123-130.

Dirac, P.A.M., 1958. *The Principles of Quantum Mechanics*, 4th ed. London: Oxford.

Donachie, W.D., 1968. Relationship between cell size and time of initiation of DNA replication. *Nature, London 219*, 1077-1079.

Donachie, W.D., N.C. Jones, and R. Teather, 1973. The bacterial cell cycle. *Symposium of the Society for General Microbiology 23*, 9-44.

Dougherty, E.C., 1957. Neologisms needed for structure and primitive organisms. I. Types of nuclei. *Journal of Protozoology 4* (*Suppl.*), 14.

Draper, N.R., and H. Smith, 1966. *Applied Regression Analysis*. New York: Wiley.

Eakman, J.M., 1966. *Models for the Statistics and Dynamics of Microbial Populations*. Ph.D. thesis, University of Minnesota.

Eakman, J.M., A.G. Fredrickson, and H.M. Tsuchiya, 1966. Statistics and dynamics of microbial cell populations. *Chemical Engineering Progress 62,* 37-49.

Eberhard, A., 1972. Inhibition and activation of bacterial luciferase synthesis. *Journal of Bacteriology 109,* 1101-1105.

Elderton, W.P., and N.L. Johnson, 1969. *Systems of Frequency Curves.* London: Cambridge.

Feller, W.P., 1957. *An Introduction to Probability Theory and its Applications,* Vol.I, 2nd ed. New York: Wiley.

Fisz, M., 1963. *Probability Theory and Mathematical Statistics,* 3rd ed. New York: Wiley.

Gregg, E.C., and K.D. Steidley, 1965. Electrical counting and sizing of mammalian cells in suspension. *Biophysical Journal 5,* 393-405.

Grover, N.B., J. Naaman, S. Ben-Sasson, and F. Doljanski, 1969. Electrical sizing of particles in suspension. I. Theory. *Biophysical Journal 9,* 1398-1414.

Gudas, L.J., and A.B. Pardee, 1974. Deoxyribonucleic acid synthesis during the division cycle of *Escherichia coli* : a comparison of strains B/r, K-12, 15, and 15T⁻ under conditions of slow growth. *Journal of Bacteriology 117,*1216-1223.

Hamming, R.W., 1962. *Numerical Methods for Scientists and Engineers.* New York: McGraw-Hill.

Harris, T.E., 1959. A mathematical model for binary fission. In *The Kinetics of Cellular Proliferation.* Ed. F. Stohlman, pp.368-381. New York: Grune and Stratton.

Harvey, J.D., 1972a. Synchronous growth of cells and the generation time distribution. *Journal of General Microbiology 70,* 99-108.

Harvey, J.D., 1972b. Parameters of the generation time distribution of *Escherichia coli* B/r. *Journal of General Microbiology 70,* 109-114.

Harvey, R.J., 1968. Measurement of cell volumes by electric sensing zone instruments. In *Methods in Cell Physiology.* Vol.III, pp.1-23. Ed. D.M. Prescott. New York: Academic Press.

Harvey, R.J., and A.G. Marr, 1966. Measurement of size distributions of bacterial cells. *Journal of Bacteriology 92,* 805-811.

Harvey, R.J., A.G. Marr, and P.R. Painter, 1967. Kinetics of growth of individual cells of *Escherichia coli* and *Azotobacter agilis.* *Journal of Bacteriology 93,* 605-617.

Helmstetter, C.E., 1967. Rate of DNA synthesis during the division cycle of *Escherichia coli* B/r. *Journal of Molecular Biology 24,* 417-427.

Helmstetter, C.E., 1969. Methods for studying the microbial division cycle. In *Methods in Microbiology*. Vol.1, pp.327-363. Eds. J.R. Norris and D.W. Ribbons. New York: Academic Press.

Helmstetter, C.E., 1974. Initiation of chromosome replication in *Escherichia coli*. II. Analysis of the control mechanism. *Journal of Molecular Biology 84*, 21-36.

Helmstetter, C.E., and S. Cooper, 1968. DNA synthesis during the division cycle of rapidly growing *Escherichia coli* B/r. *Journal of Molecular Biology 31*, 507-518.

Helmstetter, C.E., S. Cooper, O. Pierucci, and E. Revelas, 1968. On the bacterial life sequence. *Cold Spring Harbor Symposia on Quantitative Biology 33*, 809-822.

Helmstetter, C.E., and D.J. Cummings, 1963. Bacterial synchronization by selection of cells at division. *Proceedings of National Academy of Sciences, U.S.A. 50*, 767-774.

Helmstetter, C.E., and D.J. Cummings, 1964. An improved method for the selection of bacterial cells at division. *Biochimica et Biophysica Acta 82*, 608-610.

Henrici, P., 1964. *Elements of Numerical Analysis*. New York: Wiley.

Hoffman, H., and M.E. Frank, 1965. Synchrony of division in clonal microcolonies of *Escherichia coli*. *Journal of Bacteriology 89*, 513-517.

Howard, A., and S.R. Pelc, 1953. Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. *Heredity, London (Suppl.) 6*, 261-273.

Hughes, W.H., 1955. The inheritance of differences in growth rate in *Escherichia coli*. *Journal of General Microbiology 12*, 265-268.

Johnson, N.L., and S. Kotz, 1970. *Continuous Univariate Distributions - 1*. Boston: Houghton-Mifflin.

Keeping, E.S., 1962. *Introduction to Statistical Inference*. Princeton: Van Nostrand.

Kelly, C.D., and O. Rahn, 1932. The growth rate of individual bacterial cells. *Journal of Bacteriology 23*, 147-153.

Kempner, E.S., and F.E. Hanson, 1968. Aspects of light production by *Photobacterium fischeri*. *Journal of Bacteriology 95*, 975-979.

Kendall, D.G., 1948. On the role of variable generation time in the development of a stochastic birth process. *Biometrika 35*, 316-330.

Kendall, D.G., 1952. On the choice of mathematical model to represent normal bacterial growth. *Journal of the Royal Statistical Society B 14*, 41-44.

Koch, A.L., 1966a. On evidence supporting a deterministic process of bacterial growth. *Journal of General Microbiology 43*, 1-5.

Koch, A.L., 1966b. Distribution of cell size in growing cultures of bacteria and the applicability of the Collins-Richmond principle. *Journal of General Microbiology 45,* 409-417.

Koch, A.L., and G. Blumberg, 1976. Distribution of bacteria in the velocity gradient centrifuge. *Biophysical Journal 16,* 389-405.

Koch, A.L., and M. Schaechter, 1962. Model for statistics of the cell division process. *Journal of General Microbiology 29,* 435-454.

Kubitschek, H.E., 1958. Electronic counting and sizing of bacteria. *Nature, London 182,* 234-235.

Kubitschek, H.E., 1960. Electronic measurement of particle size. *Research 13,* 128-135.

Kubitschek, H.E., 1962a. Normal distribution of cell generation rate. *Experimental Cell Research 26,* 439-450.

Kubitschek, H.E., 1962b. Loss of resolution in Coulter counters. *Review of Scientific Instruments 33,* 576-7.

Kubitschek, H.E., 1964. Apertures for Coulter counters. *Review of Scientific Instruments 35,* 1598-9.

Kubitschek, H.E., 1966. Generation times : ancestral dependence and dependence upon cell size. *Experimental Cell Research 43,* 30-38.

Kubitschek, H.E., 1967. Cell generation times : ancestral and internal controls. *Fifth Berkeley Symposium on Mathematical Statistics and Probability.* Vol.4, pp.549-572. Eds. L. Le Cam and J. Neyman. Berkeley: University of California Press.

Kubitschek, H.E., 1968a. Linear cell growth in *Escherichia coli. Biophysical Journal 8,* 792-804.

Kubitschek, H.E., 1968b. Constancy of uptake during the cell cycle in *Escherichia coli. Biophysical Journal 8,* 1401-1412.

Kubitschek, H.E., 1969a. Counting and sizing microorganisms with the Coulter counter. In *Methods in Microbiology.* Vol.1, pp.593-610. Eds. J.R. Norris and D.W. Ribbons. New York: Academic Press.

Kubitschek, H.E., 1969b. Growth during the bacterial cell cycle : analysis of cell size distribution. *Biophysical Journal 9,* 792-809.

Kubitschek, H.E., 1970. Evidence for the generality of linear cell growth. *Journal of Theoretical Biology 28,* 15-29.

Kubitschek, H.E., 1971a. Control of cell growth in bacteria : experiments with thymine starvation. *Journal of Bacteriology 105,* 472-476.

Kubitschek, H.E., 1971b. The distribution of cell generation times. *Cell Tissue Kinetics 4,* 113-122.

Kubitschek, H.E., 1974. Constancy of the ratio of DNA to cell volume in steady-state cultures of *Escherichia coli* B/r. *Biophysical Journal 14,* 119-123.

Kubitschek, H.E., H.E. Bendigkeit, and M.R. Loken, 1967. Onset of DNA synthesis during the cell cycle in chemostat cultures. *Proceedings of National Academy of Sciences, U.S.A. 57,* 1611-1617.

Kubitschek, H.E., and M.L. Freedman, 1971. Chromosome replication and the division cycle of *Escherichia coli* B/r. *Journal of Bacteriology 107,* 95-99.

Kubitschek, H.E., M.L. Freedman, and S. Silver, 1971. Potassium uptake in synchronous and synchronized cultures of *Escherichia coli*. *Biophysical Journal 11,* 787-797.

Lark, K.G., and O. Maaløe, 1954. The induction of cellular and nuclear division in *Salmonella typhimurium* by means of temperature shifts. *Biochimica et Biophysica Acta 15,* 345-356.

Lebowitz, J.L., and S. Rubinow, 1974. A theory for the age and generation time distribution of a microbial population. *Journal of Mathematical Biology 1,* 17-36.

Maaløe, O., 1962. Synchronous growth. In *The Bacteria.* Vol.IV, pp.1-32. Eds. I.C. Gunsalus and R.Y. Stanier. New York: Academic Press.

MacLean, F.I., and R.J. Munson, 1961. Some environmental factors affecting the length of *Escherichia coli* organisms in continuous cultures. *Journal of General Microbiology 25,* 17-27.

Marr, A.G., R.J. Harvey, and W.C. Trentini, 1966. Growth and division of *Escherichia coli*. *Journal of Bacteriology 91,* 2388-2389.

Marr, A.G., P.R. Painter, and E.H. Nilson, 1969. Growth and division of individual bacteria. *Symposium of the Society for General Microbiology 19,* 237-261.

Maruyama, Y., 1956. Biochemical aspects of cell growth of *Escherichia coli* as studied by the method of synchronous culture. *Journal of Bacteriology 72,* 821-826.

Maruyama, Y., and T. Yanagita, 1956. Physical methods for obtaining synchronous culture of *Escherichia coli*. *Journal of Bacteriology 71,* 542-546.

Mel, H.C., and D.W. Ross, 1975. Biophysics of cell separations. *Quarterly Reviews of Biophysics 8,* 421-438.

Miller, R.G., 1973. Separation of cells by velocity sedimentation. In *New Techniques in Biophysics and Cell Biology.* Vol.1, pp.87-112. Eds. R.H. Pain and B.J. Smith. New York: Wiley.

Miller, R.G., and R.A. Phillips, 1969. Separation of cells by velocity sedimentation. *Journal of Cell Physiology 73,* 191-202.

Minor, P.D., and J.A. Smith, 1974. Explanation of degree of correlation of sibling generation times in animal cells. *Nature, London 248,* 241-243.

Mitchison, J.M., 1971. *The Biology of the Cell Cycle.* London: Cambridge.

Mitchison, J.M., and W.S. Vincent, 1965. Preparation of synchronous cell cultures by sedimentation. *Nature, London, 205,* 987-989.

Mitchison, J.M., and K.M. Wilbur, 1962. The incorporation of protein and carbohydrate precursors during the cell cycle of a fission yeast. *Experimental Cell Research 26,* 144-157.

Nachtwey, D.S., and I.L. Cameron, 1968. Cell cycle analysis. In *Methods in Cell Physiology.* Vol.III, pp.213-259. Ed. D.M. Prescott. New York: Academic Press.

Nealson, T.H., T. Platt, and J.W. Hastings, 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *Journal of Bacteriology 104,* 313-322.

Nurse, P., and P. Fantes, 1977. Transition probability and cell-cycle initiation in yeast. *Nature, London 267,* 647.

Painter, P.R., 1975. Generation times of bacteria. *Journal of General Microbiology 89,* 217-220.

Painter, P.R., and A.G. Marr, 1968. Mathematics of microbial populations. *Annual Review of Microbiology 22,* 519-548.

Papoulis, A., 1962. *The Fourier Integral and its Applications.* New York: McGraw-Hill.

Papoulis, A., 1966. Error analysis in sampling theory. *Proceedings of the Institute of Electrical & Electronics Engineers 54,* 947-955.

Pierucci, O., 1972. Chromosome replication and cell division in *Escherichia coli* at various temperatures of growth. *Journal of Bacteriology 109,* 848-854.

Powell, E.O., 1955. Some features of the generation times of individual bacteria. *Biometrika 42,* 16-44.

Powell, E.O., 1956a. Growth rate and generation time of bacteria, with special reference to continuous culture. *Journal of General Microbiology 15,* 492-511.

Powell, E.O., 1956b. An improved culture chamber for the study of living bacteria. *Journal of the Royal Microscopical Society 75,* 235-243.

Powell, E.O., 1958. An outline of the pattern of bacterial generation times. *Journal of General Microbiology 18,* 382-417.

Powell, E.O., 1964. A note on Koch & Schaechter's hypothesis about growth and fission of bacteria. *Journal of General Microbiology 37,* 231-249.

Powell, E.O., 1969. Generation times of bacteria : real and artificial distributions. *Journal of General Microbiology 58,* 141-144.

Powell, E.O., and F.P. Errington, 1963. Generation times of individual bacteria : some corroborative measurements. *Journal of General Microbiology 31,* 315-327.

Princen, L.H., and W.F. Kwolek, 1966. Coincidence corrections for particle size determinations with the Coulter Counter. *Review of Scientific Instruments 36,* 646-653.

Pritchard, R.H., P.T. Barth, and J. Collins, 1969. Control of DNA synthesis in bacteria. *Symposium of the Society for General Microbiology 19,* 263-297.

Prescott, D.M.,1959. Variations in the individual generation times of *Tetrahymena geleii* HS. *Experimental Cell Research 16,* 279-287.

Rackham, S.J., 1977. *A Pulse Height Analyser for Displaying Coulter Counter Particle Size Distributions.* M.Sc. thesis, University of Waikato.

Rahn, O., 1932. A chemical explanation for the variability of the growth rate. *Journal of General Physiology 15,* 257-277.

Rowbury, R.J., 1972. Bacterial cell division : its regulation and relation to DNA synthesis. *Science Progress, Oxford 60,* 169-188.

Rubinow, S.I., 1968. A maturity-time representation for cell populations. *Biophysical Journal 8,* 1055-1073.

Rye, R.M., and D. Wiseman, 1967. Some factors affecting the sizing & counting of *Escherichia coli* with the Coulter counter. *Journal of Pharmacy & Pharmacology 19 (Supplement),* 9S-14S.

Schaechter, M., J.P. Williamson, J.R. Hood, Jun., and A.L. Koch, 1962. Growth, cell and nuclear divisions in some bacteria. *Journal of General Microbiology 29,* 421-434.

Schumaker, V.N., 1967. Zone centrifugation. *Advances in Biological and Medical Physics 11,* 245-337.

Shehata, T.E., and A.G. Marr, 1970. Synchronous growth of enteric bacteria. *Journal of Bacteriology 103,* 789-792.

Shields, R., 1976. New view of the cell cycle. *Nature, London 260,* 193-194.

Shilo, B., V. Shilo, and G. Simchen, 1976. Cell cycle initiation in yeast follows first-order kinetics. *Nature, London 264,* 767-770.

Smith, J.A., and L. Martin, 1973. Do cells cycle? *Proceedings of the National Academy of Sciences, U.S.A. 70,* 1263-1267.

Smith, J.A., and L. Martin, 1974. Regulation of cell proliferation. In *Cell Cycle Controls.* Eds. G.M. Padilla, I.L. Cameron, and A. Zimmerman, pp.43-60. New York: Academic Press.

Smither, R., 1975. Use of a Coulter counter to detect discrete changes in cell numbers and volume during growth of *Escherichia coli. Journal of Applied Bacteriology 39,* 157-165.

Stárka, J., and J. Koza, 1959. Nephelometric determination of cell count in synchronously dividing cultures of bacteria. *Biochimica et Biophysica Acta 32,* 261-262.

Sykes, J., 1971. Centrifugal techniques for the isolation and characterization of subcellular components from bacteria. In *Methods in Microbiology*. Vol.5B, p.84. Eds. J.R. Norris and D.W. Ribbons. New York: Academic Press.

Topping, J., 1962. *Errors of Observation and their Treatment*. 3rd ed. London: Chapman & Hall.

Tsuchiya, H.M., A.G. Fredrickson, and R. Aris, 1966. Dynamics of microbial cell populations. *Advances in Chemical Engineering 6*, 125-206.

Wheals, A.E., 1977. Transition probability and cell-cycle initiation in yeast. *Nature, London 267*, 647-648.

Wood, L.C., 1968. A review of digital pass filtering. *Reviews of Geophysics 6*, 73-97.

Yule, G.U., 1925. A mathematical theory of evolution, based on the conclusions of Dr J.C. Willis, F.R.S. *Philosophical Transactions B 213*, 21-87.

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Koch, A.L., 1966c. The logarithm in biology. 1. Mechanisms generating the log-normal distribution exactly. *Journal of Theoretical Biology 12*, 276-290.