

METHODS IN CELL PHYSIOLOGY

Volume III

David M. Prescott

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VOLUME III

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Methods in Cell Physiology

Edited by

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INSTITUTE FOR DEVELOPMENTAL BIOLOGY
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VOLUME III



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PREFACE

Volume III of this treatise continues to present techniques and methods in cell research that have not been published or have been published in sources that are not readily available. Much of the information on experimental techniques in modern cell biology is scattered in a fragmentary fashion throughout the research literature. In addition, the general practice of condensing to the most abbreviated form materials and methods sections of journal articles has led to descriptions that are frequently inadequate guides to techniques. The aim of this volume is to bring together into one compilation complete and detailed treatment of a number of widely useful techniques which have not been published in full detail elsewhere in the literature.

In the absence of firsthand personal instruction, researchers are often reluctant to adopt new techniques. This hesitancy probably stems chiefly from the fact that descriptions in the literature do not contain sufficient detail concerning methodology; in addition, the information given may not be sufficient to estimate the difficulties or practicality of the technique or to judge whether the method can actually provide a suitable solution to the problem under consideration. The presentations in this volume are designed to overcome these drawbacks. They are comprehensive to the extent that they may serve not only as a practical introduction to experimental procedures but also to provide, to some extent, an evaluation of the limitations, potentialities, and current applications of the methods. Only those theoretical considerations needed for proper use of the method are included.

Finally, special emphasis has been placed on inclusion of much reference material in order to guide readers to early and current pertinent literature.

DAVID M. PRESCOTT

September, 1968

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Chapter 1

Measurement of Cell Volumes by Electric Sensing Zone Instruments

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I. Introduction

The measurement of particle concentrations and volumes by electric sensing zone instruments has found wide application in recent years. Such instruments are capable of rapid and very accurate measurement of particle concentrations, and of more or less accurate determinations of volume distributions of particle populations.

The original biological application of the technique was in routine blood cell counting (Brecher *et al.*, 1956). Kubitschek (1958, 1960) introduced modifications which permitted counting of bacterial cells, and

pointed out that electric sensing zone instruments were capable of measuring cell volume distributions as well as numbers. With instruments currently available, particles ranging in volume from about 0.1 to 5×10^5 μ^3 (for corresponding spherical particles, a range of diameters from 0.6 to 100 μ) can be counted and measured, provided only that the particles can be suspended in a medium of suitable electrical conductivity. This size range includes a wide variety of types of cells, including bacteria, the smaller protozoa, red and white blood cells, and tissue culture cells, all of which have been successfully studied by this method.

A recent review by Kubitschek (1967) provides a comprehensive description of the methodology and the underlying theory of the electric sensing zone technique. In an earlier review Berg (1965) describes electric, optical, and sonic sensing zones for particle counting and measurement.

The present paper is concerned with the accuracy of measurement of volume distributions by electric sensing zone instruments now in use. These instruments are: (1) The Coulter Counter Model B (Coulter, 1953, Coulter Electronics Inc., Hialeah, Florida); (2) The instrument described by Harvey and Marr (1966); (3) The Celloscope 101 (Particle Data Inc., Elmhurst, Illinois); (4) The Nuclear Chicago particle measurement system (Nuclear Chicago Corp., Des Plaines, Illinois). There is little question of the accuracy of all these instruments in the counting of cells. The accuracy and fidelity of measurement of volume is by no means as certain nor is it easily determined. Faced with this uncertainty most workers have evidently been content to accept the apparent volume distributions measured by their instrument as accurately representing the real distribution. More rigor is shown by Kubitschek (1967) who applied the criterion that measurements should be made under conditions where the variance of a standard suspension of particles is at a minimum.

The only attempt at rigorous evaluation of the fidelity of measurement of volume distribution is that of Harvey and Marr (1966). The volume distribution of a suspension of latex spheres was measured by electron microscopy. The electron microscopic measurements were taken as a primary standard, and statistical comparisons of distributions measured by the electric sensing zone instrument with the primary standard were used as the criteria for fidelity and accuracy of measurement. Their results are recapitulated in this paper, and the technique is used to evaluate the performance of instruments not tested in their original study.

The value of electron microscopic measurements as a primary standard is not completely established. Bonse and Hart (1966) have shown that the mean diameters of latex spheres measured by electron microscopy agree

very well with values calculated from X-ray diffraction measurements. Smaller particles (diameters $< 0.5\ \mu$) tended to be measured as too large, and larger particles (diameters $> 2\ \mu$) were measured as too small by electron microscopy, but in the range of diameters 0.5–2 μ the agreement was within 1%. Since the X-ray diffraction data were shown to be free of any detectable systematic errors these results provide reasonable evidence of the validity of electron microscopic data as a primary standard.

II. Design of Equipment

A. The Electric Sensing Zone Transducer

The transducer is a cylindrical aperture separating two electrode chambers filled with electrolyte. The aperture is commonly fabricated from ruby or glass, and may have a diameter of 10 μ to 1 mm, the length usually being about equal to the diameter.

The particles being measured are suspended in the outer electrode chamber and pumped through the aperture. When a dc voltage is applied between the electrodes, the aperture and a small hemispherical volume at either end of it provide the major resistance to flow of current and form the sensing zone of the transducer. During the passage of a cylindrical particle of volume v through the aperture, the resistance R of the sensing zone will be increased. The magnitude, ΔR of the resistance increase is a function of the particle volume (Kubitschek, 1958);

$$\frac{\Delta R}{R} = \frac{v}{V}\left(\frac{\rho}{\rho - \rho_0} - \frac{a}{A}\right)^{-1} \tag{1}$$

where V = the volume of the sensing zone
A = area of the aperture normal to its axis
ρ_0 = resistivity of the electrolyte
ρ = resistivity of the particle
a = cross-sectional area of the particle normal to the axis of the aperture

For particles with $a \ll A$ Eq. (1) reduces to:

$$\frac{\Delta R}{R} \simeq \frac{v}{V} \cdot \frac{\rho - \rho_0}{\rho} \tag{2}$$

If the resistivity of the particle is much greater than the resistivity of the electrolyte, the response will become independent of the resistivity of the particle:

$$\frac{\Delta R}{R} \simeq \frac{v}{V} \tag{3}$$

Thus for particles with diameters much smaller than that of the aperture the resistance change produced by the particle is proportional to its volume. When the diameter of the particle is 10% of the aperture diameter ($a/A = 0.01$) the deviation from linearity will be 1%, and this can be taken as setting a practical upper limit for the particle size which can be measured with a given aperture.

This analysis holds for particles in the shape of a right cylinder. Under some circumstances particles of different shapes but equal volumes can give rise to different resistance changes. Gregg and Steidley (1965) showed that the resistance change produced by a disk with its face normal to the axis of the aperture was three times the resistance change produced by a sphere of equal volume. This is due to the fact that current flow lines will not follow flat surfaces normal to the flow of current, as in the case of the disk. Thus a volume of high effective resistivity is established greater than the volume of the disk. This will not be the case for particles which have no sharp edges or flat faces, and have smooth surfaces, a description which will fit most biological cells. For such particles current flow lines will closely follow the surface, and the resistance change will not be greatly influenced by particle shape. Kubitschek (1967) has calculated that, when compared to a sphere of the same volume, a prolate spheroid with an aspect ratio of 4:1 will give rise to a resistance change only 3% greater.

The increase in resistance produced by the passage of a particle results in a transient change in the voltage drop across the sensing zone, proportional to the resistance change, and this voltage pulse constitutes the output of the transducer. The form of the pulse would ideally be a square wave, but the actual pulse will not have this shape, primarily because of the gradient of the electrical field and the capacitance of the aperture. From the measurements of Gregg and Steidley (1965) this capacitance is of the order of 100 picofarads, giving the aperture a time constant of 1–5 μsec, depending upon its resistance. Hence the minimum rise time of the pulse would be 2–10 μsec. The capacitance of the particle could also affect the rise time. However, Gregg and Steidley (1965) have shown that for typical mammalian cells the time constant due to this capacitance is about 0.4 μsec, making this effect relatively unimportant.

After the rise, the pulses may be flat-topped, but a slight concavity is often observed (Kubitschek, 1967), which can possibly be attributed to the capacitances of the particle and the aperture. The pulse has a finite decay time, due again to the field gradient and aperture capacitance. Under normal conditions of operation, the mean transit time of particles through the sensing zone will be 10–30 μsec, depending on the dimensions of the aperture and the pressure applied across it. The overall pulse duration will be of this order of magnitude.

The transducer described above is used in all instruments with only minor modifications. The pulses produced are amplified and measured and it is in the amplification and measurement systems that the instruments differ.

B. Pulse Amplification and Measurement

1. Coulter Counter Model B

This instrument employs simple amplification of pulses by a vacuum tube amplifier. The time constant of the amplifier is about 30 μsec, which results in about a 10-fold increase in the duration of pulses during amplification. The amplified pulses are measured by a single-channel analyzer, which allows manual measurement of size distributions.

2. Instrument Described by Harvey and Marr (1966)

The pulses from the transducer are first amplified by a Tennelec 100B preamplifier (Tennelec Instrument Co., Inc., Oak Ridge, Tennessee). The pulses have a duration of 200 to 300 μsec at the output of the preamplifier, which is about 10 times greater than that expected from the mean transit time of particles through the sensing zone used. Since the preamplifier has a rise time of less than 1 μsec, this distortion of the pulses must result from the design of the constant current device used to provide the voltage between the electrodes. The pulse shapes are restored by differentiation and integration using a Tennelec TC200 linear amplifier (Tennelec Instrument Co., Inc.). This procedure reduces pulse widths to about 10 μsec, and the amplitude of the resulting pulse is proportional to the true amplitude of the input pulse above any arbitrary baseline. As a result the true amplitudes of coincident pulses can be measured. A final differentiation produces pulses of suitable form for acceptance by a Nuclear Data 180-FM 512-channel pulse-height analyzer (Nuclear Data, Inc., Palatine, Illinois). The analyzer measures each pulse and stores it as a count in a channel of the memory unit, the channel number being proportional to the amplitude of the pulse.

3. Celloscope 101

Simple amplification of pulses by a close-coupled solid-state amplifier is used. The amplifier is linear to about 80% of maximum output, and has a time constant of about 10 μsec. The duration of pulses is thus increased 2–3 times during amplification. The amplifier pulses can be measured by a single-channel analyzer incorporated in the instrument, but are of suitable shape for acceptance by a multichannel pulse-height analyzer. A Nuclear Data 110 128-channel analyzer is used in the present study.

4. Nuclear Chicago Particle Measurement System

Pulses are amplified by a solid-state linear amplifier. Output pulses from the amplifier have a 5–20 μsec rise time. They pass to a discriminator circuit, and if the pulse amplitude exceeds the chosen threshold value, a monostable multivibrator circuit with a variable cycle period is triggered. This permits the original pulse to enter a sampling circuit which charges to the maximum voltage reached by the pulse during the cycle period. At the end of the cycle period the sample gate closes, and the sampling circuit discharges, producing a pulse with amplitude proportional to the maximum voltage reached during the sampling period, and with a shape suitable for acceptance by a 400-channel pulse-height analyzer.

III. Evaluation of Fidelity of Measurement of Particle Volume

A. Methods of Measurement

1. Measurement by Electron Microscopy

Polyvinyltoluene latex spheres with nominal diameter 2.051 μ and polystyrene latex spheres with nominal diameter 1.099 μ were obtained from Dow Chemical Co., Midland, Michigan. The 2.051 μ spheres were used in the evaluation of the Coulter Counter and the apparatus of Harvey and Marr (1966), the 1.099 μ spheres in the evaluation of the Celloscope and the Nuclear Chicago apparatus. A suspension of cells of *Escherichia coli* strain ML30 was also used for evaluation of the apparatus of Harvey and Marr (1966). For measurement by electron microscopy 0.1% formaldehyde was added to a sample of culture, which was then centrifuged, washed once in 0.04% formaldehyde, and suspended in a solution containing 0.04% formaldehyde and 0.1% serum albumin at a concentration

of 2×10^8 cells/ml. Latex spheres were prepared for electron microscopy by dilution into 0.1% serum albumin. Suspension of cells and spheres were sprayed onto Formvar-coated grids and air-dried.

Electron micrographs of 2.051 μ latex spheres and cells of *E. coli* ML30 were made with an RCA EMU-3E microscope. Magnification was controlled to within $\pm 0.75\%$. Micrographs were printed to give a final magnification of 10,000 $\times$. Electron micrographs of 1.099 μ latex spheres were made with a Siemens Elmiskop IA. Close control of magnification was obtained by making all micrographs from a single grid. Barrel distortion, estimated from parallel micrographs of a carbon replica of a standard silver grid, was less than 0.2%. Micrographs were printed at a final magnification of 17,000 $\times$. Dimensions of spheres and cells were estimated from the prints to within ± 0.1 mm. The volumes of spheres were calculated from the measured diameters. Measurement of length is sufficient to estimate relative volume of cells of *E. coli* (Marr *et al.*, 1966).

2. Measurement by Electric Sensing Zone Instruments

Latex spheres were diluted in filtered 0.85% sodium chloride or 0.1 *N* hydrochloric acid to a final concentration of 2 to 5×10^5 particles/ml. Cells of *E. coli* were diluted to the same concentration in 0.85% sodium chloride containing 0.04% formaldehyde.

Transducers with an aperture diameter of 30 μ and aperture length close to 30 μ were used in the evaluation of all instruments. The Coulter Counter and the apparatus of Harvey and Marr (1966) used transducers manufactured by Coulter Electronics, Inc. The aperture in these transducers is fabricated from glass and the rate of flow through the aperture is approximately 0.2 ml/minute. Transducers manufactured by Particle Data, Inc. were used in the Celloscope and the Nuclear Chicago system. The aperture in these transducers is fabricated from ruby, and the rate of flow through the aperture is approximately 0.12 ml/minute.

B. Comparison of Electric Sensing Zone and Electron Microscopic Measurements

1. Criteria Used in Comparison

In comparing the distributions of particle volume measured by the electric sensing zone method with those measured by electron microscopy two criteria were used:

(a) The coefficients of variation (C.V.) of the two distributions should not be significantly different by the *t* test.

(b)The probability that the value of chi-square for the difference between the distributions is due to chance should be equal to or greater than 0.05. Chi-square was calculated taking the electron microscopic measurements as the observed distribution and the electric sensing zone measurements as the expected distribution. This convention was adopted since the distributions measured by the electronic sensing zone method are based on measurements of 5000–10000 particles, whereas electron microscopic measurements included only 250–350 particles.

2. Coulter Counter

Figure 1 compares the distribution of volume of 2.051 μ nominal diameter latex spheres measured by electron microscopy with the distribution measured by the Coulter Counter Model B. The latter shows a strong positive skewness, compared with the slight negative skewness of the distribution measured by electron microscopy, and its C.V. is about twice as great (0.134 versus 0.0688). The probability that the value of

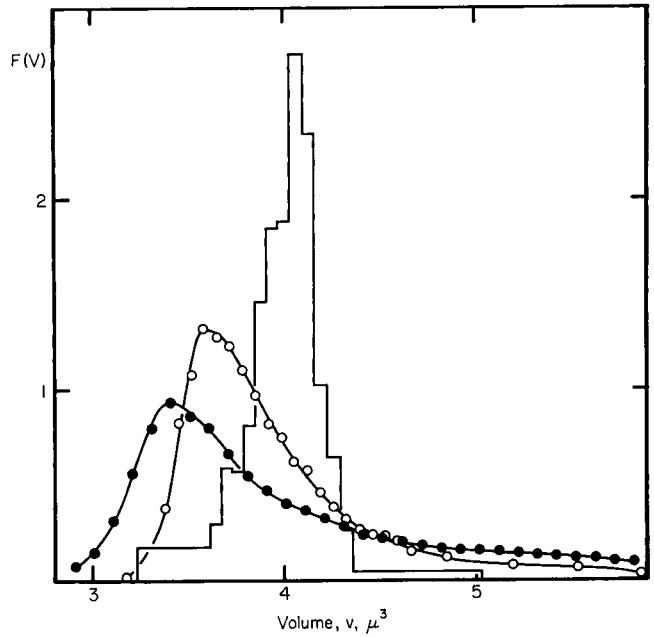


Fig. 1. Frequency functions of volume distributions of latex spheres with nominal diameter of 2.051 μ. Histogram, measured by electron microscopy; ○, measured by Coulter Counter Model B; ●, measured by the instrument of Harvey and Marr (1966) without differentiation and integration of pulses. The distributions are transformed to obtain equal means. Reprinted with permission from *J. Bacteriol.* **92**, 805 (1966).

chi-square for the difference is due to chance is less than 0.0001. Measurements of volume distributions by the Coulter Counter are thus seriously distorted, the frequency of larger volumes being greatly exaggerated.

3. Apparatus of Harvey and Marr (1966)

Figure 1 also shows the distribution of volumes of 2.051 μ diameter latex spheres measured by the apparatus of Harvey and Marr (1966) without differentiation and integration of pulses. The result is similar to that obtained with the Coulter Counter. The C.V. of the electric sensing zone measurement is 0.18, more than twice the C.V. of the electron microscopic measurement, and the probability that the value of chi-square for

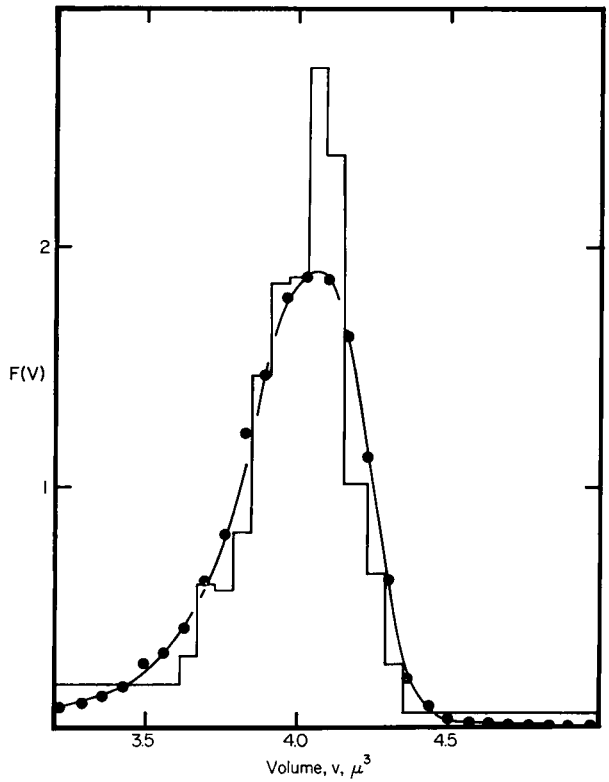


FIG. 2. Frequency functions of volume distributions of latex spheres with nominal diameter of 2.051 μ. Histogram, measured by electron microscopy; ●, measured by the instrument of Harvey and Marr (1966) with differentiation and integration of pulses. The distributions are transformed to obtain equal means. Reprinted with permission from *J. Bacteriol.* **92**, 805 (1966).

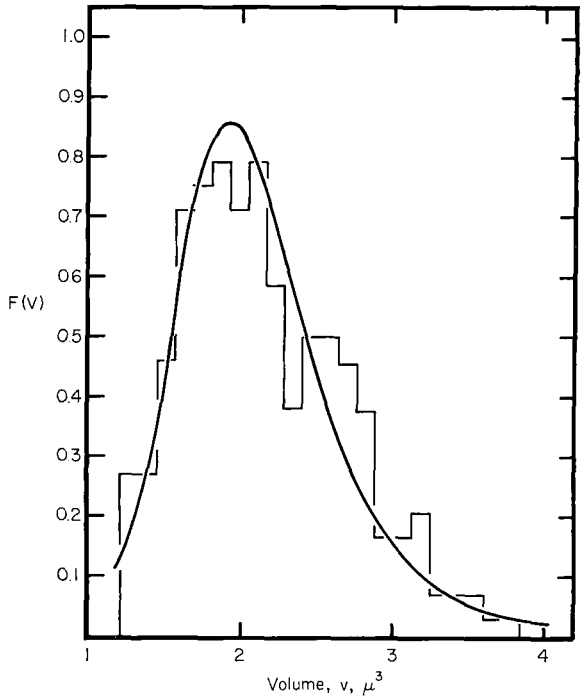


FIG. 3. Frequency function of the distribution of length of cells of *E. coli*, measured by electron microscopy (histogram), compared with the distribution of volume of the same sample measured by the instrument of Harvey and Marr (1966) with differentiation and integration of pulses (solid curve). Distributions are transformed to obtain equal means. Reprinted with permission from *J. Bacteriol.* **92**, 805 (1966).

the difference is due to chance is less than 0.0001. The distortion again consists of an exaggeration of the frequency of larger particles.

The distribution measured with differentiation and integration of the pulses is compared with the distribution measured by electron microscopy in Fig. 2. The coefficients of variation are not significantly different; 0.0613 ± 0.002 for the electric sensing zone measurement, versus 0.0688 ± 0.003 for the electron microscopic measurement. The probability that the value of chi-square obtained for the difference between the distributions was due to chance was 0.165.

A comparison between electric sensing zone and electron microscopic measurement of the volume distribution of cells of *E. coli* was also made. These cells have the form of cylinders with hemispherical ends, and a wide range of axial asymmetry is found within a single population. The results of the comparison (Fig. 3) show that this variation in shape

does not measurably distort the measurement of the volume distribution by the electric sensing zone apparatus. The coefficients of variation were 0.247 ± 0.009 for the electron microscopic measurement and 0.233 ± 0.009 for the electric sensing zone measurement; the difference is not significant. The probability that the value of chi-square calculated for the differences between the distributions was due to chance was 0.78.

The technique of differentiation and integration of pulses from the electric sensing zone thus appears to yield faithful measurements of particle volume distributions.

4. Celloscope 101

The distribution of volumes of 1.099 μ latex spheres measured by electron microscopy is compared with distributions measured by the

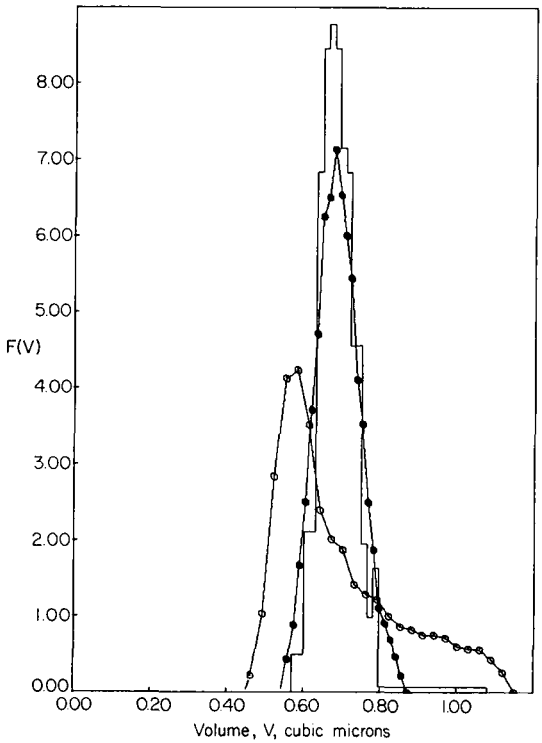


FIG. 4. Frequency functions of the distribution of volumes of latex spheres with 1.099 μ nominal diameter. Histogram, measured by electron microscopy; ○, measured by the Celloscope with normal operating conditions; ●, measured by the Celloscope with amplifier time constant and flow rate through the sensing zone modified as described in Section IV,C. Distributions are transformed to obtain equal means.

Celloscope in Fig. 4. When measured by the Celloscope with normal operating conditions, an exaggeration of the frequency of larger particles is again observed. The C.V. of the Celloscope measurement is 0.23, more than twice the C.V. of the electron microscope measurements, and the probability that chi-square for the difference is due to chance is less than 0.0001.

If the amplifier time constant of the Celloscope and the rate of flow through the sensing zone are modified as described in Section IV,C of this paper, very good agreement between the Celloscope and electron microscope measurements is obtained (Fig. 4). The distribution of volume of 1.099 μ diameter latex spheres measured by the Celloscope then has a C.V. of 0.0838 ± 0.001, versus 0.0804 ± 0.0106 for the electron microscopic measurements. The probability that the value of chi-square obtained for the difference between the distributions is due to chance is 0.28.

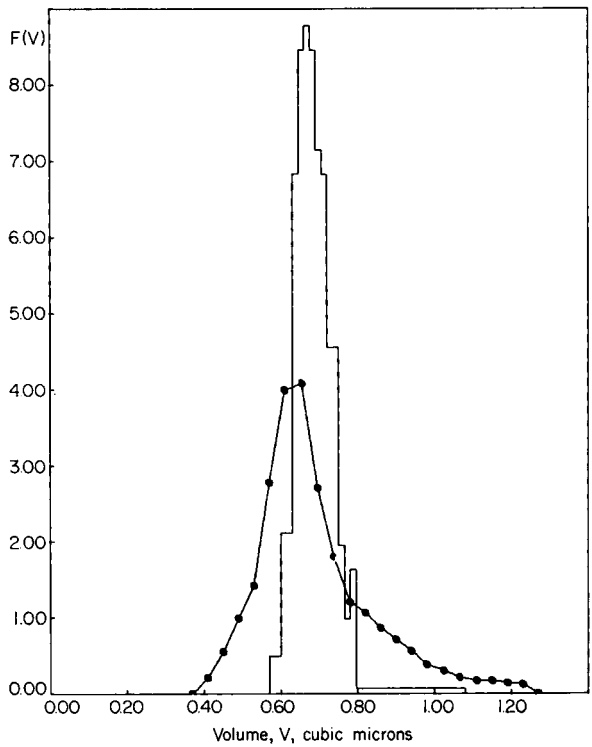


FIG. 5. Frequency functions of the distribution of volume of latex spheres with 1.099 μ nominal diameter. Histogram, measured by electron microscopy; ●, measured by the Nuclear Chicago particle measurement system. Distributions are transformed to obtain equal means.

This result shows that simple amplification and direct measurement of pulses from the electric sensing zone can give faithful measurement of particle volume distributions. The conditions which must be satisfied to obtain this result will be discussed in Section IV,C.

5. Nuclear Chicago Particle Measurement System

Comparison of measurements of the volume distribution of 1.099 μ diameter latex spheres by this instrument with the distribution measured by electron microscopy is shown in Fig. 5. The C.V. measured by the electric sensing zone instrument is 0.216, again more than twice that of the electron microscopic measurement, and the probability that chi-square for the difference is due to chance is less than 0.0001. The distortion takes the same form as that observed with the Coulter Counter and unmodified Celloscope. Measurements of similar latex spheres by Kubitschek (1967), using a 15 μ diameter aperture, instead of the 30 μ aperture used for this measurement, do not show this type of distortion. It is suggested (Kubitschek, personal communication) that poor noise characteristics of the Nuclear Chicago amplifier may result in this instrument being incapable of accurate measurement of particles of 1 μ diameter when used with a 30 μ diameter aperture.

IV. Causes and Remedies of Distortion in Electric Sensing Zone Volume Measurements

A. Inherent Linearity of Electric Sensing Zone Instruments

Direct amplification and measurement of the pulses produced by the passage of particles through the electric sensing zone can result in considerable distortion in the measurement of volume distributions. This is evident in the results from all instruments (Figs. 1, 4, and 5). Though the exact form of the distortion varies with different instruments, its principal feature is the same for all; that is, an exaggeration of the frequency of particles of larger volume. This produces an increase in the apparent coefficients of variation and skewness.

This distortion is not due to any inherent nonlinearity in the operation of the sensing zone or amplifier, as is shown in Fig. 6. Here the mean volumes measured by the Coulter Counter of three samples of latex spheres are plotted against the mean volumes determined electron microscopically by the manufacturer. Although the Coulter Counter produces severe distortion in the measurement of a single volume distribution (Fig. 1), the measurement of mean volume is linear to within $\pm$ 0.5% over a

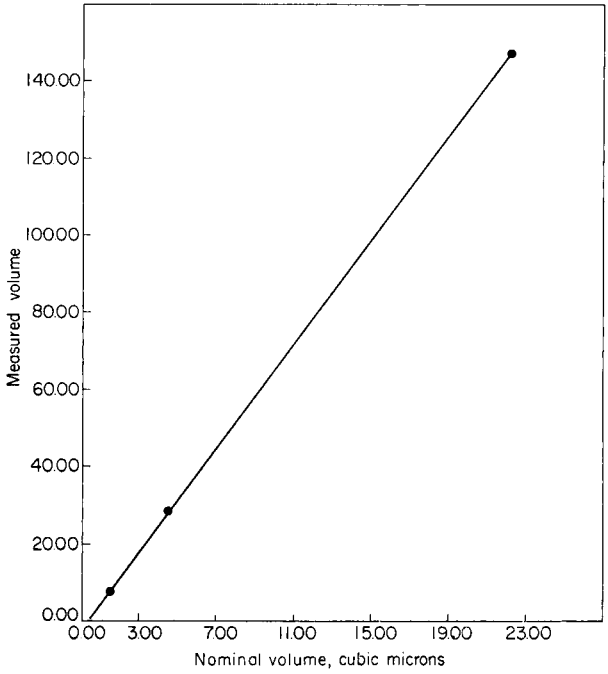


FIG. 6. Mean volume of latex spheres measured by the Coulter Counter Model B as a function of nominal volume. Standard error of the slope is 0.5%, and the intercept is not significantly different from zero.

20-fold range of volumes, and extrapolates to zero as expected. This result can be obtained, usually with greater accuracy, with all of the instruments described. It is evident, therefore, that at least the mean amplitude of the pulses produced by the passage of particles through the electric sensing zone is a linear function of the volume of the particles. On this basis there can be little doubt that Eq. (1) is valid, and that the causes of the distortion must be sought elsewhere. The results indicate that whatever the cause, it must be completely random with respect to particle volume.

B. Effect of Particle Coincidence

1. Physical Coincidence in the Sensing Zone

The simultaneous presence of two or more particles within the sensing zone will produce a resistance change roughly proportional to the sum

of the volumes of the particles. The coincident particles will be then measured as one spuriously large particle, and since two-particle coincidence will occur most frequently the apparent volumes of these spuriously large particles should be concentrated at twice the modal volume of the population. This is not precisely the form of the observed distortion in volume measurement, but physical coincidence could nonetheless contribute.

The frequency of coincident passage is determined by a Poisson distribution, and can be calculated if the volume of the sensing zone, and the particle concentration are known. The former is difficult to determine however, and the frequency of coincidence is more readily determined empirically, by comparison of measured with known concentration as the known concentration is increased. As measured by this method the frequency of coincidence in all volume measurements reported here is less than 1%. It is clear that the frequency of particles measured as spuriously

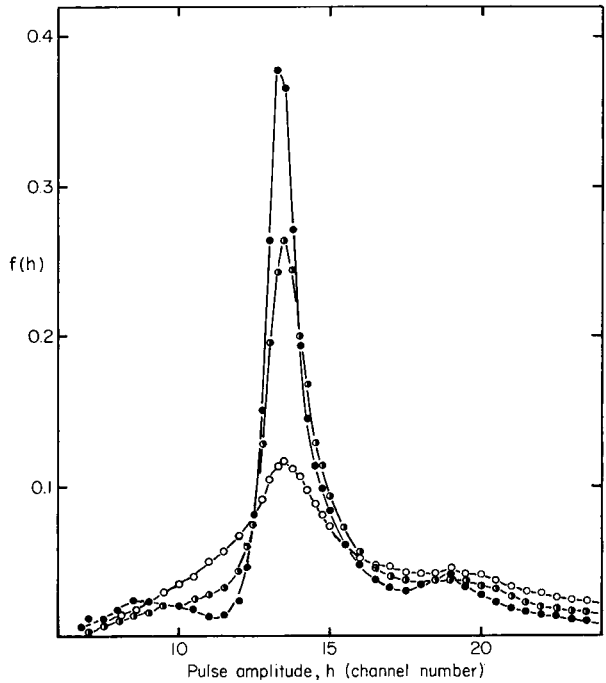


FIG. 7. Frequency functions of distribution of volume of latex spheres, 2.051 μ nominal diameter, measured at different counting rates by the instrument of Harvey and Marr (1966) without differentiation and integration of pulses. The counting rates were: ●, 1.34×10^4; ◑, 9.86×10^4; ○, 3.04×10^5 particles per minute. Reprinted with permission from *J. Bacteriol.* **92**, 805 (1966).

large in Figs. 1, 4, and 5 is much greater than this, hence physical coincidence in the sensing zone can be eliminated as a source of distortion.

2. Electronic Coincidence

In the Coulter Counter, and in the instrument of Harvey and Marr (1966), the time of passage of a particle through the sensing zone is between 10 and 20 μsec, but the duration of pulses in the preamplifier is increased to 200–300 μsec. Under conditions where coincidence in the sensing zone is eliminated, coincidence in the amplifier can still occur. Its effect on pulse amplitude differs from that of physical coincidence.

The greater part of the 200–300 μsec pulse duration is due to a long decay from peak to zero voltage. If a second pulse occurs during this period its apparent peak amplitude will be increased by an amount equal to the residual amplitude of the first pulse. Depending upon the timing of the two pulses, the second could have an apparent amplitude ranging from its true amplitude to an amplitude equal to the sum of the true amplitudes of the two. Harvey and Marr (1966) showed that this type of

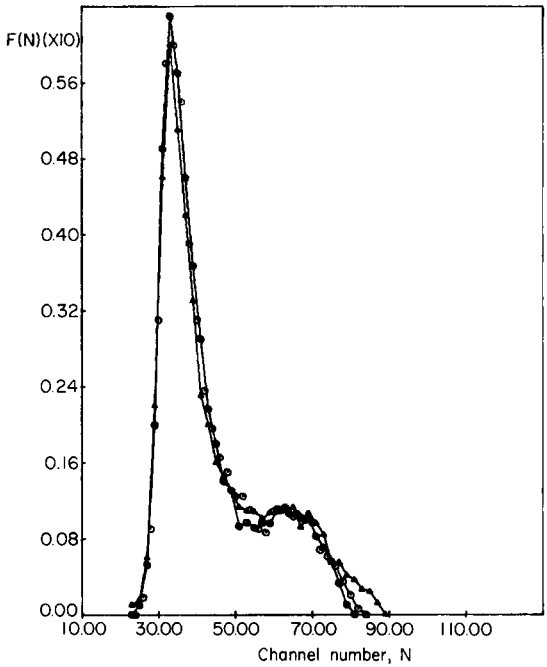


Fig. 8. Frequency functions of the distribution of volume of latex spheres, 1.099 μ nominal diameter, measured at different counting rates by the Celloscope. The counting rates were: ●, 4.5×10^4; ○, 6.6×10^4; ▲, 3.1×10^5 particles per minute.

coincidence could produce the type of distortion which is obtained in the measurement of volume distributions (Fig. 7). As counting rate, and hence the frequency of coincidence, was increased the apparent frequency of large particles increased.

In the Celloscope, however, pulse durations are only about 30 μsec, and it would be expected that this type of coincidence would contribute very little to the distortion. This is shown to be the case in Fig. 8; a 7-fold increase in counting rate results in only a very small increase in the frequence of the very largest apparent volumes.

Electronic coincidence can thus contribute to the observed distortion, but only where the pulses from the electric sensing zone are themselves extremely distorted.

C. Effect of the Distribution of Transit Times through the Sensing Zone

The 30 μ diameter aperture under normal operating conditions has a Reynolds number (Reynolds, 1883) of about 100, far below the critical value of 2000. Flow through the aperture is thus laminar, and if steady flow is assumed the velocity of flow will thus be distributed as (Streeter, 1962):

$$u = \frac{\Delta P}{4\mu L}(r_0^2 - r^2) \tag{4}$$

in which

r_0 = radius of the aperture
u = velocity at a distance r from the axis
ΔP = pressure drop along the aperture
L = length of the aperture
μ = viscosity of the liquid

The velocity varies parabolically. It is zero at the circumference of the aperture, and has a maximum value at the axis equal to twice the mean velocity.

The distribution of the volume of liquid passing through an aperture as a function of velocity can be derived from Eq. (4) and is:

$$Q = 1 - (u/u_m)^2 \tag{5}$$

in which Q is the fraction of the total flow passing with velocity equal to or greater than u, and u_m is the maximum velocity.

Equation (5) shows that for 99% of the particles passing through the aperture there can be a 10-fold range of velocities and thus of transit

times, and that 75% of the total can have a velocity greater than the mean velocity. Under the normal operating conditions, the range of velocities in a 30 μ diameter aperture will not be quite this large, since Eqs. (4) and (5) are not valid at the entrance to the aperture. The velocity at the entrance is uniform over the entire area of the aperture and equal to the mean velocity, and the length required to obtain the stable velocity distribution is dependent on the diameter of the tube and the Reynolds number (Langhaar, 1942). For the 30 μ aperture the range of velocities will actually be about 7-fold.

Kubitschek (1962) pointed out that the distribution of transit times of particles through the aperture could result in a distortion of the measured volume distribution. The results of Spielman and Goren (1967) support this contention. They found that reducing the distribution of transit times by confining the trajectories of particles to essentially the same streamline considerably reduced the spread of the measured distribution.

The amplifier of an instrument such as the Celloscope has a rise time of about 25 μsec, but the mean transit time of particles through the aperture is about 12 μsec. The amplifier is thus unable to respond fully to the voltage pulses produced at the sensing zone by particles with transit times less than the rise time of the amplifier, and the measured volumes of these particles will be a function of transit time as well as actual volume. Equation (5) shows that a large fraction of the particles will indeed have transit times less than 25 μsec. On this basis the peak of the distorted volume distribution measured by the Celloscope would be expected to correspond to particles passing through the aperture with transit times shorter than the rise time of the amplifier. The rather clearly defined tail of the measured distribution would be expected to correspond to particles passing through the aperture with transit times longer than the rise time of the amplifier. The amplitude of these pulses would be measured faithfully, thus the tail of the apparent distribution is expected to include the true distribution of volumes. If this hypothesis is correct, increasing the mean transit time of particles should result in improved fidelity of volume measurement. These predictions are confirmed by the results in Fig. 9.

Figure 9A shows the distribution of volume of 1.099 μ diameter latex spheres measured by the Celloscope under normal operating conditions; that is, with a pressure drop across the aperture of 16.8 cm of mercury, corresponding to an average time of transit through the aperture of 12 μsec. The measurement was made with a different 30 μ aperture from that used to obtain the data in Fig. 4, and a slight difference is observed between the two distributions; the distribution in Fig. 9A has a more pronounced positive skewness, and the tail has more the form of a

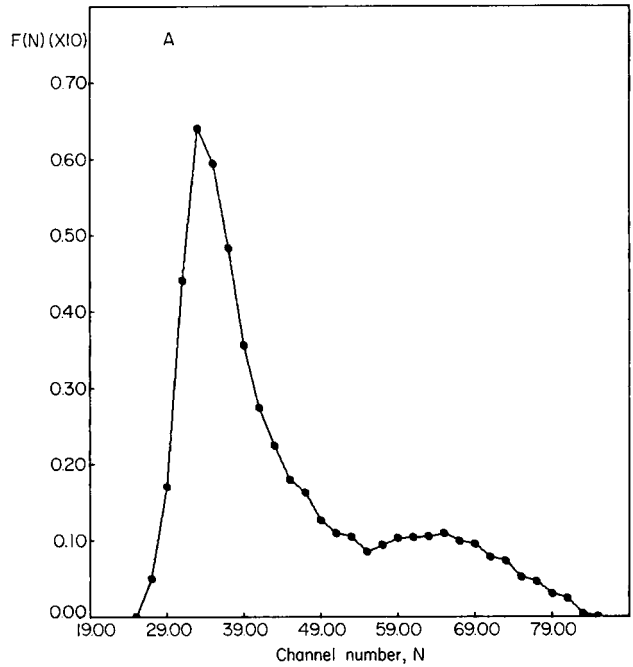
second peak. Treatment of the suspension of latex spheres with Triton-X nonionic detergent or brief sonic oscillation had no effect on the magnitude of this second peak, showing that it does not arise from aggregation of spheres to form doublets.

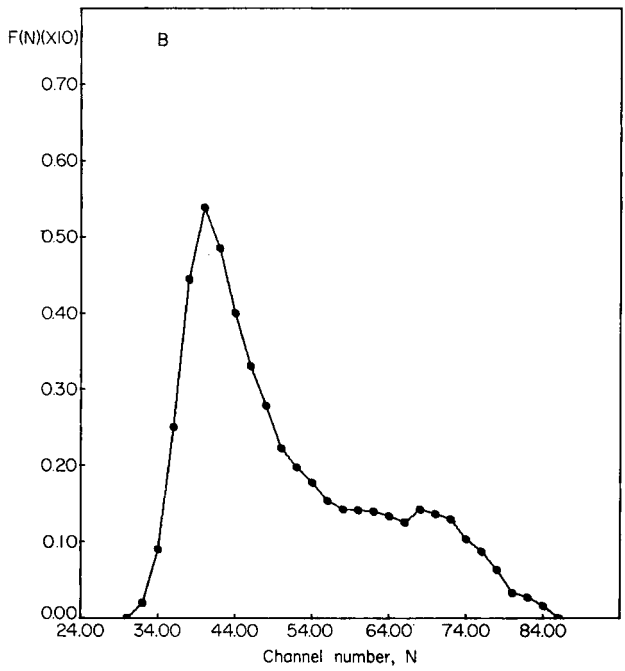
Figures 9B, C, and D show the effect of increasing the mean time of transit through the aperture. This was accomplished by reduction of the pressure drop across the aperture. As transit time is increased the mode of the upper peak remains stationary, and the fraction of the population contained within this peak increases. The apparent volume at the mode of the lower peak increases, and the two peaks coincide when the mean transit time is increased to 66 μsec (Fig. 9D). The distribution shown in Fig. 9D has a C.V. of 0.098 ± 0.001, which is not significantly different from the C.V. of the distribution measured by electron microscopy (0.804 ± 0.0106). However, the probability that the value of chi-square for the difference between the two distributions is due to chance is only 0.01, insufficient to meet the criteria set up in Section III,B,1.

The results in Fig. 9 support the hypothesis that the distribution of transit times of particles is a major source of distortion in electric sensing zone volume measurements. However the comparison with the electron microscopic measurements shows that some distortion remains. This might be expected, since with a 66 μsec mean transit time, the shortest transit times are only just greater than the rise time of the amplifier. If this distortion could be eliminated by a further increase in the mean transit time, the hypothesis would be fully confirmed. However, longer transit times cannot be used because then electrical heating produces unacceptable noise levels.

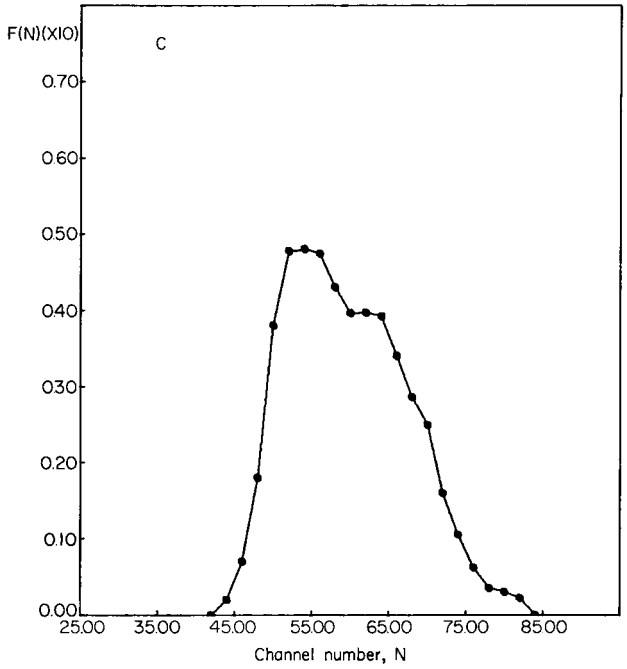
The hypothesis also predicts that improved fidelity of volume measurement should be obtained if the rise time of the amplifier were reduced. This can be easily done in the case of the Celloscope by reducing the capacitance of one condenser. It was found that, with the mean transit time held constant, reduction in the rise time of the amplifier produced identical results to those produced by increase in transit time shown in Fig. 9. The minimum rise time of the amplifier is limited by the capacitance of other circuit components to about 5 μsec and at the normal mean transit time this is insufficient to completely eliminate the distortion, since the shortest transit times are then only just greater than the rise time.

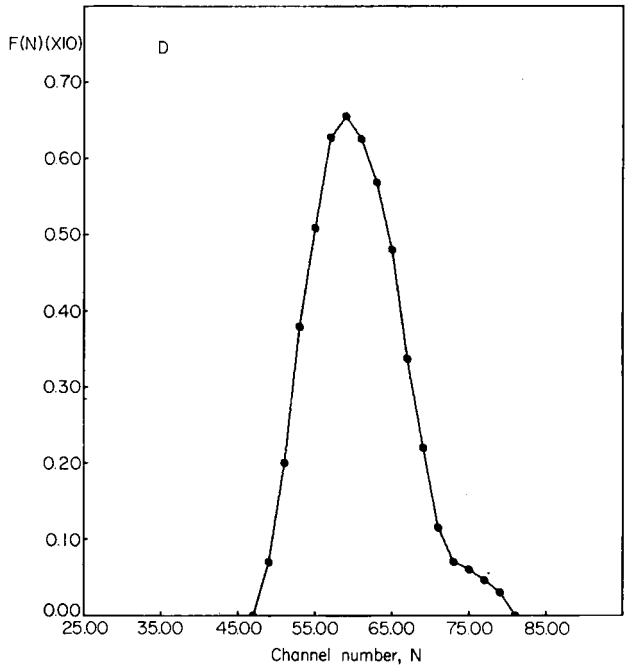
FIG. 9. Effect of increase in mean transit time of particles through the aperture on the measurement of latex spheres, 1.099 μ nominal diameter, by the Celloscope. Transit times were varied by alteration of the pressure drop across the aperture. The mean transit times are: A, 12 μsec; B, 20 μsec; C, 44 μsec; D, 66 μsec. Figures follow on pp. 20 and 21.





FIGS. 9A and B. For legend see p. 19.





FIGS. 9C and D. For legend see p. 19.

However, the combination of increased transit time with reduced amplifier rise time completely eliminates the distortion. The volume distribution of 1.099 μ latex spheres, measured by the Celloscope with mean transit time increased to 60 μsec, and the amplifier rise time reduced to 5 μsec are shown in Fig. 4. As described in Section III,B,4, there is no detectable distortion.

The hypothesis that the distortion produced by direct measurement of the pulses produced in the electric sensing zone is due to the distribution of transit times through the sensing zone is fully confirmed. The validity of Eqs. (1)–(3) is also demonstrated by these results.

The conditions for faithful and accurate measurement of volume distributions by the electric sensing zone can thus be defined. It is necessary that the pulse amplifier have a rise time of at least 4–5 times less than the shortest transit times of particles through the sensing zone. The practical minimum rise time of the amplifier will be limited by the signal-to-noise ratio and by the circuit components to a relatively high value. Also, since the sensing zone itself has a rise time of 2 to 10 μsec little advantage will be gained by reducing the amplifier rise time much below this range. With these limitations on amplifier rise time the shortest permissible transit times are of the order of 10 to 50 μsec, corresponding to mean transit times of approximately twice those values. The maximum practical mean transit times are limited by the extent of electrical heating within the sensing zone. Transit times can be increased either by reduction of head pressure or lengthening of the sensing zone (Kubitschek, 1962, 1967).

The operation of the instrument of Harvey and Marr (1966) must be considered. Their technique of differentiation and integration of pulses was designed to eliminate the distortion due to electronic coincidence, but quite fortuitously the effects of the distribution of transit times was also eliminated. The probable explanation is that with a given set of differentiator and integrator time constants the amplifier accepts pulses with only a narrow range of shapes and durations. Thus the distribution of transit times of those pulses actually measured would be greatly reduced, leading to reduced distortion. Whether or not this is the correct explanation it does seem that where direct measurement of pulse height yields faithful volume measurement the use of such pulse shaping techniques is undesirable.

While the majority of measurements used for the evaluation of electric sensing zone instruments have been made on spherical latex particles, there is little doubt that the results are applicable to measurements of biological cells. This conclusion is supported by the theoretical considerations and the actual evaluation of cell measurements discussed in this

paper. The resistivity, the capacitance, and the shape of most cells is such that their volume can be measured accurately by electric sensing zone instruments.

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Chapter 2

Synchronization Methods for Mammalian Cell Cultures

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I. Introduction

Over the past decade, as techniques for cell culture have improved workers in a variety of areas have been attracted by the method because cell culture provides an isolated system for experimental work. In the fields of cytogenetics, radiation biology, virology, developmental biology, biochemistry, and many others, cell culture has made it possible to do experiments that are impractical or impossible to do in whole organisms, especially where human cells are involved. However, with time and

experience, the workers in each area rapidly reach the point where even the cell culture system begins to look complex and inadequate. After isolating cloned strains of cells, developing uniform culture techniques, and carefully excluding contaminants like PPLO, viruses, etc., one is usually faced with the problem of variations in cell response through a cell cycle. The growing cell culture contains cells in all phases of their reproductive cycle, and in each phase the cells exhibit differing sensitivities to radiation, virus infection, enzyme induction, etc. Thus, although the culture may contain only one cell type, the random distribution of cells in different phases of the reproductive cycle (asynchrony) makes the culture effectively heterogeneous for many kinds of experiments. A variety of approaches have been developed to overcome this problem, and it is my purpose in this report to review and compare for the benefit of those who may be unaware of the distinct advantages of synchronized cell cultures.

II. Cell Reproductive Cycle

Although a growing cell culture may appear to increase continuously at the macroscopic level, it is obvious that the actual reproductive process is discontinuous at the cellular level. An individual cell will exist for a finite period of time and then abruptly fission into two cells, each of which gradually grows in mass but divides again only after a certain period of time. This interval from one cell division to the next we call the generation time, a term applicable only to individual cells. A related term, the doubling time, refers to the period required for a population of cells to double in number; the two terms are not properly used interchangeably, although they are frequently misused in current literature.

In the period from one cell division to the next a series of events are known to occur, some morphological and some biochemical in nature, which are listed in Table I as we currently understand them for the Chinese hamster cell strain Don-C. The cell cycle can be divided into four major periods: (1) G_1, the interval before replication of the genetic apparatus of the cell, (2) S, the period of DNA synthesis during which the genetic apparatus is duplicated, (3) G_2, the interval following DNA synthesis lasting until the onset of visible changes in the cell which mark the start of (4) M, mitosis, the period of cell division during which chromosomes are demonstrable in the cell. For most mammalian cells grown at 37°C, the periods of S, G_2, and M are relatively constant, with

TABLE I
MORPHOLOGICAL AND BIOCHEMICAL EVENTS IN THE CELL REPRODUCTIVE CYCLE OF CHINESE HAMSTER CELL DON-C

Event	Period	Initial time (hour)	Duration (hours)	Reference
Initiation of major RNA synthesis	G_1	0.5	10.7	Stubblefield *et al.*, 1967
Decrease in thymidine kinase activity	G_1	0.5	2.0	Stubblefield and Murphree, 1967
Initiation of chromosomal DNA synthesis	S	2.0	7.0	Stubblefield *et al.*, 1967
Maturation of daughter centrioles	S	3.0	—	Stubblefield, 1968
Return of thymidine kinase activity	S	3.5	9.0	Stubblefield and Murphree, 1967
Acceleration of total RNA synthesis rate	S	4.5	6.7	Klevecz and Stubblefield 1967; Stubblefield *et al.*, 1967
Acceleration of total protein synthesis rate	S	5.5	5.7	Stubblefield *et al.*, 1967
Centriole reproduction begins	S	6.0	—	Stubblefield, 1968
Synthesis of late-replicating chromosome segments	S	8.0	1.0	Hsu, 1964; Stubblefield *et al.*, 1967
DNA synthesis decreases, cells enter G_2	G_2	9.0	2.0	Hsu, 1964; Stubblefield *et al.*, 1967
Centrioles separate into 2 pairs	G_2	11.0	0.2	Stubblefield, 1968; Brinkley *et al.*, 1967
Prophase	M	11.2	0.2	Stubblefield, unpublished data
Metaphase	M	11.4	0.2	Stubblefield, unpublished data
Anaphase	M	11.6	0.1	Stubblefield, unpublished data
Telophase and nuclear membrane reformation	M	11.7	0.3	Stubblefield, unpublished data

values of 6 to 9 hours for S, 2 to 5 hours for G_2, and 0.2 to 1.0 hours for M. G_1 is the most variable, lasting 30 hours or more in some cell lines, and lacking entirely in at least one line of Chinese hamster cells (Robbins and Scharff, 1967).

III. Synchrony by Analysis

There are several broad approaches to the problem of asynchrony in a culture. In the simplest case, the experiment is performed using an asynchronous culture, but only those cells in a particular part of the cell cycle are included in the analysis. For example, the concept of G_1, S, and G_2 was established in just this way. Tritiated thymidine given to an asynchronous culture is incorporated into the DNA of only the cells in S, and the temporal relationship of the S phase to the cell cycle can be established by autoradiagraphic assay for isotope in the chromosomes of cells in mitosis at various times after the isotope is administered. Thus, the morphologic marker of mitosis can be used as a reference point for analysis of other events in the cell cycle. As a first step in the synchronization of any cell culture, the approximate values of G_1, S, G_2, and M should be determined as follows.

Experiment I. Cell Cycle Analysis

Prepare 15 replicate monolayer cultures of about 1×10^6 cells each in 10 ml of growth medium. After the cultures begin exponential growth, add tritiated thymidine (2 μc/ml and 5–10 c/mmole) to the medium and continue incubation at 37°C for 15 minutes. Then change all cultures to a medium containing unlabeled thymidine (5 μg/ml). (Use conditioned medium from similar cultures, and try not to allow a temperature drop when the medium is changed.) At hourly intervals remove a culture, harvest the cells, and prepare them for autoradiographic analysis (see Schmid and Carnes, 1965). After 10 hours remove a culture every 2 hours (at greater intervals if the cells are known to grow slowly). For each culture determine the percentage of cells in mitosis and the percentage of mitotic cells with radioactive chromosomes. The results of such an experiment with Chinese hamster fibroblast cells is shown in Fig. 1.

Although a more sophisticated analysis is possible (Sisken and Morasca, 1965), for our purpose it is sufficient to estimate the values as indicated in Fig. 1. If the percentage of mitotic cells in the samples was fairly uniform then the mean fraction of mitotic cells (λ) can be used to compute the mean duration of mitosis as a fraction (θ) of the total cell doubling time:

$$\theta = \frac{\log_e (1 + \lambda)}{\log_e 2} \tag{1}$$

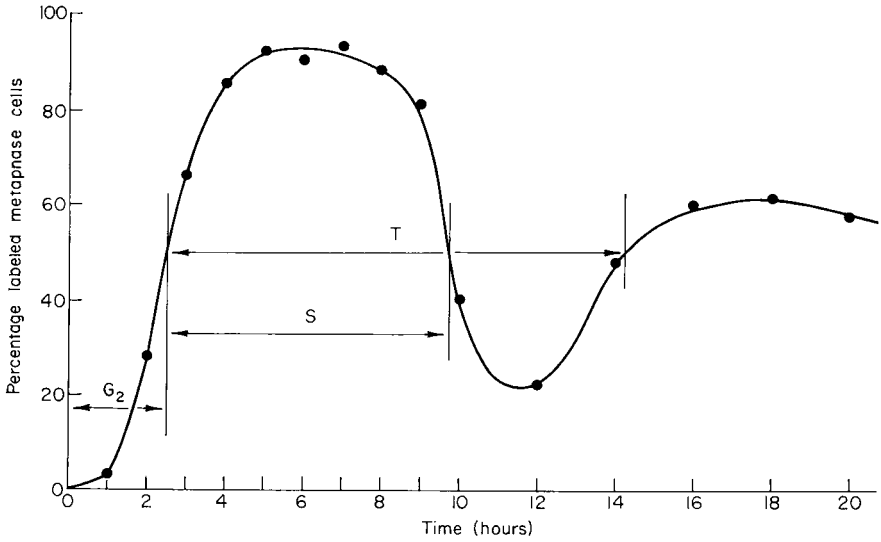


FIG. 1. Cell cycle analysis performed as in Experiment I using Chinese hamster fibroblasts. Note that the time relationships in the graph are reversed so that the first interval measured is the G_2 period. Estimates are based on the points at which 50% of the metaphase cells are labeled.

In the case shown in Fig. 1, the average fraction of mitotic cells was 0.032, so we may compute that:

$$\theta = \frac{\log_e 1.032}{\log_e 2} = \frac{0.0315}{0.693} = 0.0454 \tag{2}$$

Since the doubling time was about 12 hours (Fig. 1) then the mitotic interval was:

$$M = \theta T = 0.0454 \times 12 = 0.54 \text{ hours} \tag{3}$$

IV. Synchrony by Inhibition and Release of DNA Synthesis

A second broad approach to the problem of culture asynchrony is to attempt the synchronization of the whole culture. The methods used are analogous to the stopping of a steady flow of automobiles at a traffic light and then releasing them all simultaneously when the signal turns green. In these synchrony methods, the steady flow of cells around the cell cycle is interrupted by agents which block DNA synthesis, and

the synchronized population is then released either by removal of the blocking agent or addition of a missing essential metabolite. These systems are most useful in cell lines where the doubling time is more than twice the length of the S phase (e.g., cells with a long G_1 period). The reasons for this restriction will become apparent as we proceed.

Several agents have been used successfully in such an approach. Amethopterin [methotrexate, Rueckert and Mueller (1960); or aminopterin] and 5-fluorodeoxyuridine (Littlefield, 1962) inhibit DNA synthesis by interfering with the methylation of thymidine. High concentrations of thymidine (Xeros, 1962), deoxyadenosine, or deoxyguanosine (Mueller and Kajiwara, 1966) inhibit DNA synthesis because of feedback effects on the synthesis of other nucleotide precursors. These latter three agents can be effectively reversed by either omitting the agent or by supplying the missing nucleoside(s).

Because the agents will block the progress of any cell at any stage of DNA synthesis, it is not possible to accumulate all of the cells at any one point in the replication cycle. However, all of the cells in G_2, M, and G_1 should eventually be blocked at the start of S, so if the duration of S is a small fraction of the whole cell cycle, then the part of the population blocked at various points in the S phase will be negligable compared with the synchronous population. The use of these agents is illustrated in the following experiments.

A. Experiment II. Amethopterin Block of DNA Synthesis

Prepare 10 replicate cultures of about 1×10^6 cells each. After the cultures begin exponential growth, add to each of 9 cultures a one-tenth volume of medium containing amethopterin ($10\times = 10$ μg/ml) and adenosine ($10\times = 100$ μg/ml). To the tenth culture and to one of the amethopterin-treated cultures add tritiated thymidine (2 μc/ml and 5–10 c/mmole) at this same initial time. After 10 minutes at 37°C, harvest the two isotope-treated cultures and prepare slides for autoradiography as in Experiment I. Treat an additional culture with tritiated thymidine for 10 minutes every 2 hours (shorter intervals if the cells grow rapidly). Prepare slides for autoradiography from each sample. For each sample determine the percentage of cells in mitosis and the fraction of cells with radioactive nuclei. The results of such an experiment using HeLa cells is shown in Figs. 2 and 3.

From the plot of mitotic fraction versus time in Fig. 2 a fair estimate of the length of the G_2 period can be obtained. From the plot of the fraction of cells with labeled nuclei one can readily estimate how long the

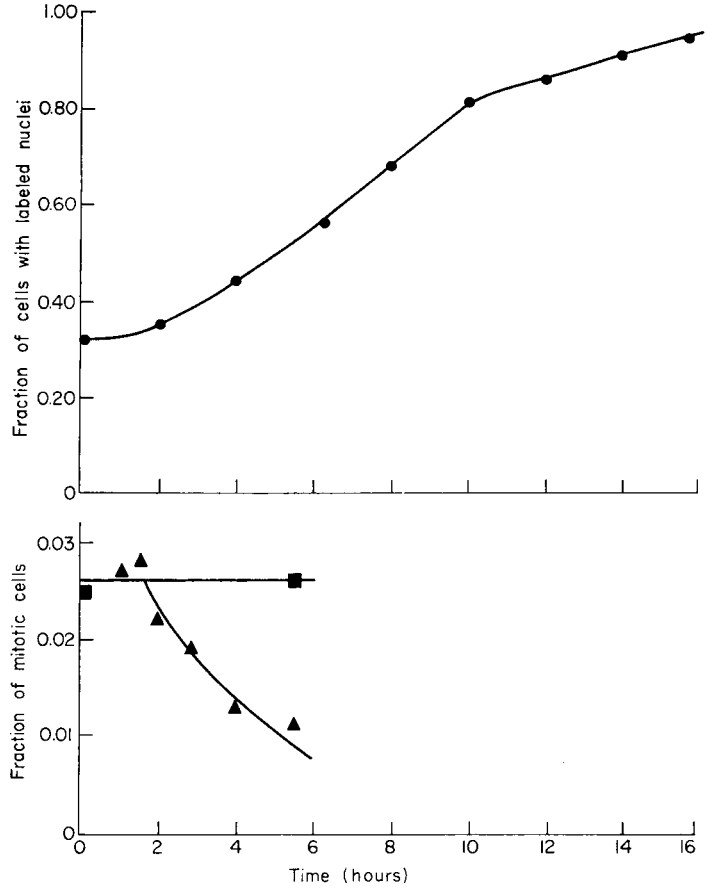


FIG. 2. Experiment II, the accumulation of nuclei in the S phase following addition of amethopterin to HeLa cell cultures (top curve), and the effect of the same drug on the fraction of mitotic cells (lower curves). The mitotic fraction drops to about one-half the normal value by 4 hours; allowing 0.7 hours for M, the G_2 period can be estimated as about 3 hours. Thus, beginning at 4 hours, the points in the upper curve can be plotted as in Fig. 3. Amethopterin, ▲ and ●; control, ■.

drug treatment must last in order to accumulate most of the cells in the S phase.

Plotting the data in the fashion described by Puck and Steffen (1963) allows a good determination of both the S period and the average cell doubling time, if the G_2 period is already known. In the case shown the G_2 period is about 3 hours (allowing an additional hour for the mitotic period). The first two time points cannot be used because they require

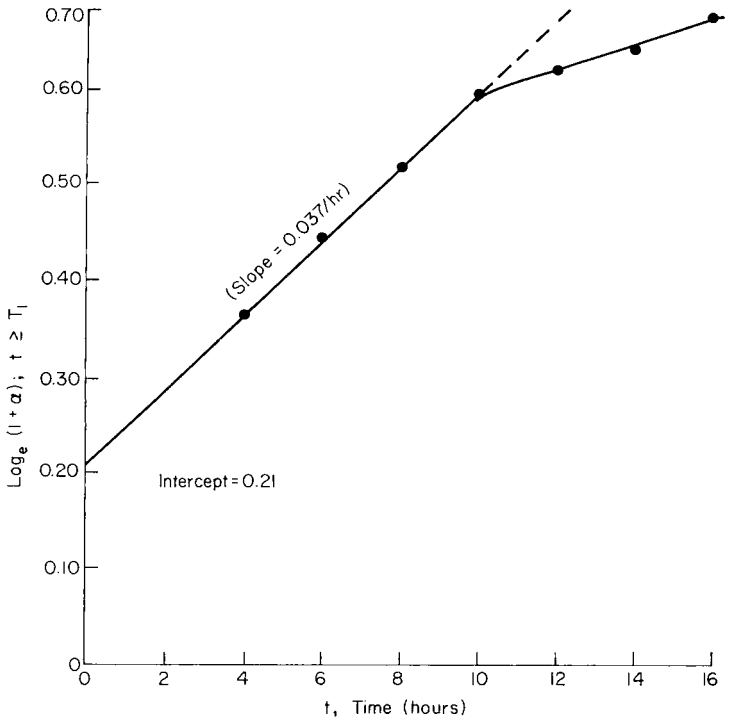


FIG. 3. Display of the data of Fig. 2 according to the formulation of Puck and Steffen (1963). See text for details of the calculations involved.

prior knowledge of the doubling time (T), but values can be calculated for cases where $t \geqslant T_1$, where T_1 is the G_2 interval. Equation (4), described by Puck and Steffen (their Eq. 14b), shows the relationship involved, where α is the fraction of cells with labeled nuclei and $[T_2 - T_1]$ is the S period:

$$\log_e (1 + \alpha) = \frac{\log_e 2}{T} ([T_2 - T_1] + t) \tag{4}$$

Thus, a plot of $\log_e (1 + \alpha)$ versus t should give a straight line with a slope of $\log_e 2/T$ which is the growth constant, k.

$$k = \frac{\log_e 2}{T} \tag{5}$$

We can then calculate from the slope value of 0.037/hour that

$$T = \frac{\log_e 2}{\text{slope}} = \frac{0.69315}{0.037/\text{hour}} = 18.7 \text{ hours} \tag{6}$$

From the extrapolated intercept value of 0.21, the interval $[T_2 - T_1]$, which is the S period, can be calculated by dividing the intercept by the growth constant, k.

$$\text{S period} = \frac{\text{intercept}}{\text{slope}} = \frac{0.21}{0.037/\text{hour}} = 5.7 \text{ hours} \quad (7)$$

For a different approach consult the report of Okada (1967).

Experiments using FUdR (0.1 μg/ml) or high thymidine concentration can be run analogously. If the concentration of the inhibitor is too low, the mitotic fraction will not decrease, and the labeled nuclei fraction will not accumulate as in Fig. 2. In a fast growing population, the fraction of the population initially in S phase may be so high that it will be difficult to get a good slope calculation of the doubling time; in such a case it is usually better to use another synchrony method or a modification to be suggested later.

B. Experiment III. Synchronization with Amethopterin Block and Thymidine Release

Once the length of time necessary for an inhibitor to accumulate 90 + % of the cells in S phase has been determined, then a test of the reversibility of the block can be performed. Prepare 20 cultures as in Experiment II, and when in exponential growth add the amethopterin and adenosine as before to all cultures. Add thymidine (to 5 μg/ml) to 4 cultures at the starting time to serve as controls. Harvest one control culture immediately and every 7 hours thereafter and assay for cell number. Incubate the remaining 15 cultures for the period of time needed to accumulate at least 90% of the cells in S phase (in the case of HeLa, 14–16 hours). Harvest 1 culture and add thymidine (to 5 μg/ml) to all the remaining cultures; these should then be assayed at hourly intervals for cell number.

The number of cells per culture can be determined by any of several methods. If a Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida) is available, cell suspensions can be assayed after trypsinization of the monolayers. Alternatively, carefully standardized procedures give reasonably reliable data using a standard hemocytometer, but the microscopy is rather tedious. Phase-contrast, time-lapse cinematography makes everything simple, and reduces the experiment to only two cultures, the control and the synchronized population. We routinely use this latter method, photographing the cells in a standard culture vessel, modified to permit phase microscopy (Stubblefield, 1964), while the rest of the replicate cultures are used for experimental work. Figure 4 shows

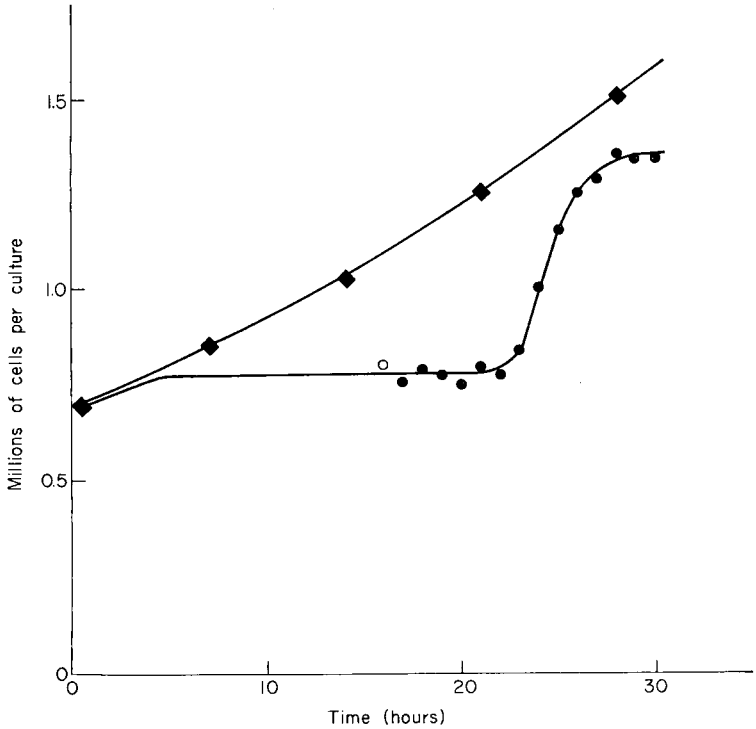


FIG. 4. Synchronization of HeLa cells by amethopterin block and thymidine release (Experiment III). Thymidine added at time zero, ◆; at 16 hours, ●; no thymidine, ○.

Coulter Counter data from such an experiment using HeLa cells. Assay of DNA replication for this system has been published by Mueller *et al.* (1962).

If a burst of cell division does not occur 6–10 hours after release of the inhibition, then a lower inhibitor concentration should be tried. One must also be certain that the culture medium is not metabolically spent, since the cultures will probably be 50–60 hours old. If this proves troublesome, the medium should be changed at the start of the experiment, and not at the point of reversal.

Cells with a short doubling time (10–12 hours) usually have 50–60% of the cells in S phase at any time during exponential growth. Other synchrony methods usually work better, but for some types of experiments it is advantageous to be able to synchronize by DNA synthesis block. In such cases a preliminary phasing of the cells makes a great difference. Cells taken from a culture which have just reached stationary phase are predominately in G_1, so they can be subcultured directly into

fresh medium containing amethopterin, FUdR, or excess thymidine, and a larger percentage of cells will accumulate at the start of S, rather than being distributed throughout the S phase. Alternatively, another synchrony method can be used to phase the cells first, and the DNA inhibitor added at an appropriate point to catch the cells in the second cycle.

The major disadvantage of DNA inhibition methods of synchronization centers around a problem called "unbalanced growth" in the literature. Although cells are stopped in their reproductive cycle by DNA inhibitors, they continue to make RNA and protein and increase in cell mass (Rueckert and Mueller, 1960). For many experiments this is not a serious difficulty; however, studies of enzyme activities through the cell cycle, for example, are not reliable using these techniques (Stubblefield and Mueller, 1965; Stubblefield and Murphree, 1967). Care must be taken not to hold the cells in the blocked state for more than one cell doubling time, since the cells then become subject to "thymineless death" (Rueckert and Mueller, 1960) presumably as a result of their unbalanced growth.

V. Synchrony by Selection of Mitotic Cells

The direct approach to synchronization of a cell population is to mechanically select from the population all of those cells that are in a certain brief phase of their reproductive cycle. Prescott (1956) hand-selected ameba in mitosis to provide a synchronous population, but obviously this is impractical when large populations are needed. In the laboratory of Dr. Gerald C. Mueller at the University of Wisconsin in 1959, we reasoned that it should be possible to collect mitotic cells from tissue culture monolayers since in the mitotic phase the cells are more loosely attached than in interphase. We demonstrated to our own satisfaction that this was indeed the case, but since the yield of mitotic cells that could be removed from a monolayer was always small—less than 1%—the method did not appeal to us, and we turned our attention to the DNA inhibition methods which were discussed in Section IV.

In 1963 Terasima and Tolmach published the first studies of HeLa cells synchronized by selection of mitotic cells. In spite of low yields of cells, they demonstrated that the method was useful for certain types of experiments. Further modification of the method by Robbins and Marcus (1964) and by Lindahl and Sörenby (1966) has increased the usefulness of the approach. By collecting periodically from the same

cultures or by collecting continuously from a single large culture, it has been possible to increase the yield of mitotic cells to a more practical level. However, these methods still require quite a large amount of culture medium, glassware, and hard work to produce gram quantities of mitotic cells.

The difficulty of obtaining large quantities of viable mitotic cells has been met in our own laboratory by using the drug Colcemid to arrest cells in mitosis. The discovery that Colcemid inhibition was reversible in Chinese hamster cells (Stubblefield, 1964) led not only to better synchrony techniques, but also to a better understanding of the mechanism of Colcemid inhibition.

Chinese hamster cells blocked in mitosis can be treated for 2 to 3 hours with 2×10^{-7} *M* Colcemid before any permanent damage is inflicted; longer exposure results in multipolar mitoses (Stubblefield, 1968). However, with only 3 hours exposure to Colcemid the percentage of mitotic cells in the culture rises from around 3% to 20%, and the yield of mitotic cells increase from 1% to better than 10% of the total culture. This makes it possible to recover enough mitotic cells from 10 monolayers to seed 2 synchronized monolayers at the same density (each mitotic cell immediately produces 2 cells). This return makes the technique more useful for biochemical studies. With a few extra tricks, gram quantities of mitotic cells of 98% purity can be collected daily with a minimum of work and equipment.

A. Experiment IV. Colcemid Block

Prepare 10 replicate exponential cultures. Test for sensitivity to Colcemid by treating for 4 hours with concentrations ranging from 10^{-4} *M* to 10^{-8} *M* as follows: control (no Colcemid), 1×10^{-8} *M*, 3×10^{-8} *M*, 1×10^{-7} *M*, 3×10^{-7} *M*, etc. Prepare slides for assay of percentage of mitotic cells. *Caution:* In trypsinizing monolayers with an abundance of mitotic figures, be careful not to dislodge mitotic cells in rinsing solutions.

A plot of the percent mitotic cells versus $\log_{10}$ of Colcemid concentration should plateau at the minimal effective Colcemid concentration. For Experiment V choose a Colcemid dose of twice this value.

B. Experiment V. Colcemid Synchronization

Prepare 20 exponential monolayer cultures and treat them all with twice the minimal effective Colcemid concentration for 3 hours. Carefully pour off and pool the medium from the cultures; from a 10-ml aliquot remove any suspended cells by centrifugation and save the pellet

for assay of percent mitotic cells. Cover each monolayer with 5 ml of cold trypsin solution (0.1% in Hanks saline omitting calcium and magnesium salts) and shake the cultures horizontally (2 cps with a 10 cm amplitude) for exactly 60 seconds. Immediately remove the trypsin with suspended mitotic cells and remove them from the trypsin by centrifugation. Several stacked cultures can usually be handled together in this procedure, so the harvesting of mitotic cells should be completed in 10 minutes or less, especially if several people assist in shaking the cultures. Resuspend and pool all of the mitotic cell pellets in 10.5 ml of growth medium; recentrifuge 0.5 ml and assay the pellet for percent mitotic cells. Dilute the 10 ml cell suspension to 57 ml, plant 10 cultures of 5 ml each, and begin incubation at 37°C. Assay cultures for percent mitotic cells and total cell number at 0 hours, 0.5 hours, 1 hour, 2 hours, 3 hours, and harvest the remaining 6 cultures at 2-hour intervals beginning 4 hours before one doubling time from the start of the experiment. Data for the Chinese hamster cell Don-C is given in Fig. 5.

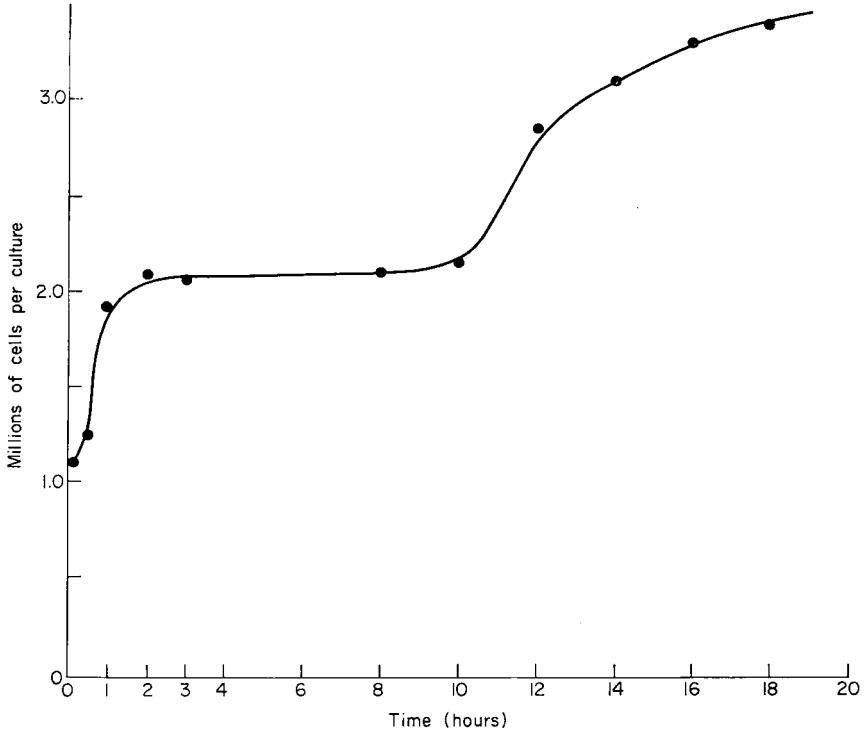


FIG. 5. Synchronized growth of Chinese hamster Don-C cells following release from Colcemid inhibition of metaphase cells (Experiment V).

In some cell lines, mitotic cells are so loosely attached that the cells suspended in the medium are of high enough purity and number to warrant saving them. In such cases trypsin may be replaced by fresh medium or saline. On the other hand, some cells may require trypsinization for longer than 1 minute to remove a maximum yield of mitotic cells. It may be helpful to try a series of collection times to find the method giving the highest yield and maximum purity.

Should the cells fail to divide after the Colcemid is removed, there are

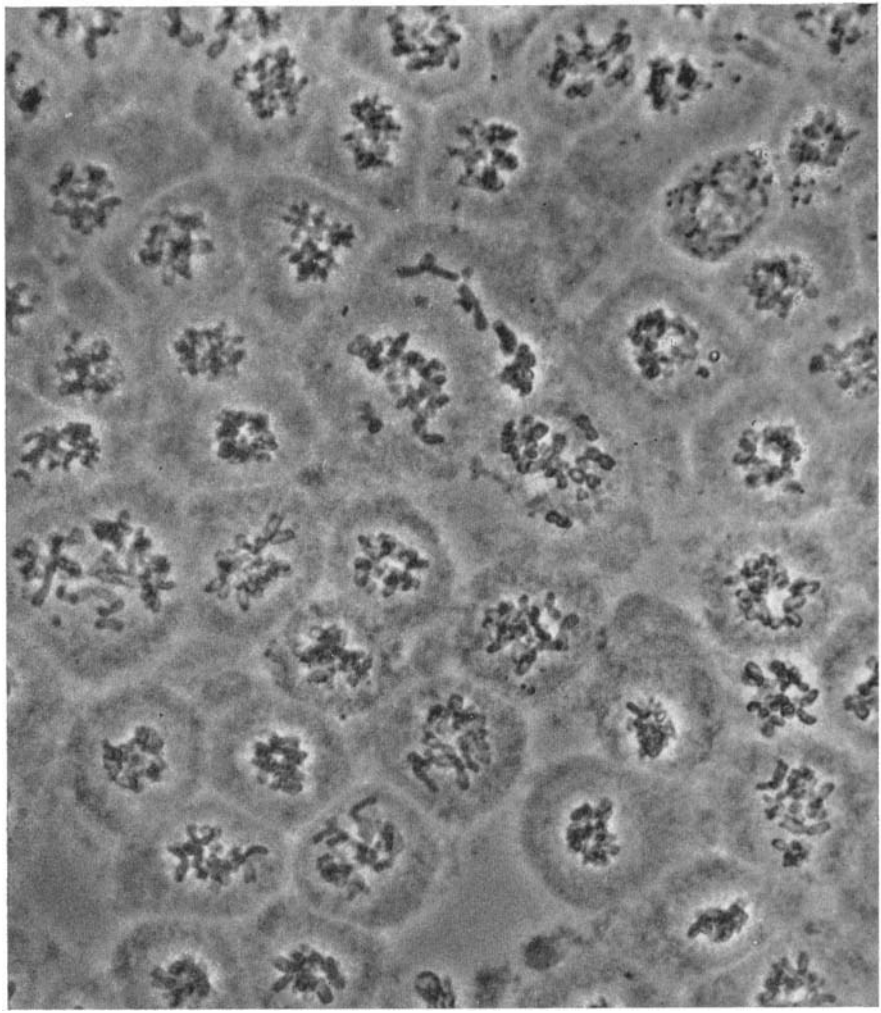


FIG. 6. Metaphase Don-C cells differentially trypsinized from monolayers after 3 hours of Colcemid treatment.

several possible remedies. Try a lower Colcemid concentration or block for only 2 hours. Some HeLa lines do not recover well from Colcemid treatment, but our experience has been that clones of cells which did recover can be used with success. The defect in HeLa is not common to all human cells, since no difficulty has been encountered in synchronizing other human tumor and normal diploid cells (Romsdahl, 1968).

A recent improvement of the method in our laboratory has been to grow the monolayer cultures in 600-ml serum bottles that are rotated once every 5 minutes packed horizontally in a large fiber drum. About 3×10^7 cells in 60 ml of medium are planted in each bottle. The advantages come from two considerations: first, the cultures give a maximum surface area for the monolayers but are still compact; and second, the purity of the mitotic cells collected from the monolayers after Colcemid treatment increased from 90 to 98% (Fig. 6). The latter effect seems to indicate that loosely attached interphase cells, which contaminate the mitotic fraction in static monolayers, never have an opportunity to attach at all in rotating monolayers and are discarded with the Colcemid medium. Mitotic cells are collected by rolling the bottles with 15 ml of trypsin in them back and forth on a table top for 1 minute.

VI. Factors Affecting the Loss of Synchrony

The loss of synchrony with time is due to variation in the generation time among the individual cells of a population. In an earlier study (Stubblefield *et al.*, 1967) we demonstrated a good correlation between the distributions of generation times in individual cells in stock culture and in Colcemid-synchronized culture. Once the method adequately selected an initial fraction that was quite pure, our attention was directed toward the reduction of heterogeneity in the population. The use of a clone instead of a mixed stock population reduced heterogeneity considerably. However, a study of synchrony in individual clones has led to the conclusion that heterogeneity can be reduced even more.

In Fig. 7, which depicts data from a time-lapse motion picture, the first 5 generations of a developing clone of Chinese hamster fibroblasts (line Don) is shown. The amazing thing about this particular case is the continued synchrony of the system over so long a time period in the absence of environmental fluctuations. The outstanding characteristics of these cells were their rapid growth and uniform small size; the cells had so little cytoplasm that they could not flatten well on the glass sub-

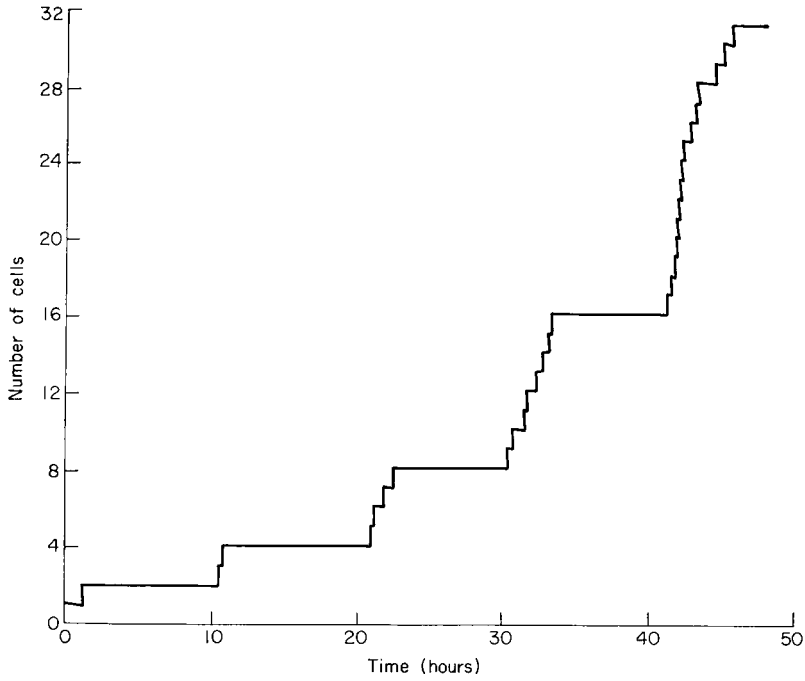


FIG. 7. Development of a clone of small Chinese hamster fibroblasts. Data was obtained by time-lapse motion picture analysis.

strate. Since strain Don-C contained smaller cells than the parent line Don and demonstrated less variation in generation time from cell to cell, we wondered if perhaps even smaller cells might be better suited for synchrony studies. For the case shown in Fig. 7, the mean generation time was 637 minutes (average of 29 cells) with a standard deviation of 72 minutes compared with 760 minutes ± 140 for Don-C (Stubblefield *et al.*, 1967). The Don-C calculation ignores a 10% fraction of the population which did not divide, however.

By coincidence, we happened to have a Chinese hamster cell clone which was very similar in appearance to the small cells described above; we called this strain B14-C59a. B14-C59a was synchronized by the method used in Experiment V, and the results (from movie analysis data) are shown in Fig. 8. The better synchrony in the second mitotic wave is evident in the B14-C59a when compared to Don-C. However, the average generation time was longer, 920 minutes ± 136. The standard deviation calculation is more meaningful in this case (compared to Don-C) since all of the cells but two divided in the second mitosis. Thus, the

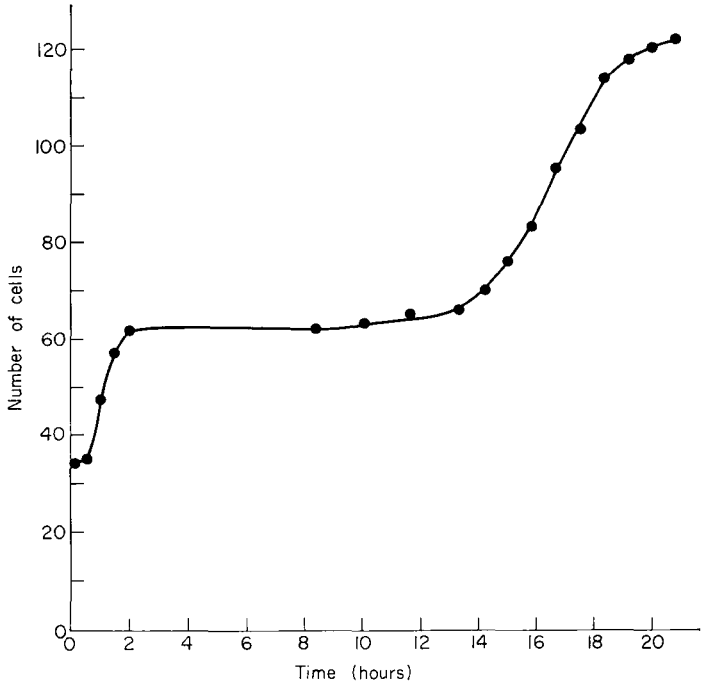


FIG. 8. Synchronized growth of B14-C59a cells following release from Colcemid inhibition as in Experiment V.

smaller cells appear to be more uniform in behavior than the Don-C clone even though the generation time is longer.

Although we do not know why the smaller cells seem to maintain synchrony better than the larger cells, there is an indication in these results that it is helpful to be selective in choosing a cell line for synchrony studies. Our experience has been that a clone of rapidly dividing and uniformly small cells always gives better synchrony than the parent cell line.

VII. Multiple Synchronization

Perhaps the most important point of the discussion in Section VI is that the art of cell synchronization may be well enough developed to allow us to begin thinking in terms of permanently synchronized mammalian cell cultures. In order for a system to be useful there are certain obvious

prerequisites: (1) the forces used to keep the cells in synchrony must not permanently damage the cells, (2) the cells which maintain good synchrony should be somehow selected *for* in the system, and (3) the cells which are selected *against* must be appreciably less than half of the population each generation. Under these circumstances it should be possible to maintain a productive synchronized culture.

In a crude way routine subculture can provide just the proper circumstances for permanent synchronization of a cell culture. We were somewhat surprised to find that Don-C cells subcultured at 24-hour intervals grew in "synchronous" steps (Stubblefield *et al.*, 1967). The original motive for daily subculture was to keep the cells in "fresh" medium. The accidental fact that the subculture interval was twice the cell doubling time was the key to the procedure. The exposure to cold, or trypsin, or fresh medium seems to select against cells in G_1 phase and to favor cells in early S phase. We routinely use such staged cells for Colcemid block 18 hours after subculture to boost the yield of mitotic cells. Such an experience points up the very real difficulty of producing truly exponential cultures when they are needed.

There is the distinct possibility, then, that periodic fluctuations in temperature, nutrients, etc., could be used to keep a synchronized culture in phase. The extreme methods of cold shock first used by Wildy and Newton (1958) are probably too severe for these purposes (the effects of such treatment are quite variable in different cell lines). Fluctuations of 10°C will probably be sufficient if applied at the appropriate points in the cell cycle. Although the techniques we have described in this report are quite useful for short-term synchronization and thus help us to understand the cell reproductive cycle, they are probably too severe to be useful for repeated synchronization of the same culture.

Acknowledgments

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Chapter 3

Experimental Techniques for Investigation of the Amphibian Lens Epithelium

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I. Introduction

In his recent monograph, "Adaptive Growth," Goss (1964) remarked that, "the lens also offers singular opportunities for investigations, for in addition to the classic role it has played in the elucidation of phenomena like embryonic induction, the lens has continued to be a tissue of such exceptional qualities that it has served as a model *par excellence* for the biochemical analysis of histological structures in general. With the possible exception of cartilage, erythrocytes, epidermis and sperm, few other

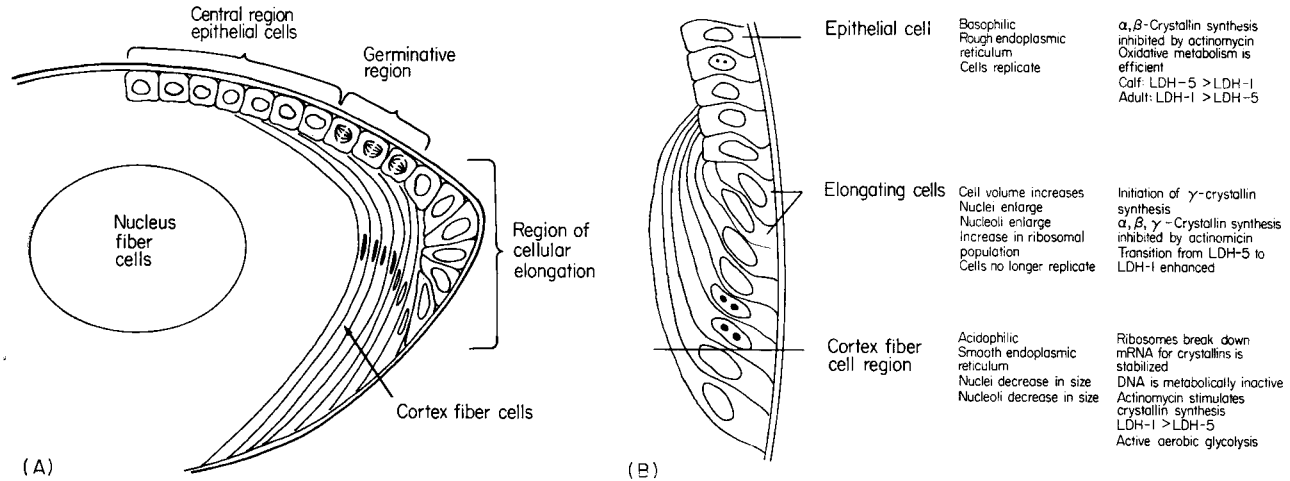


FIG. 1. (A) Diagram of the lens of the adult vertebrate. The lens is surrounded by an external noncellular capsule. Beneath the capsule are the lens epithelial cells. The region of cellular elongation is in the peripheral area. This is the region of transition where the epithelial cells begin to elongate into fiber cells. The fiber cells that are newly laid down constitute the cortex region; the fiber cells laid down during the early growth period of the lens compose the nucleus region of the adult lens. (B) Diagram of the region of cellular elongation in the vertebrate lens. The major morphological and biochemical characteristics associated with lens cell differentiation are listed. [From Papaconstantinou (1967). Copyright 1967 by the Am. Assoc. Advan. Sci.]

tissues in the body are available in as pure a form as is the lens which is naturally uncontaminated by a blood supply, innervation or the ubiquitous fibroblast."

In addition to the characteristics just emphasized, the lens also contains a population of epithelial cells which easily qualifies as one of the most convenient systems for studies of certain aspects of cell division. These cells are found immediately subjacent to the anterior portion of the lens capsule, where they exist as a monolayer (Fig. 1). Normally, cell division is restricted to the so-called germinative zone which approximates, but does not coincide with, the equator of the organ. These cells

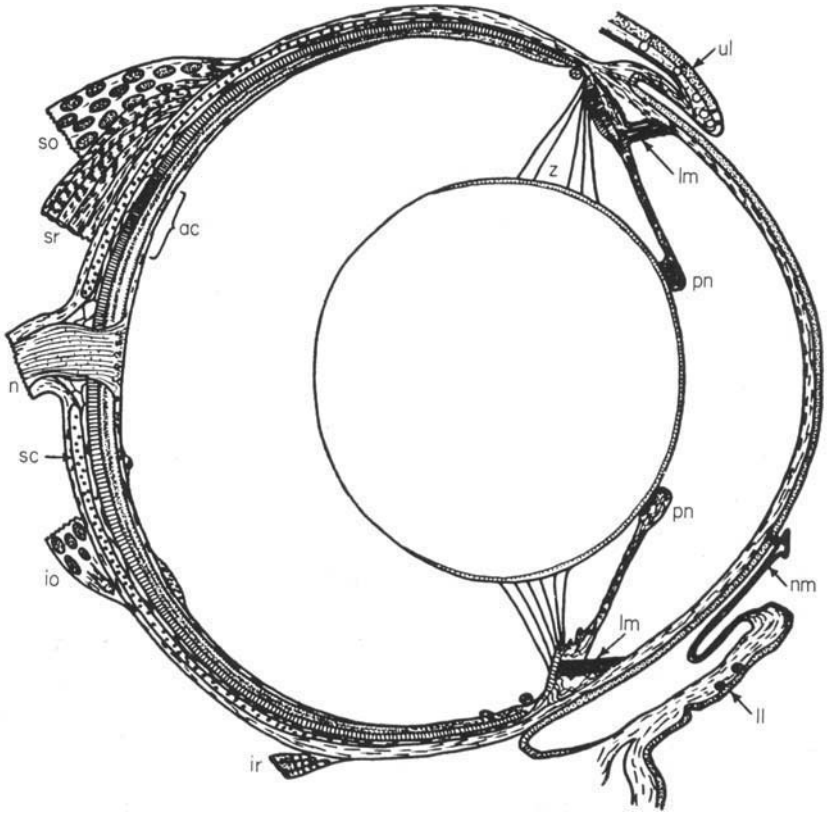


FIG. 2. The anuran eye in vertical section (semidiagrammatic) based largely upon the leopard frog, *Rana pipiens*. ×11½. ac, Area centralis; io, inferior oblique; ir, inferior rectus; ll, lower lid; lm, lens muscles; n, optic nerve; nm, "nictitating membrane"; pn, pupillary nodules (attached to lens); sc, scleral cartilage; so, superior oblique; sr, superior rectus; ul, upper lid; z, zonule. [By permission from Hafner Publ., New York. (Walls, 1942).]

eventually become lens fibers—a process that goes on throughout the life of the organism—after which the capacity to divide is irrevocably lost. Typically, in the adult vertebrate, the cells which lie nearer to the anterior pole than to the equator, do not divide either. However under appropriate experimental conditions they can be stimulated to do so in parasynchronous fashion. During the past few years we have been investigating the alterations, both biochemical and morphological, which are associated with the transformation from relative reproductive quiescence to active mitosis. Our system of choice has been the amphibian lens (Fig. 2). This material is convenient from several points of view, namely, size, availability, spectrum of temperatures through which experimentation is practical, and relative hardiness under *in vitro* conditions.

In this chapter, no attempt will be made to discuss the many and varied methods which have been used in conjunction with problems of lens differentation and regeneration. Our concern will be related chiefly to the handling of the amphibian lens epithelium. It should be emphasized, however, that such techniques can be applied, with very slight modifications, to investigations of lens epithelium in almost any vertebrate organism. In many cases, in fact, the methods we use are based upon those developed by Harding *et al.* (1960) and Srinivasan and Harding (1965) and others for the mammalian system.

II. Methods for Fixed Material

A. Whole Mounts

The preparation of whole mounts, makes it possible to visualize the entire epithelial population on a single histological specimen. This technique was originally worked out by Howard (1952) for rabbit lenses. It is much more convenient than routine sectioning and is far more useful in many respects.

Following enucleation and removal of any adhering ocular adnexa, a circular incision (ca. 3 mm diameter) is cut through the posterior aspect of the globe. This facilitates penetration of the fixative. The specimen is then placed in Carnoy's fluid (3:1 v/v absolute ethanol; acetic acid) and is left in this fixative for 24 hours. Following this it is placed for an additional day in 70% ethyl alcohol. After having freed the fixed lens from adjoining tissue one may proceed to prepare the whole mount as follows (Fig. 3). The lens is impaled (through the posterior pole) upon a double needle (which can be kept secure in any convenient holder).

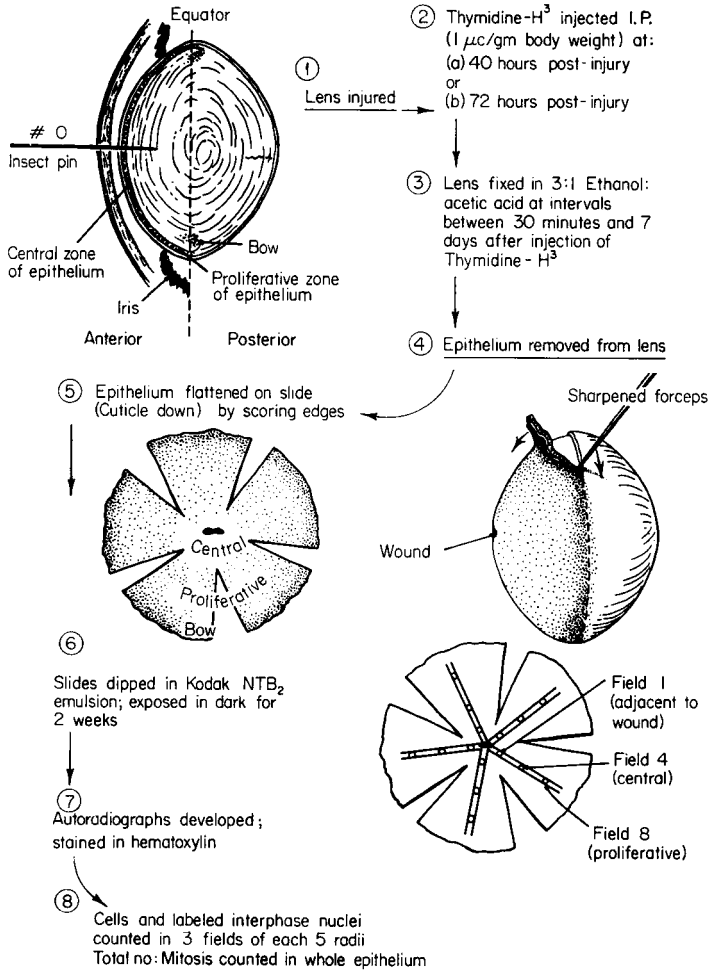


FIG. 3. Procedure used by Dr. Nancy Rafferty for labeling a population of lens epithelial cells with thymidine-H³ following injury. (From Fig. 1, Rafferty, 1967.)

Then, under the dissecting microscope, a circumferential incision is made about the equator of the organ. Though others have used sharpened forceps for this purpose we have found it easiest to use either a small von Graefe or a Beaver eye knife with the smallest blades available.[1] Once having made the incision it becomes possible to roll the entire capsule anteriorly with a small eye spatula. In order to avoid cell loss

[1] These instruments are available from The Lawton Company, 425 Fourth Avenue, New York, New York or The Pilling Company, Delaware Drive, Fort Washington, Pennsylvania, surgical supply houses.

this should be done gradually with occasional submersion of the lens in distilled water or 70% EtoH (the submersion process helps to float the capsule off the organ). In this way the capsule with adhering epithelial cells can be completely separated from the rest of the lens. At this stage the tissue is folded upon itself and is still unsuitable for histological processing.

In order to obtain a flat preparation it is convenient to employ two ½ inch 27-gauge hypodermic needles in insect needle holders. Under the dissecting microscope it is possible to remove all folds and unfurl any flaps that have arisen during the "skinning" procedure. It is usually easy to differentiate the cellular side of the capsule from the acellular one by the orientation of the flaps. Since the cells were originally sandwiched between the capsule and the lens fibers, and since the capsule was rolled forward, the flaps should tend to lie under the main body of the capsule (rather than over them) when the cell side is up.

It is critical, particularly for autoradiography with β-emitters, that the cells be on the upper surface. If the tissue is mounted the other way,

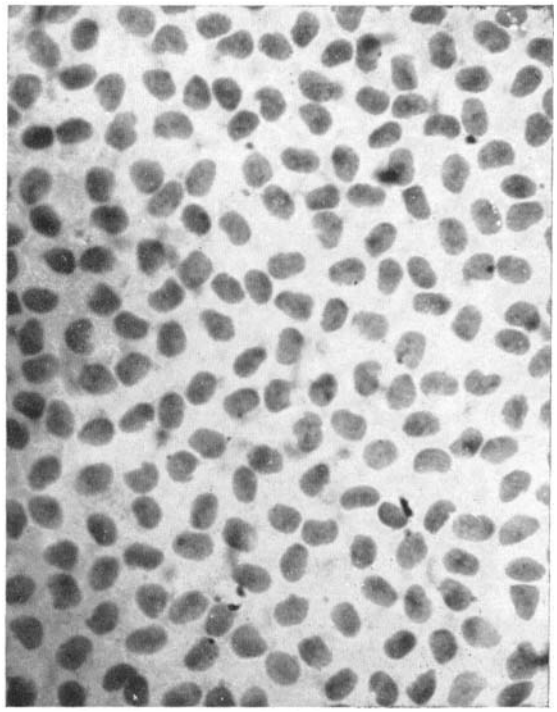


FIG. 4. Whole mount of bullfrog lens epithelium. Hematoxylin stained, approximately $\times$ 100.

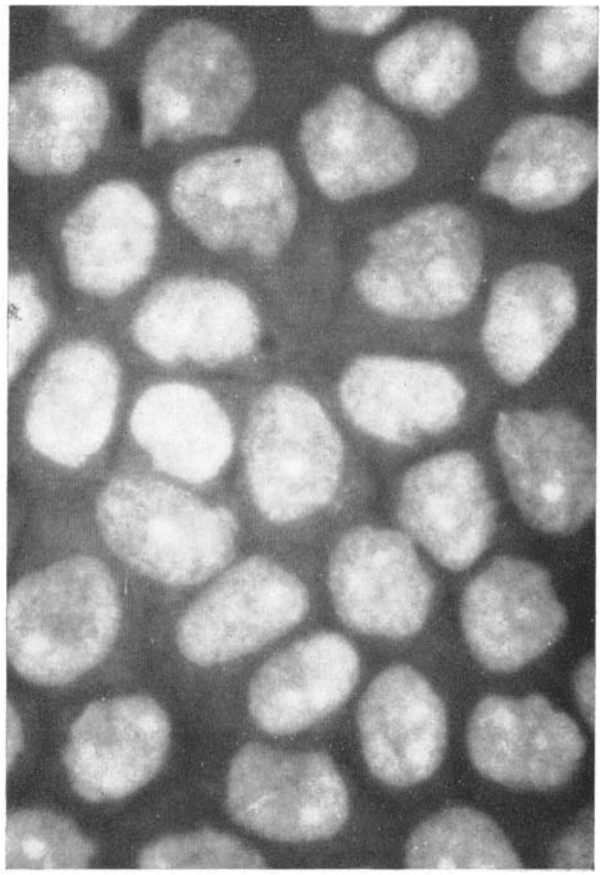


FIG. 5. Whole mount of bullfrog lens epithelium as viewed by fluorescence microscopy after staining with acridine orange.

so that the emulsion does not make contact with the cells, unsatisfactory autoradiograms result. This was specifically proved by Harding *et al.* (1960) several years ago.

Unfurling of the capsule should be carried out upon slides which have been coated with a 0.5–1% solution of gelatin. To promote flattening, the specimens should be scored with a razor blade from 4–8 times. When the tissue has dried it will adhere to the glass so that staining procedures can be carried out. A preparation produced in accord with the above description and stained with Harris' hematoxylin is shown in Fig. 4. Figure 5 is a photograph of a whole mount which was treated with acridine orange and observed by fluorescence microscopy. It can be seen that the cell population is quite homogeneous. Though not apparent in the fig-

ures, inspection of the tissue goes to show that the cells in the equatorial zone are relatively small and are packed more closely than those in the vicinity of the pole and prequatorial region.

For many staining methods the above noted techniques are satisfactory. However for the Feulgen procedure it is probably best to carry out hydrolysis and staining upon the whole lens and to make whole mounts afterward. The optimal timing of hydrolysis should be determined experimentally upon the particular material being used. As noted below, the Feulgen procedure can be used upon lens epithelium in experiments where it is desirable to employ chromosome spreads. Though we have not yet made use of the technique it should be very easy to apply microspectrophotometry to stained whole mount preparations.

B. Autoradiography

Autoradiograms can be made with either liquid emulsion or Kodak AR 10 stripping film. Since the procedure for lens epithelium is not different from that used for other tissues no detailed discussion need be presented here. In our laboratory we have thus far worked only with β-emitters (H^3- and C^{14}-thymidine, H^3-uridine, H^3-iododeoxyuridine, H^3-reconstituted protein hydrolyzate and individual amino acids, H^3-puromycin, and H^3-actinomycin D).

In *in vivo* experiments, for example in the study of injury reactions, the most convenient way to supply the isotope is by intracameral injection (i.e., injection into the anterior chamber). The radioactive material can be added to any defined mammalian tissue culture medium (e.g., Hanks, Earles, Eagles) which has been diluted with sterile distilled water (7 parts medium:3 parts water). The solution is then injected into the eye. To accomplish this a 30-gauge needle is inserted through the limbus into the anterior chamber. To avoid injection of air it is important to use a thoroughly dry hypodermic needle. After insertion of the needle into the anterior chamber one waits until emerging aqueous humor is seen to completely fill the needle, after which a syringe containing the experimental solution is affixed to it. For large anura (*Rana catesbeiana, Bufo marinus*) one can supply about 0.15 ml, for the smaller animals, such as *Rana pipiens*, roughly 0.08 ml. The animal should, of course, be anesthetized prior to injection. The most suitable material we have found for this purpose is 0.2% MS 222.[2] Anesthesia is accomplished by simply immersing the organism in a bath of the aforementioned solution for 15 to 30 minutes. To be certain that all cutaneous sensibility is also

[2] Methane sulphonate, Sandoz Pharmaceuticals, Hanover, New Jersey.

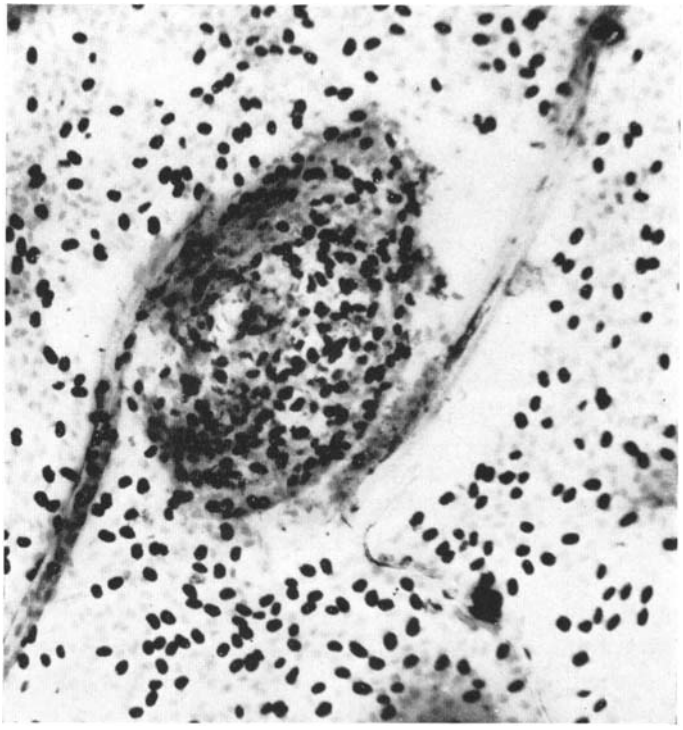


FIG. 6. Autoradiogram of a whole mount of epithelium from an animal whose lens was injured. The animal received thymidine-H^3 2 days later. Note that the epithelial cells have migrated into the wound focus and that many cell nuclei show the uptake of thymidine-H^3. (Stained with hematoxylin; $\times 75$) (From Rothstein *et al.*, 1967.)

lost we have found it useful to layer one or two drops of Ophthaine[3] between the cornea and "nictitating" membrane.

The type of results obtained by using this procedure are shown in Fig. 6. In this case the lens was mechanically injured with a 0-gauge insect needle as shown in Fig. 3. Then, 2 days afterward, thymidine-H^3 (20 μC/ml–3 C/mmole) was injected into the anterior chamber. The animal was sacrificed 2 days later; whole mounts were subsequently made, filmed, and exposed in the refrigerator for about 2 weeks. The uptake of isotope is conspicuous. And as we have shown elsewhere is a direct result of the injury which preceded it (Rothstein *et al.*, 1964, 1965b).

It is also possible, as Rafferty and others have found, to supply isotope via the intraperitoneal route (Rafferty, 1967; Thomson *et al.*, 1962). By

[3] E. R. Squibb & Sons, Inc., 745 Fifth Avenue, New York, New York.

this method we have demonstrated, in fact, that actinomycin-H^3 passes the blood-aqueous barrier and eventually comes to reside in the nuclei of the lens epithelial cells (Rothstein *et al.*, 1967). However it is our feeling that for most experimental regimens use of the intracameral route burdens subsequent interpretation of results with fewer ambiguities.

Isolated lenses in culture can, of course, also be used for autoradiographic studies by simply placing them in appropriate isotope containing solutions. *In vitro* approaches naturally allow for a closer control of concentration levels and other parameters.

Tritium thymidine autoradiography opens up the possibility of studying cell cycle kinetics in lens epithelium by either the labeled mitosis (Quastler and Sherman, 1959; Wimber, 1960) or double-labeling procedures (Pilgrim and Maurer, 1962; Wimber and Quastler, 1963). Figure 7 shows the results obtained in our laboratory for cells of the bullfrog germinative zone. This data was obtained through the labeled mitosis method. The length of S was found to be 99 hours at 24°C. To confirm

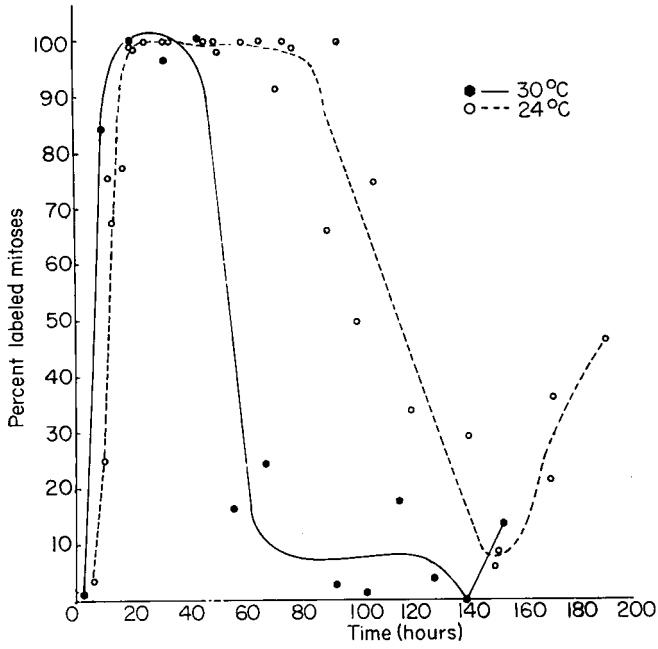


FIG. 7. Curves showing percentage of mitoses labeled with thymidine-H^3 in the germinative zone at various times after the substance had been injected into the anterior chambers of frogs maintained at either 24° or 30°C. The points represent average values gotten from five whole mount preparations. (From Fig. 4, Reddan and Rothstein, 1966.)

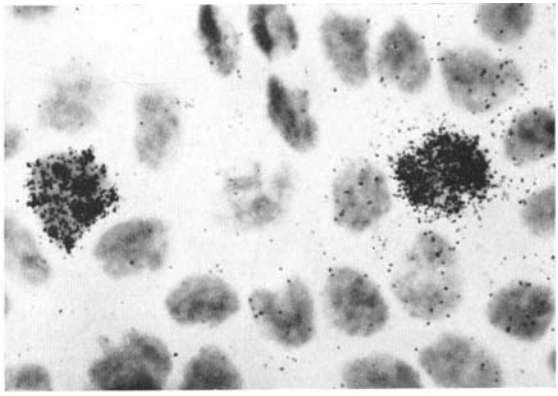


FIG. 8. Autoradiogram of lens epithelial cells which had been exposed to both H^3-(1 μC/ml, 6.6 C/mmole) and C^{14}-labeled thymidine (1 μC/ml, 3.0 C/mmole). The H^3 isotope was administered first with the C^{14} label being injected 4 hours thereafter. The C^{14}-tagged nucleus (right) is overlaid with silver grains in many planes while the H^3-tagged nucleus (left) is very discretely marked in only one. (From Fig. 5, Reddan and Rothstein, 1966.)

this value a double-label experiment with thymidine-H^3 and thymidine-C^{14} was also carried out. The figure obtained for S in this way was 100 hours. As shown in Fig. 8 it is quite simple to differentiate between nuclei containing only H^3, on the one hand, and those which contain C^{14} either alone or in combination with H^3, on the other hand. The length of synthesis is then determined by the expression (Bresciani, 1964)

$$H/C = t_a/t_s$$

where: H = number of nuclei labeled with only H^3
C = number of nuclei labeled with H^3 and C^{14} or C^{14} alone
t_a = interval between injection of H^3 and C^{14}
t_s = length of S phase

As we have pointed out elsewhere, an intracameral injection of thymidine-H^3 is essentially equivalent to a pulse label (Reddan and Rothstein, 1966). (Thus, 15 minutes after injection there is no longer sufficient isotope in the aqueous humor of one anterior chamber to produce positive autoradiograms when it is injected into another anterior chamber.) Hence one does not encounter the problem of tagging too great a proportion of the cell population. One cautionary note should however be raised. The percentage of cells that actually form part of the progenitor population is not known with certainty. Thus calculations of total cell cycle time and transit time through G_1, which are usually based upon proportionality ex-

pressions, can be erroneous. Since it is impractical (at least for *in vivo* studies) to do continuous labeling in order to ascertain the extent of the progenitor population this uncertainty will probably remain with us for some time to come.

With the aforementioned limitations in mind, it may be noted here, that we have calculated a cycle time of 83 days for the *Rana catesbeiana* germinative zone. Direct microscopic observations of lens epithelial cells in organ culture (see Section III,A below) indicate a mitotic period of no greater than 2 hours. G_2, from the labeled mitosis method, was found by Reddan and myself to amount to roughly 11 hours and, as already stated, S is in the neighborhood of 100 hours. Hence if it could be assumed that all of the germinative zone cells are truly progenitor cells the G_1 period would occupy over 90% of the total cycle time. As demonstrated in a previous piece of work (Reddan and Rothstein, 1966), changes in temperature, the production of an injury, or explantation to an *in vitro* system all exert easily perceptible influences upon kinetic parameters.

C. Chromosome Spreads

Whole mount specimens can also be employed in the preparation of chromosome spreads. These have so far been made on tissue from rabbit lenses (Figs. 9 and 10) and also from that of *R. catesbeiana.* To date the mammalian material has yielded better results than its amphibian counterpart. As shown in Fig. 10 it is possible to do autoradiography in conjunction with the procedure.

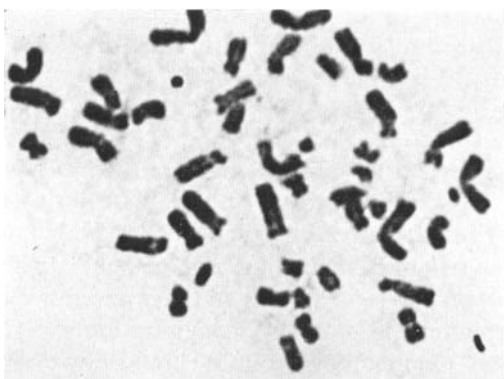


FIG. 9. Chromosomes of rabbit from injured lens epithelium. Note morphology (acetoorcein stain). [From Srinivasan and Harding, *Invest. Ophthalmol.* 4, 452 (1965).]

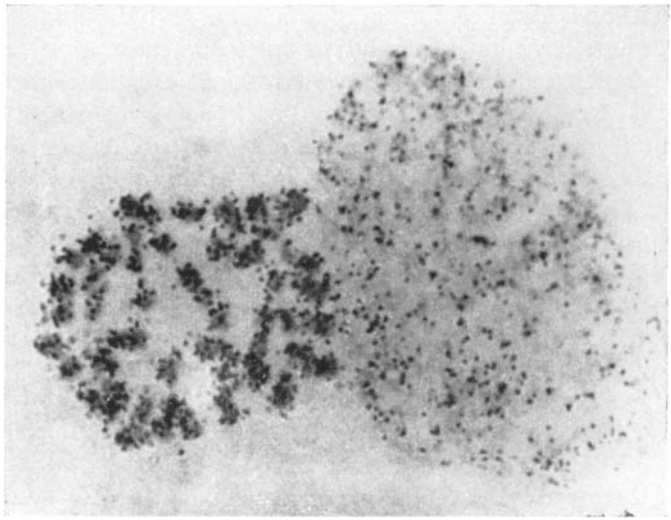


FIG. 10. Autoradiogram of chromosomes of rabbit from injured lens epithelium. Thymidine-H^3 was injected at 18 hours after injury and the eye fixed at 28 hours. Note localization of silver grains over the chromosomes and also a labeled nucleus. (From Srinivasan and Harding, 1963.)

The technique originally used by Srinivasan and Harding (1963, 1965) is basically as follows: Mitosis is stimulated by needle injury and then colchicine (2 μg/ml) is injected into the anterior chamber when the mitotic index reaches a peak. The eye is then enucleated, holes are cut in the posterior region of the globe and also through the cornea, after which the globe is placed in diluted (3:7) Eagle's basal medium, to which 2 μg/ml colchicine has been added. Following an hour's stay (36°C) in the above solution the material is fixed in Carnoy's fluid. Then, 18 hours later a whole mount is made and the area with the highest mitotic activity (i.e., the region around the injury) is cut out of the preparation. The epithelial cells are removed from the capsule with a solution containing 2 gm acetoorcein in 100 ml of 45% acetic acid. Chromosomes are spread by placing a coverslip over the cells and bringing pressure to bear on them. Once this has been done the slide, with coverslip still attached, is placed on dry ice for 10 minutes. The slip is then flipped off with a cold razor blade; following this the slide is brought through 3:1 and 9:1 alcohol-acetic acid and then 95% and absolute alcohol. At this point, Srinivasan and Harding report, tissue may be carried through hydrolysis in preparation for the Feulgen reaction. Autoradiography is, as Fig. 10 shows, also practical.

In our own laboratory we have made spreads of amphibian chromo-

somes based on a modified version of Srinivasan and Harding's technique. For our material we have found 1 μg/ml to be the optimal concentration of colchicine. Furthermore, we have had far better results with acetoorcein than with Feulgen. For the hypotonic solution we have employed 30 ml Wolf and Quimby's amphibian culture medium (Wolf and Quimby, 1964) plus 15 ml sodium chloride-free Earle's medium. Moreover, it is possible, if sufficient pressure is brought to bear on the coverslip, to make spreads directly on whole mounts. The results obtained in this way are admittedly not as good as those gotten with the cell dispersal technique but are much easier to produce. Furthermore, there are experimental situations in which it is desirable to retain some semblance of the original intercellular relationships.

D. Scintillation Counting Procedures

One of the most useful techniques that we have applied in our studies of macromolecular syntheses in lens epithelium is that of scintillation counting. This method naturally allows one to score many more preparations in a shorter amount of time then does grain counting. The prepara-

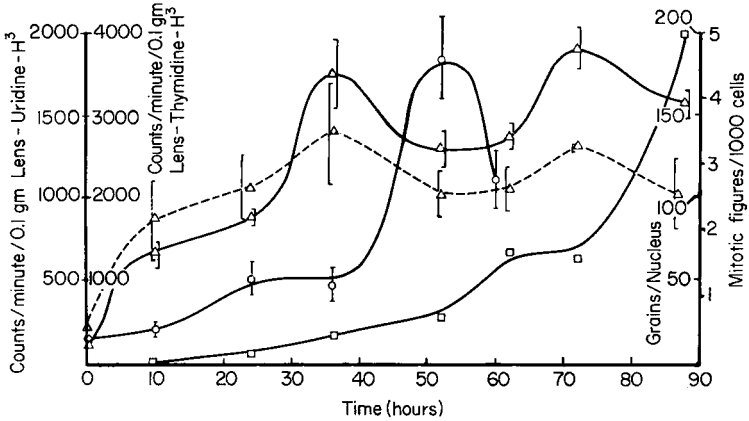
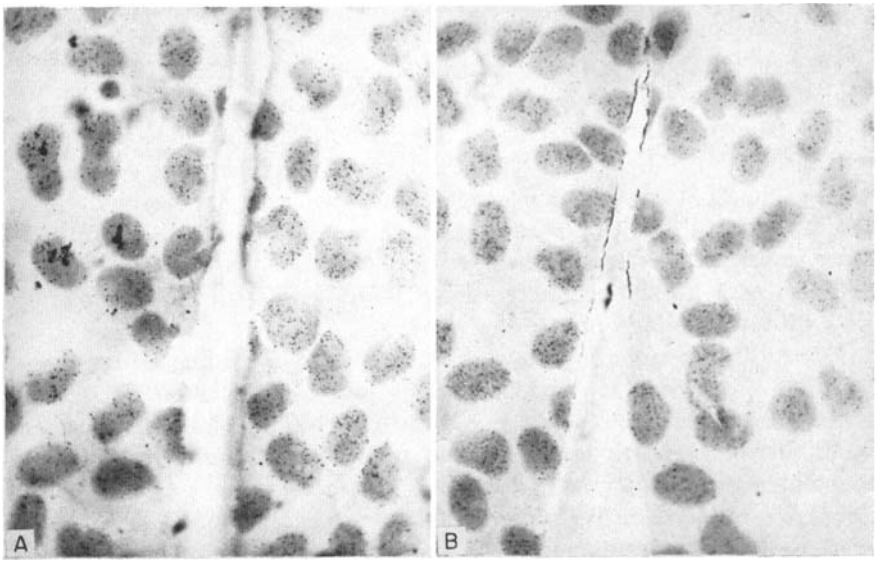


FIG. 11. Chronological relationships between the synthesis of RNA and DNA and the onset of mitotic activity in epithelial cells of the cultured bullfrog lens. Lenses were maintained *in vitro* for varying periods of time and were then incubated either in uridine-H^3 (40 minutes) or in thymidine-H^3 (1 hour) or were simply fixed and scored for mitotic figures. Each point represents an average of at least three preparations. Grain counts were made upon autoradiograms which were exposed for 1 week. Standard deviations are indicated. □—□, Mitotic figures/1000 cells; △—△, counts/minute/0.1 gm lens—uridine-H^3; △ - - △, grains/nucleus—uridine-H^3; ○—○, counts/minute/0.1 gm lens—thymidine-H^3. (From Rothstein *et al.*, 1966b.)

tion of tissue for counting is comparatively simple. At the termination of the experiment whole mounts are prepared in the usual way (see Section II,A above) except that the capsule is not scored or allowed to dry. It should, in fact, be emphasized, that total dessication of the sample prevents it from dissolving in solubilizer. The tissue is kept moist with 70% ethanol. Since water in the alcohol does produce some quenching it is necessary to avoid completely drenching the sample. We have typically found it easiest to use a piece of filter paper to soak up excess alcohol so that when solubilization is carried out the capsule is moist but not overly so. It is probably best to determine the optimum degree of moisture experimentally on the particular system being investigated. It is also critical to remove all extraneous tissue, particularly fibers, from the specimen. These fibers, it should be pointed out, are really cells that are in the process of elongating. They are not inert cellular products. Thus



1388 cpm 1948 cpm 5453 cpm 2714 cpm

FIG. 12. The tissues in these autoradiograms originated from two animals. In (A) (left) one lens was incubated in uridine-H^3 right after isolation; the other (right) 30 hours thereafter. Half of each whole mount from an individual lens was then mounted upon a slide and autoradiogrammed; the other halves being employed for scintillation counting. The figures beneath the tissues represent counts per minute for the aforementioned material. (B) The tissue to the left was incubated in uridine-H^3 30 hours post-isolation. The one to the right was handled in the same manner but had been exposed to actinomycin D for the first 4 hours of culture. (From Rothstein *et al.*, 1966b).

they tend to incorporate many of the substances (i.e., nucleic acid precursors, etc.) that are frequently used for experimental purposes. In making the whole mount one should naturally avoid detaching cells from the capsule as far as possible. If cells are lost one should estimate the percentage and incorporate it as a correction factor in the final results. Since it is usually not possible to arrive at anything better than an estimate it may become important to cross-check results by another technique such as grain counting.

After all debris has been removed from the specimen and it is appropriately moistened it may be placed in 0.25–1 ml of NCS solubilizer[4] (room temperature) for 12–24 hours. If H^3 is being counted, polyethylene vials are to be chosen over glass ones. After solubilization, one adds to the vials scintillation cocktail consisting of 0.4% PPO[5] and 0.025% dimethyl POPOP[6] in toluene. A convenient alternative scintillation fluid can be prepared with omnifluor[7] which is available as a dry powder consisting of 98% PPO and 2% MSB.[8] A 0.4% solution in toluene is satisfactory for counting purposes. We have generally worked with a final volume of 15 ml. Samples are finally placed in a liquid scintillation spectrometer, are brought down to 0°C, and are then counted.

The lens tissue which remains after whole mounts have been prepared should be dessicated and weighed. Thus it becomes practical to express the final data as counts per minute per unit dry weight of lens. Because the cell loss correction factor is, as emphasized, only an estimate some uncertainty is usually injected into the data. In order to establish confidence in the procedure we have therefore cross-checked our findings with autoradiography.

Figure 11, for example, shows the time course of uridine-H^3 incorporation into frog lenses at various periods following isolation. It is to be noted that grain counting in this case bears out, at least in a general way, the changes suggested by the scintillation procedure. This correspondence is also reflected in the autoradiograms and scintillation counts reproduced in Fig. 12 and 13. In these cases one-half of a capsule was used for autoradiography, the other for scintillation spectrometry. It is therefore feasible to use both techniques on each eye and have one lens of a particular organism serve as a control for the other. Since the tissue has been fixed in Carnoy's fluid almost all acid-soluble precur-

[4] Nuclear Chicago Company, Des Plaines, Illinois.
[5] 2,5-Diphenyloxazole.
[6] 1,4-Bis-2-(4-methyl-5-phenyloxazolyl)-benzene.
[7] New England Nuclear Corporation, Boston, Massachusetts.
[8] Bis-(*O*-methyl steryl)-benzene.

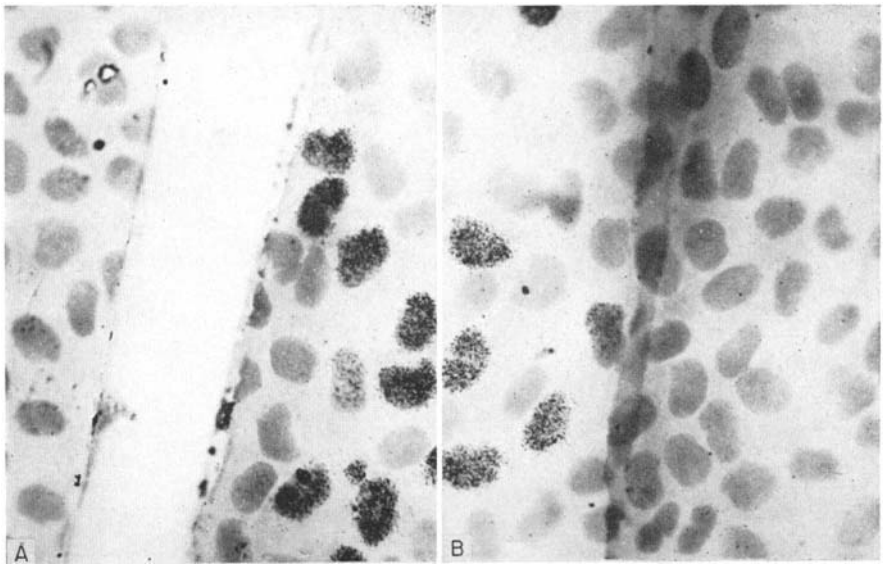


FIG. 13. These specimens were prepared just like those described in Fig. 12 (each "pair" originated from the same animal) with thymidine-H^3 being used in place of uridine. In (A) the tissue on the left was placed in isotope solution immediately following isolation; the one to the right after it had been in culture for 50 hours. In (B) the material on the left was again cultured for 50 hours and then incubated in thymidine-H^3. The one on the right received the same treatment but was exposed to actinomycin D for the first 4 hours of culture. (From Rothstein *et al.*, 1966b.)

sors are removed directly. In fact the amount of radioactivity in the fixative is often quite considerable so that it may be beneficial to wash the lens several times. Of course, if it is desirable, trichloroacetic acid (TCA) or any other appropriate substance may be employed either in advance of making the whole mount or just after it has been prepared.

E. Electron Microscopy

The first electron micrographs of the lens epithelium of *Rana catesbeiana* were recently prepared by M. Ravitz of the Albert Einstein College of Medicine. Following enucleation the capsule, together with adhering cells, is separated from the fiber mass by removing the latter through a longitudinal slit in the postetrior aspect of the lens. The capsule and cells are then fixed for 2 hours at pH 7.2 in phosphate buffered osmium.



FIG. 14. Electron micrograph of lens epithelial cells of *Rana catesbeiana*. × 12,800. Nu, nucleolus; En, Nuclear envelope; N, Nucleus. Mitochondria are also plentiful. Courtesy M. Ravitz.

After treatment with Epon 812 sectioning is carried forward on a Porter-Blum MT-2 ultramicrotome with diamond knife. Staining of sections is accomplished with uranyl acetate and then lead citrate.

Figures 14 and 15 are electron micrographs, prepared by Ravitz, which show the ultrastructure of the amphibian lens epithelial cells quite clearly. One of the interesting features of these cells is the complex interdigitation of the plasma membranes (Fig. 15). Judging from evidence we have obtained at the light microscope level (Gierthy *et al.*, 1968) it appears almost certain that these membranes undergo dramatic changes when the lens epithelial cells are stimulated to divide by either mechanical injury or explantation.

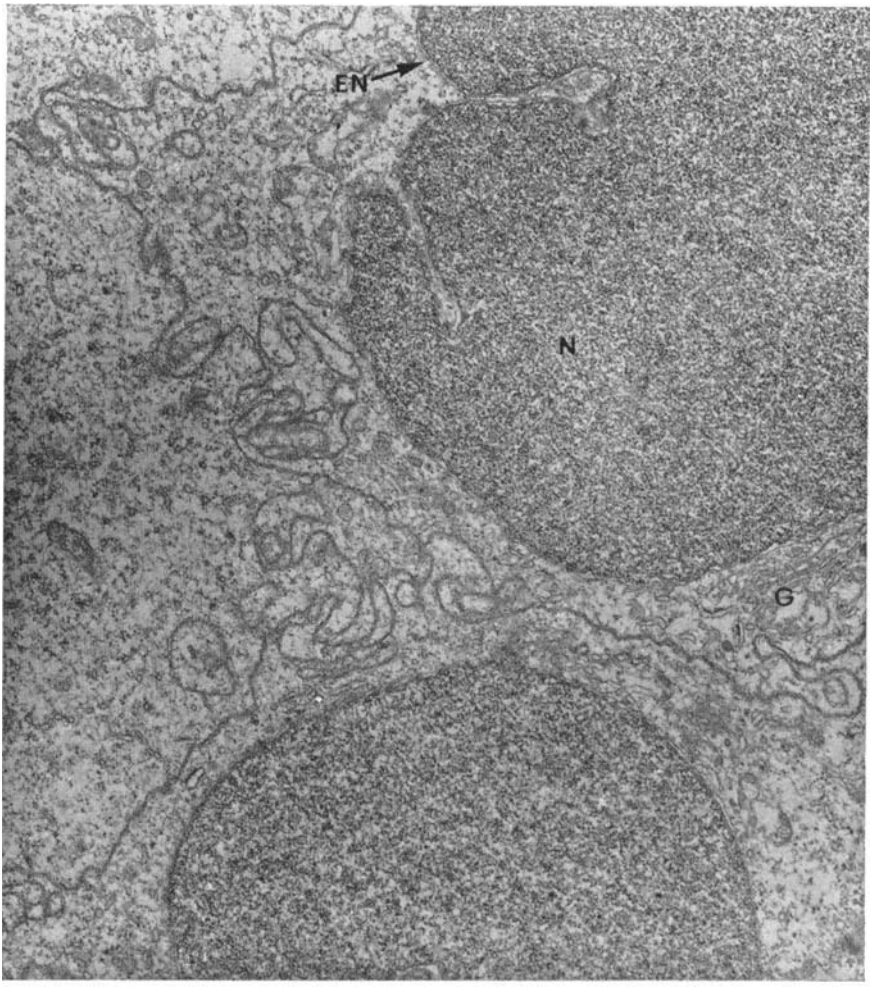


FIG. 15. Electron micrograph of lens epithelial cells of *Rana catesbeiana*. × 31,000. N, Nucleus; En, nuclear envelope; G, golgi zone. Note interrelationships of cell membranes. Courtesy M. Ravitz.

III. Methods for Live Material

A. Organ Culture and Cell Culture

Mammalian lenses have been successfully cultured by Bakker (1936), Schwartz (1960), Bito (1963), Harding *et al.* (1962), Kinsey *et al.*

(1955), and others. In recent years we have developed a very simple technique for the maintenance of amphibian material as well (Rothstein *et al.*, 1965a).

Following enucleation, a circumferential cut is made midway between the two poles of the globe. This serves to expose the posterior aspect of the lens. Then with two strabismus ("mouse tooth") forceps one separates the lens and adjacent pigmented tissue from the sclera. This is accomplished by grasping the sclera with one forceps and the choroid and retina with the other. While the former instrument is held stationary the latter is used to pull away (gradually!) the pigmented tissue. When it is free the lens can be lifted away from the remaining cornea and sclera. A small amount of iris should be left attached to the lens for easy manipulation. The organ is then placed in an appropriate medium.

The culture fluid we have found most suitable is that developed by Wolf and Quimby (1964) for growth of *Rana catesbeiana* tongue fibroblasts.[9] The medium consists of the following:

25% sodium chloride-free Earle's balanced salt solution
10% fetal bovine serum
55% Eagle's minimal essential medium
10% whole egg ultrafiltrate

To the above, one may add penicillin and/or streptomycin. It has been our practice to use relatively high concentrations of antibiotics (e.g., 1000 units/ml penicillin) and antifungal agents (Mycostatin) in the initial isolation medium but much lower ones (200 units/ml penicillin) in the final culture fluid. Needless to say all of the procedures used to guarantee sterility in routine tissue culture should be adhered to in work with whole lenses.

For culture vessels it is possible to use a wide variety of products currently on the market. In our laboratory we have found it simplest to employ 30-ml specimen jars fitted with silicone rubber stoppers. The lens is placed in the dry container posterior side down, and then 25 ml culture medium is introduced by means of a sterile syringe. (It is important that not too large an air space be left in the vessel since this will allow carbon dioxide to escape from the medium causing its pH to rise.) If the tissue is to be maintained for one week or less it is not necessary to change the medium. For periods longer than this the fluid should be renewed once each week.

We have not yet determined the absolute length of time for which a lens can be kept in our system. However, with roughly similar methods

[9] This can be obtained commercially from Grand Island Biological Company, 3175 Staley Road, Grand Island, New York.

Bito (1963) succeeded in maintaining rabbit lenses for several months. In all probability even this time can be extended. We have not found gassing or perfusion to be required for supporting the cells (although such procedures can be used if the experimental program calls for them). If the technique is carried out properly the pH of the culture fluid should not change by more than 0.1–0.2 pH units in a week's time.

In both mammalian (Harding *et al.*, 1962) and amphibian (Rothstein *et al.*, 1965a) lenses explantation stimulates the central (ordinarily non-proliferating) epithelial cells to undergo DNA synthesis followed by mitosis. Though we do not yet know why this happens we have been able to take experimental advantage of the phenomenon in order to investigate the chemical events which lead to division in cultured material. Thus it seems, from our studies and those of Reddan *et al.* (1966; Bagchi *et al.*, 1968; Rothstein *et al.*, 1966a,b), that both RNA and protein

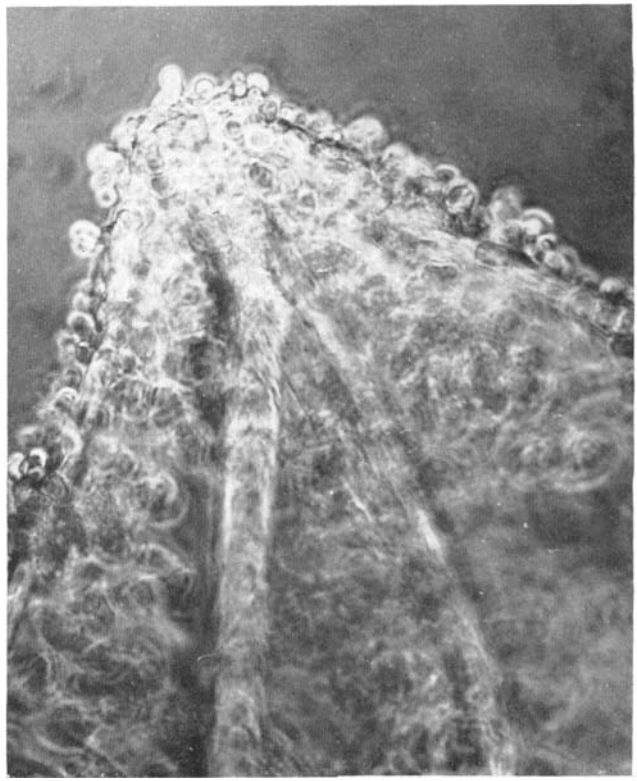


FIG. 16. Outwandering of lens epithelial cells from a capsule which was explanted to a Rose chamber as discussed in text. Phase contrast. Approximately × 160.

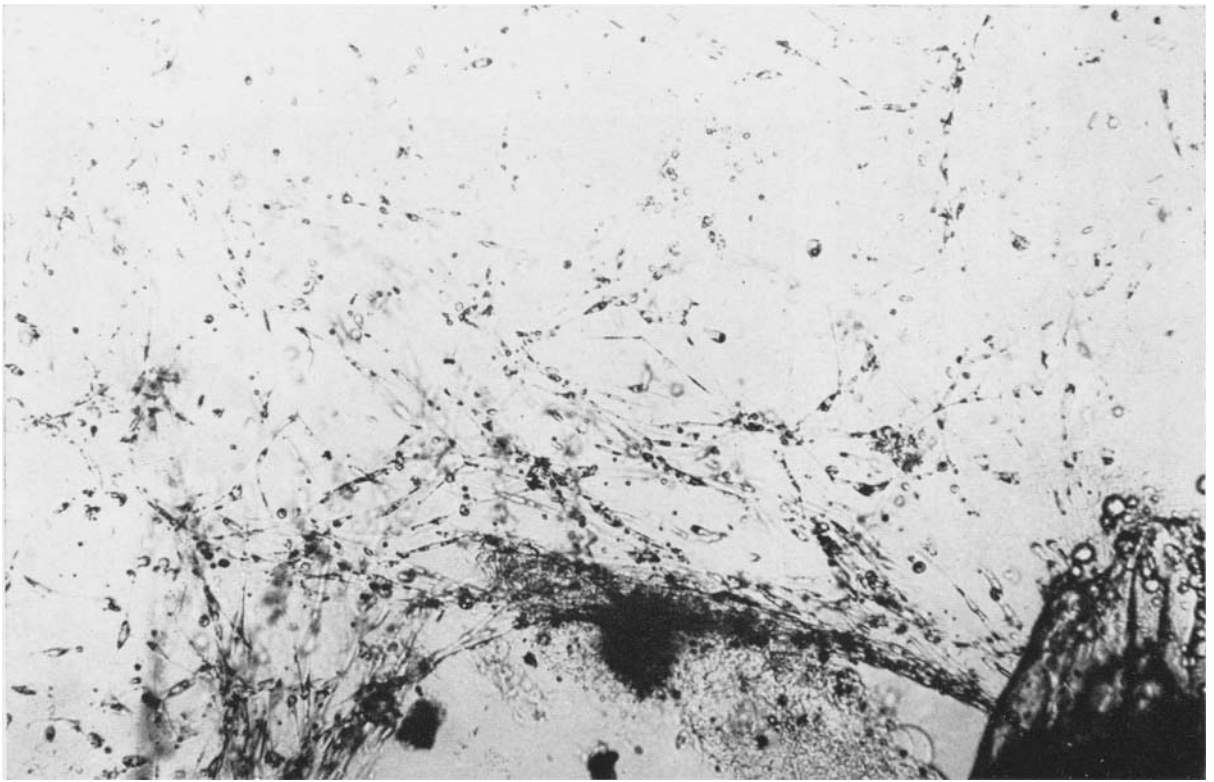


FIG. 17. In this case a capsule with a small amount of iridial tissue was explanted. The tissue shown is thus probably a mixture of iridial and lens epithelial cells.

synthesis are enhanced prior to that of DNA. If these syntheses are retarded with drugs like actinomycin or puromycin mitosis does not materialize.

That lens epithelial cells can be kept alive for comparatively long periods of time in the above mentioned culture medium suggests that it might also be appropriate for ordinary cell culture purposes. Initial experiments with embryonic avian and mammalian material also shows that it may be practical to grow lens epithelial cells as such (Kirby, 1926; Kirby *et al.*, 1929, 1932; Mamo and Leinfelder, 1958; Mann, 1948; Philpott and Coulombre, 1965). We have begun some tissue culture experiments in our laboratory, and they imply that the technique will be feasible. In these experiments the capsule is removed from a freshly isolated lens and is transplanted to a Rose (1957), Prior (Trevan and Roberts, 1962), or Sykes-Moore (1959) chamber. The cell layer is obtained by making a longitudinal cut in the posterior capsule. Most of the fibers are then drawn out through the resultant hole with a forceps. This leaves the anterior capsule in place with the epithelial cells and some fibers attached. Cover glasses (the size of these depends upon the chamber used) are coated with a few drops of frog blood plasma (we have tried to use plasma which originated from the same animal as did the experimental tissue but this is probably unnecessary), and the capsule is inverted over the prospective coagulum so that the cell side is up. The clot serves to hold the tissue in place. Following this the chamber is filled with Wolf and Quimby's medium. Progress of cell growth can be followed by phase-contrast microscopy (a long-working distance condenser is required) (Fig. 16). As Fig. 17 shows, the medium also supports the growth of iridial cells.

B. Observation of Cells in Organ Culture

In a paper which was published from our laboratory several years ago it was speculated that the stimulation of DNA synthesis and cell division by mechanical injury might be caused by changes in cell-cell relationships (Rothstein *et al.*, 1965b). To pursue studies which could help in evaluating the hypothesis just mentioned it was necessary to make direct observations of living lens epithelium since fixation so drastically changes the natural morphology of the cells.

With all of the work that has been done on lenses over the years it came as something of a surprise that the epithelium had never been observed in the living state while upon the lens itself. The chief handicap in making such observations stems from the fact that the organ, as such; acts as an optical component in the microscopic system and so

makes phase-contrast studies, as they are conventionally carried out, impractical. Thus even with long-working distance condensers the ring in the annular stop cannot generally be made to coincide with that in the phase plate of the objective. As we have shown, however, when appropriate measures are taken the epithelial cells can be discerned upon the intact lens in culture (Gierthy *et al.*, 1968).

In order to make such observations we employ a Zeiss achromatic-aplanatic phase, dark-field, phase-fluorescence condenser in conjunction with a front lens of 0.63 numerical aperture. This front lens is ordinarily used with the standard long-working distance condensers. In addition it is absolutely essential to use either a number 1 phase objective with the number 2 annulus of the condenser or a number 2 objective in conjunction with the number 3 stop. At present number 3 objectives cannot be used.

The lens is placed in a Rose multipurpose chamber (Rose, 1957) with a 0.3 cm gasket, and suitable culture medium is then introduced. It is, of course, necessary that contact be made between the anterior capsule and the uppermost coverslip (No. 1; 45 mm $\times$ 50 mm). Each time the stage is moved to view a new field the phase rings must also be realigned. This stems from the fact, already noted, that the tissue is in itself acting as a lens in the microscopic system.

In addition to ordinary phase contrast one can also apply interference-contrast (Nomarski) microscopy. This is accomplished by using the number III position on the Nomarski condenser again in conjunction with a 0.63 numerical aperture front lens. The objectives that give the best results are 16, 40, and 100 $\times$ planachromats (with phase contrast, on the other hand, one is limited to 10–40 $\times$ objectives).

A photograph of a lens which was cultured for 3-hours and then viewed in the Rose chamber by phase interference is shown in Fig. 18. Our preliminary studies of cells upon intact *in vitro* lenses have shown that cell-cell relationships begin to change about the same time that RNA synthesis starts to increase but before protein formation is augmented. As shown in Fig. 19, by about 48 hours many of the cells have changed from nearly polygonal in conformation to spindle type affairs. These cells actually seem to "creep" about on the surface of the lens and pseudopod-like structures have been observed from time to time. The spindle configuration is not, however, preserved indefinitely. Thus as Fig. 20 indicates most of the cells have rounded up by 72–90 hours and some of them are engaged in mitosis.

To show that the epithelial cells were alive during the period of observation we removed lenses from culture vessels at the time when mitosis was known to be afoot (72–90 hours post-isolation). They were



FIG. 18. Photomicrograph of epithelial cells on a lens which was cultured for 3 hours. Interference contrast. Approximately ×400. [From J. F. Gierthy *et al.*, *Exptl. Cell Res.* (1968).]

subsequently placed in Rose chambers and observed by phase-contrast and interference-contrast microscopy. Under these conditions I have seen cells move from mid metaphase to interphase. This takes approximately three quarters of an hour at 24°C ± 2°C. Figure 21 shows several photomicrographs (taken with Nomarski optics) from such a sequence. Thus the epithelial cells apparently survive, at least for some time, under the conditions employed. In any case it is relatively easy to tell when cell death has occurred. The nuclei become very grainy and in general stand out much more boldly than they do when the cells are living. In fact the opacity that develops is such that cells can be readily seen even under bright field illumination. Such observation is not possible when the tissue is in good condition.

We have been able to observe living epithelial cells in four amphibian lenses (*Rana catesbeiana*, *Rana pipiens*, *Rana clamitans*, and *Bufo marinus*) and that of one mammal (*Oryctolagus cuniculus*, the American Dutch rabbit). Best results have so far been obtained with the *R. pipiens* material. In general the amphibian lens is easier to work with because

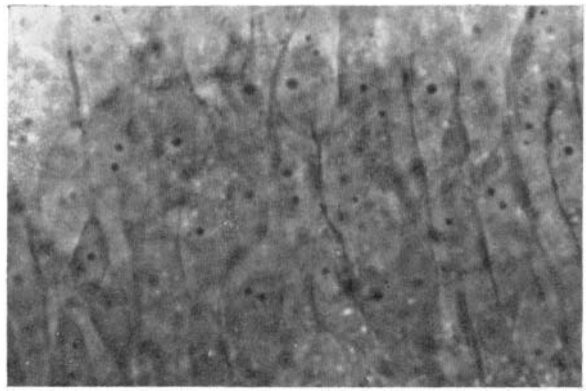


FIG. 19. After 48 hours in organ culture the epithelial cells assume a spindle-type configuration as shown in this figure. [From J. F. Gierthy *et al., Exptl. Cell Res.* (1968).]

the nucleoli stand out very prominently in phase contrast and interference contrast. However by altering the size of the gasket in the Rose chamber it should be feasible to do microscopy upon most sorts of vertebrate lenses.

Such microscopy should, incidently, be practical in conjunction with time lapse procedures which would in turn permit direct investigation of cell cycle kinetics as described by Sisken (1964). Moreover circumfusion methods can be expected to lengthen the duration during which the tissue can be maintained. Recently we have also been able to make some direct observations by fluorescence and phase fluorescence with

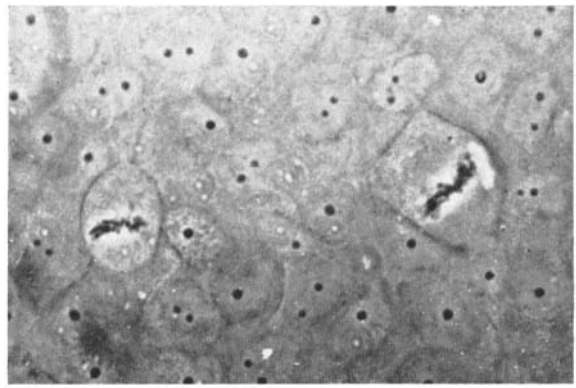


FIG. 20. This is a photograph of cells associated with a lens which had been in culture for 3 days. Note the mitotic figures. Most of the cells have begun to round up by this time. [From J. F. Gierthy *et al., Exptl. Cell Res.* (1968).]

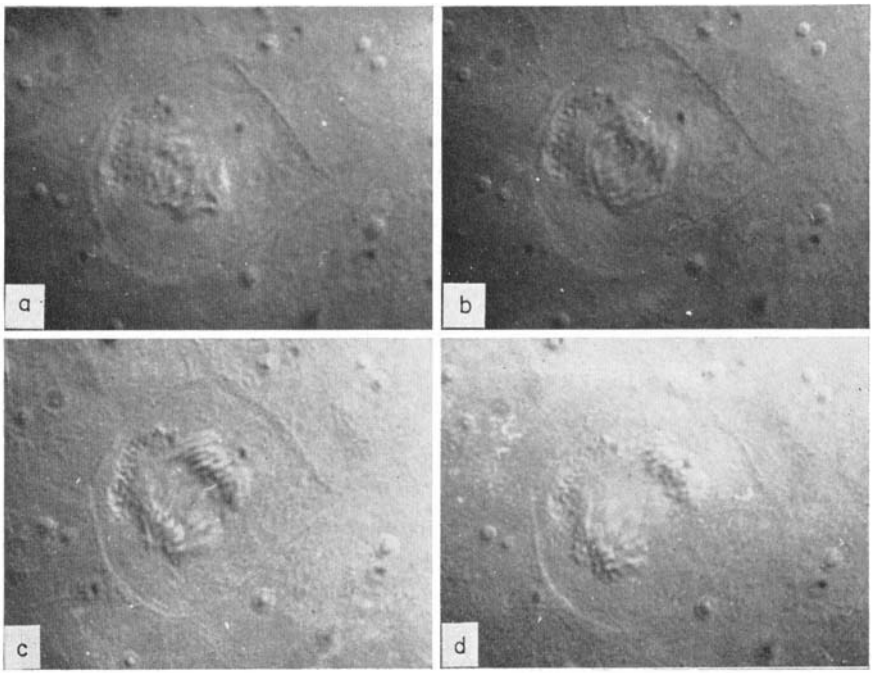


FIG. 21. (a–d) Mitotic figure which was observed from metaphase through telophase. Selected stages of the sequence are shown. Interference contrast. [From J. F. Gierthy *et al., Exptl. Cell Res.* (1968).]

acridine orange serving as the fluorochrome. Undoubtedly the most important feature of this general approach is that it enables one to study cells which are still an integral part of the organ with which they are normally associated in the *in vivo* condition.

IV. Assessment of Isozyme Activity

Though isozyme activity has been assessed in bovine lens epithelium by Papaconstantinou and his associates (1967) little has been done with the amphibian counterpart. Rafferty has performed several interesting experiments in this regard but her measurements were pursued chiefly upon material acquired from whole *R. pipiens* lenses (Rafferty, 1963). Recently Arthur Hessler, working in our laboratory, has been able to perform effective separations of the five lactate dehydrogenase (LDH) isozymes from *R. catesbeiana* lens epithelium by disc electrophoresis.

The methods employed were essentially those described previously by Davis (1964) and Dietz and Lubrano (1967).

Following removal of the capsule, homogenization at 5°C is carried out in a solution composed of 30 μl 0.1 *M* tris-glycine buffer at pH 9.4 and 30 μl 10% sucrose. After centrifugation at 5860 *g* for 5 minutes, the supernatant (4–12 μl) is electrophoresed in tubes of diameters varying from 2–6 mm.

The current employed is varied with the tube diameter and ranges from 1.0–2.5 mA per tube. With nitroblue tetrazolium (1 mg/ml) as the

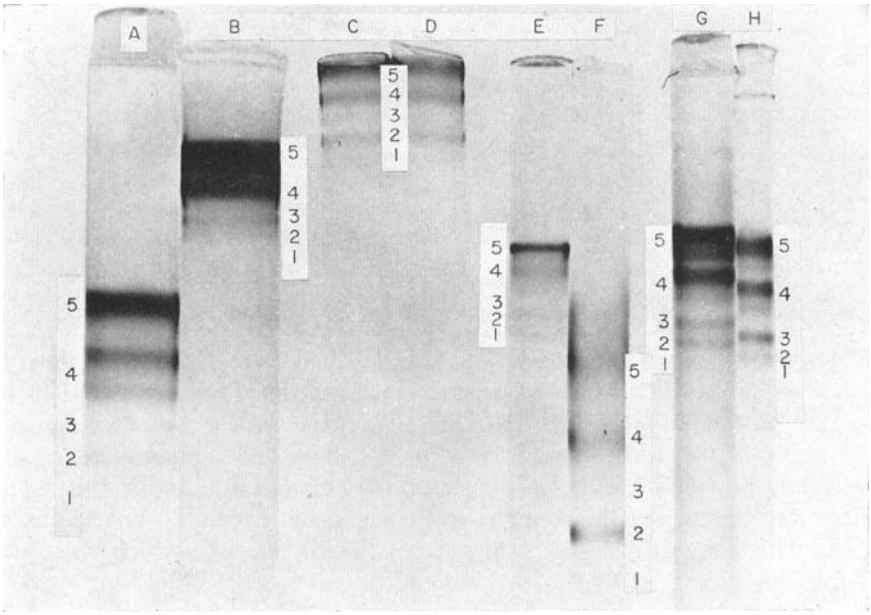


FIG. 22. Separation of lactate dehydrogenase isozymes which occur in the lens epithelium of *Rana catesbeiana* in gel tubes of varying dimensions (2–6 mm). (Courtesy of A. Hessler.)

stain, results such as are shown in Fig. 22 are routinely obtained. Tests with oxalate, oxamate, and iodoacetate imply that the bands shown do in fact represent the LDH isozymes. Though work on proteins other than LDH has not been pursued in our laboratory the initial results suggest that it should be possible with malate dehydrogenase (MDH) and other equally interesting substances. It ought to be reemphasized that the enzymes whose separation has been discussed originated from a practically pure (except for capsular substance) suspension of lens epithelium.

V. Concluding Remarks

It is clear that many techniques can be applied to investigation of the lens epithelium of amphibians (and other animals as well), and undoubtedly newer approaches will become applicable with the passage of time. More important, however, is the question of whether such techniques can pave the way for a more acute understanding of cellular activities. It is my impression that the lens epithelial population provides a powerful tool for the acquisition of such an understanding.

It is a homogeneous group of cells existing in as simple an array as has been found in any multicellular organism. Thus when one stimulates proliferation, for example, by mechanical injury, one does not need to trouble oneself about the possibility of other types of cell complicating the picture. The cells that divide as a result of the procedure are and can only be lens epithelial cells. Yet despite this simplicity we can, as shown above, deal also with problems as complex as those bearing on intercellular interrelationships. Routine tissue culture should render possible the investigation of almost any question relating to cellular function, for example, work on membrane transport. The lens, therefore, emerges as an extraordinarily versatile kind of experimental system, a system upon which it is possible to execute biochemical, histochemical, and microscopic investigations at almost all technical levels currently in use.

We feel that in the last 5 years we have made some worthwhile progress in studying problems of cell division in the lens. Such problems are obviously so complex, however, that it surpasses the power of any single group to adequately discuss much less investigate them. The whole area of cytophotometry for which the lens epithelial monolayer is so well suited has not even been touched, nor has density gradient methodology been utilized. Cell culture is in the infant stage. Perhaps it is not too much to hope that this presentation will help to direct the attention of others to the singular advantages offered by lens epithelium, a heretofore little used experimental system.

Acknowledgment

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Chapter 4

Cultivation of Tissues and Leukocytes from Amphibians

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I. Introduction

Because there is a basic similarity in the structure and function of cells from all forms of life, cell biologists have often selected test objects which appear to offer representative data with the greatest ease of observation. It is within this context that the techniques for culturing

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the amphibian cell have been explored. Amphibian tissue is composed of cells which are generally larger than those from avian or mammalian sources. In addition, they frequently contain fewer and larger chromosomes. Moreover, since the growth of these poikilothermic tissues is optimal at room temperature, successful maintenance of amphibian cultures can be achieved without an incubator.

The specialized tissue culture methods used successfully with homeothermic tissues were applied to amphibian cells in early trials. While these procedures offered a low degree of cellular proliferation, it was apparent that modifications of the culture methods were required before the amphibian tissue could be utilized as a suitable test object for cell studies.

This article summarizes current methods of tissue and blood culture of amphibians based on our experiments, as well as those of other workers, in an attempt to establish more precisely the optimal conditions for maintenance and cell growth. It is hoped that this information will provide the framework for a better understanding of the reports that have appeared recently.

II. Culture Technique for Adult Tissues

A. Materials

In the tissue culture of cold-blooded vertebrates, the epidermis of frog embryos and various organs of tadpoles have been used exclusively by previous workers for a long period of time. Since tissues from adult frogs have a lower growth potency than those of larvae, they could not proliferate adequately in the relatively poorly developed *in vitro* conditions used 20 years ago.

Advances in methodology have made it possible to grow adult amphibian tissues in culture satisfactorily. In our laboratory, a variety of soft-organ tissues were used in an attempt to compare their growth rates *in vitro* (Seto, 1964). In work with adult frogs, successful cultures were obtained from kidney, heart, and lung tissues. Newt lung tissue was also found to be an excellent material; whereas, liver, spleen, and intestine of both animals gave poor results. Newt heart tissue has been frequently used for the study of mitotic chromosomes by other workers (Bloom *et al.*, 1955; Boss, 1954; Amenta, 1961). Due to this improved growth *in vitro*, and the ease with which material could be isolated, frog kidney and salamander lung have been most frequently employed as

culture material. Besides these more commonly used tissues, frog tongue, lenses, and newt coracoid cartilages have been occasionally used in culture, and have also proved to be very satisfactory materials (Boss, 1955; Wolf and Quimby, 1964; Rothstein *et al.*, 1965).

B. Sterilization of Tissues

The difficulty in maintaining primary cultures of poikilothermic tissues is attributed to the frequency of bacterial contamination in the original preparation of materials. Since greater amounts of bacteria, fungi, and parasites are present in these tissues than in the mammalian internal organs that have direct communication with the exterior, as well as on the body surface, extensive precautions must be taken to ensure that no bacteria are introduced into the culture vessel with the tissue.

Prior to the surgical operation, in the culture room, the animal is rinsed in dilute potassium permanganate solution (approximately 5–10 mg/liter) for 30 minutes, and the entire body surface is wiped with 80% alcohol. After the animal is narcotized with 1 part of Tricaine Methanesulphonate (MS 222-Sandoz) in 2000 parts of spring water, or is immobilized by severing the spinal column, the selected organ is removed by careful aseptic techniques. Sterilized washing solution (Ca-free amphibian Ringer's solution containing high concentration of antibiotics[2]) is used repeatedly to rinse the tissue, which is held with sterile forceps. Then the tissue is minced into small pieces (1–2 mm^3) with knife blades or scissors, and tissue fragments are then suspended in a fresh balanced salt solution in order to remove blood clots, adherent mucus, or toxic chemical substances. Petri dishes containing the salt should not be used for more than one rinsing procedure.

C. Explantation of Tissue Fragments

Primary explantation techniques that were principally developed for mammalian tissues have also been applied to amphibians. The majority of previous workers, culturing embryonic or larval tissues, employed the slide or coverslip culture technique with the plasma clot system. This traditional "hanging drop" method, with a few exceptions, was almost the exclusive technique employed for lower vertebrates until recent years. Details of the procedure have been described in the textbooks of Paul (1960) and Parker (1961).

[2] Sodium chloride 0.65 gm; potassium chloride 0.014 gm; glucose 0.02 gm; double-distilled water 100 ml; penicillin 100,000 units; streptomycin 50 mg; adjust to a final pH of 7.4.

The cultures are made by explanting small tissue fragments (1–2 mm^3) embedded in a cockerel plasma clot adhering to a coverslip, which is subsequently inverted over the cavity of a depression slide. The advantages of this traditional form of culture are: (1) the simplicity of the procedure, (2) the relative ease of cell spreading, and (3) the convenience of the subsequent cytological staining procedure. The disadvantages of this technique are: (1) only small amounts of tissue can be cultured, and (2) nutrients are rapidly exhausted and cannot be replenished.

As a modification of the hanging drop procedure, a more convenient method of preparing coverslip cultures, the so-called "flying coverslip" technique, is recommended. A small, narrow coverslip (11 mm $\times$ 32 mm) is used. These can be inserted into standard 12 mm $\times$ 140 mm test tubes. Cultures are prepared with the standard plasma clot system of two such coverslips, and these are inserted back-to-back into a test tube. About 2 ml of a medium is added to the tube which is then placed in a roller drum. Large numbers of cultures can be conveniently prepared in this way for histological examination or for mounting in perfusion chambers (Pomerat, 1951).

Tissue fragments can also be explanted directly into T-flasks (Earle, 1948); fragments will anchor onto the wall of each flask because of the natural adherence of the tissue. This is achieved by leaving tissue fragments on the wall for 20 to 30 minutes prior to introducing nutrient fluid. When adherent explants are seemingly dry, they become fixed in position. This permits a periodic exchange of the medium so that the tissue can be maintained in the same flask over a considerable period of time.

D. Monolayer Cell Culture

Those traditional methods, which have been widely employed by previous workers, are not very suitable for quantitative studies in cytology, cytochemistry, and cytogenetics due to the nature of growth in the persisting clot. Monolayer cultures of isolated cells can provide a better system for such studies. However, a common trypsinization method, which is routinely used for mammalian tissue dissociation was found to be almost inapplicable to poikilothermic tissues, because the 20 or 30 minutes of trypsinization at room temperature was not effective enough for these tissues, and dispersed cells seldom retained viability. Actually, the method for preparing monolayer cell cultures of poikilothermic tissues had not been well developed until the work of Wolf *et al.* (1960). They obtained isolated cells with a carefully controlled, gentle, enzy-

matic digestion mixture, using a modification of the cold-trypsinization method described by Bodian (1956). This method provides an adequate number of viable cells.

For the amphibian tissue dissociation, two media are exclusively used in our laboratory. The first consists of 0.25% trypsin in Ca-free Ringer's solution, supplemented with calf serum and glucose. This solution consists of fewer components and has been found to be effective. The second

TABLE I
COMPOSITION OF THE DISSOCIATING MEDIUM

Simple trypsin solution[a]		Shah's dissociating medium	
Compound	Gram/liter	Compound	Gram/liter
A. NaCl	6.50	A. NaCl	6.80
KCl	0.14	KCl	0.10
		Na_2HPO_4	0.11
B. $NaHCO_3$	0.25	B. $NaHCO_3$	0.20
C. Trypsin (1:250)	2.50	C. Trypsin (1:250)	2.50
D. Glucose	1.80	D. Glucose	1.00
		E. Serum globulin	1.00

[a] When a long-term treatment is undertaken, 2.5% calf serum is added to this solution before use.

All units are dissolved separately, in double-distilled water mixed, and filtered by the Seitz filter with asbestos pads or through a Millipore filter of 0.45-μ pore size by vacuum. Antibiotics concentrations: Penicillin G 100,000 units; streptomycin sulfate 100 mg; Mycostatin 50,000 units. One-half ml of a 1% phenol red solution (Difco) is added to 1000 ml of the above media, and the pH adjusted to 7.4.

medium was provided by Shah (1962), and is a better, more widely used conditioned dissociating medium. The components of this medium are shown in Table I. These two media were found to provide the greatest yields of viable cells during amphibian cell dissociation of all media tested.

With either medium, the following dissociating procedure is employed. Minced tissue fragments are washed well by suspension in a Ca-free Ringer's solution, and the washing solution is decanted by a 5-minute centrifugation at 500 rpm. Sedimented materials are transferred into an 100 ml Erlenmeyer flask together with the dissociating medium. Thirty milliliters of the medium is added to about 3 cm^3 of the material. Digestion at 4°C is facilitated by using a magnetic stirrer to agitate the suspension. Isolated cells obtained from the first hour's harvest usually result in insignificant growth. Therefore, the first digestion mixture is decanted and discarded to remove any toxic or unfavorable materials

surrounding the tissue fragments. The second digestion with a fresh dissociating medium is continued for several hours or overnight at about 4°C. The time can vary, depending on the rate of cell dispersion, but best results are obtained if the time does not exceed 20 hours.

Cells are collected from the harvested fluid by centrifugation at 600 rpm for 10 minutes and are washed once in the growth medium. Then the cells are seeded in a culture flask with a final concentration of 3–5 $\times 10^5$ cells/ml of growth medium. It was noted that the greater concentrations of cells in the inoculum produced significantly superior growth in the primary culture. It is recommended, however, that once the cells demonstrate a vigorous growth potency the initial cell population should be reduced.

The culture flask is kept in an incubator without agitation for 48 hours to facilitate the adherence of cells onto the glass surface. After 7 or 8 days at 26°C, the cells become mitotically active. The culture will usually show a uniform monolayer cell sheet over the surface of a flask within 2 weeks.

E. Continuous Observation of Cultures

If careful observation of phase-contrast microscopy or photomicrography of living cells is needed, the hanging drop method or a perfusion chamber of a coverslip culture is commonly employed. However, if continuous observation or a long-term phase cinematographic record of a living culture is required, the cells must be grown in a special chamber with the necessary optical properties.

The Rose multipurpose culture chamber (Rose, 1954; Rose *et al.*, 1958) used in our study facilitates morphological analyses of intracellular phenomena of cultured cells, as well as cytochemical investigations of cell organoids. This sandwichlike container is very useful for phase-contrast microscope studies and for the manipulation of the cell itself. The procedure employs a strip of cellophane as the agent for anchoring tissue explants to the coverglass wall of the chamber. The cellophane strip also allows clear visualization of the cell grown in the chamber.

Several tissue fragments, approximately 1–2 mm^3, are placed on a center area of a 43 $\times$ 50 mm coverglass. In assembling the chamber, a "cellophane" strip sterilized in 80% ethanol and then washed in a sterilized saline solution is laid across the tissue explants. This strip is obtained by slitting the sides of Visking dialysis tubing to provide a single layer of a semipermeable membrane. The silicone gasket is next positioned over the coverglass, and the chamber is closed with another coverglass. Two metal plates (75 mm $\times$ 50 mm), which have a center hole 25

mm in diameter, offer rigidity when positioned on both sides of the chamber. Each plate has corner holes for the accommodation of 4 screws that hold the components together. After the screws are gently tightened, the extended ends of the cellophane membrane may be carefully pulled so that the wrinkles are smoothed. The explants are then securely lodged against the coverglass wall. The chamber is filled with approximately 2 ml of nutrient fluid through an inlet needle which penetrates the gasket, while an opposite outlet needle serves as an air vent. The technique is completed by tightening the 4 screws and removing the needles.

The subsequent exchange of fluids is accomplished by inserting two opposing hypodermic needles. The expended medium is removed with negative pressure produced by one syringe, then fresh medium is injected into the chamber from a second syringe. If it is desired to fix and stain the cultures, the cells on the bottom coverslip are washed with balanced salt solution and fixed before the retaining plates have been removed.

F. Nutrient Media

Culture media obtained from natural sources were widely used in the early experiences with tissue culture of cold-blooded vertebrates. Since the time that Harrison (1907) successfully grew pieces of frog medullary cord in coagulated frog lymph, Drew (1912), Holmes (1914), Uhlenhuth (1915), and other early workers also cultivated anuran tissues in homologous plasma. In more recent years, a medium composed of chick embryonic extract and cockerel plasma has been commonly used for many purposes. However, the clot-forming media suffers from obvious disadvantages: (1) their composition is unknown and variable, and (2) it is difficult to reproduce exact conditions from one experiment to another.

Chemically defined media, now frequently used for various studies with mammalian tissues, are also available for amphibian tissue culture. The synthetic media designated as Earle's, Eagle's, Hanks', NCTC 109, Medium 199, or 1066 have been applied to amphibian cell culture by many workers. The mammalian-type media stimulate attachment and division of poikilothermic cells *in vitro*, but the number and quality of cells in culture gradually decline during subsequent cultivation. Reduction of the salt concentration of the medium, providing a more isotonic condition for amphibians, proved more effective in attaining better cell growth. We ordinarily employed Eagle's minimum essential medium (MEM) (Eagle, 1959), diluted with 25% double-distilled water and

enriched with 15% bovine serum and 5% whole egg ultrafiltrate or chick embryonic extract.

Such diluted mammalian-type media have been effectively utilized for several years in cultures of poikilothermic tissues. Auclair (1961) and Freed (1962) diluted 100 ml of NCTC 109 by adding 54 ml double-distilled water and enriched it with calf serum, chick embryonic extract, and lactalbumin hydrolyzate. Wolf and Quimby (1964) used NaCl-free Earle's balanced salt solution to dilute mammalian-type media 15%, 25%, and 35%. Their attempt revealed that a 25% or 35% dilution resulted in better growth for frog tongue cells. The medium used by Wolf and Quimby (1964) consisted of 55 ml Eagle's MEM, 25 ml NaCl-free Earle's BSS, 10 ml whole egg ultrafiltrate, 10 ml fetal bovine serum, and antibiotics.

The presence of chick egg ultrafiltrate (Microbiological Associates) or chick embryonic extract in the medium is particularly effective in securing vigorous cell growth from poikilothermic animals.

The medium, whose composition is given in Table II, was originally provided by Shah (1962). This growth medium was prepared by modifying Eagle's basal medium containing appropriately balanced salt concentrations (used by Barth and Barth, 1959, for frog embryos) enriched with calf serum and whole egg ultrafiltrate. The above medium has been preferentially used in our laboratory since it gave obviously

TABLE II

COMPOSITION OF AMPHIBIAN GROWTH MEDIUM AS MODIFIED FROM EAGLE'S BASAL MEDIUM[a,b]

Compound	Gram per liter
A. NaCl	5.150
KCl	0.075
$MgSO_4 \cdot 7H_2O$	0.204
$Ca(NO_3)_2 \cdot 4H_2O$	0.078
$CaCl_2$	0.045
B. Na_2HPO_4	0.030
KH_2PO_4	0.037
C. $NaHCO_3$	0.750
D. Lactalbumin hydrolyzate	5.000
E. Yeast extract	1.000

[a] From Shah (1962).

[b] Growth medium is made as follows: 82% above medium,[c] 5% whole egg ultrafiltrate, and 13% calf serum. Penicillin G 100,000 units, streptomycin sulfate 100 mg, Mycostatin 50,000 units, and 0.5 ml of 1% phenol red solution (Difco, TC) are added to 1000 ml of the medium. The pH 7.4 adjusted by one or two drops of 1 *N* NaOH or HCl.

[c] Sterilization is performed as shown in Table I.

better growth of adult amphibian tissue explants than the undiluted or arbitrarily diluted mammalian media.

G. Temperature of Incubation

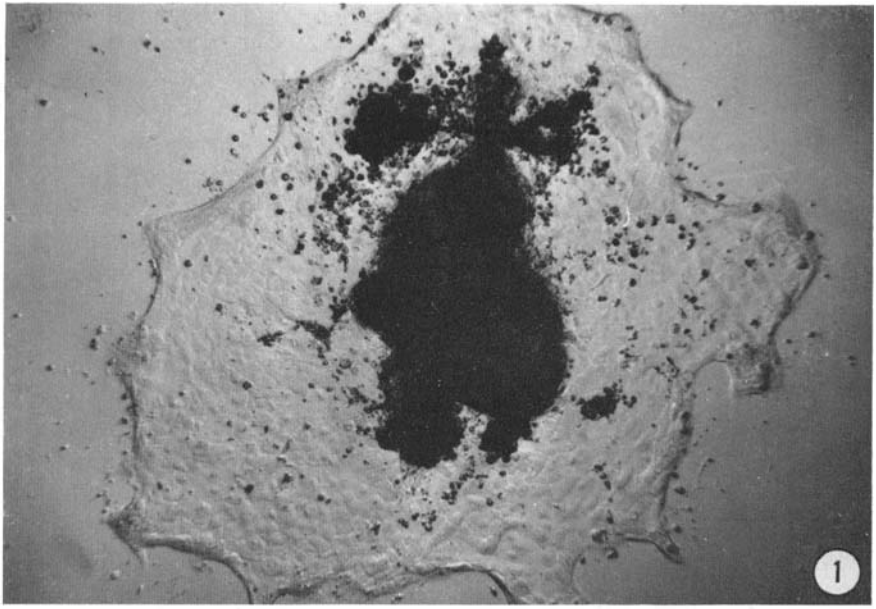
Cultures of amphibian tissue grow best at 26°C. Although the cells are viable at all temperatures from 5° to 37°C for a limited period of time, (Wolf and Quimby, 1964), they do not survive more than 1 week at 37°C (Stephenson, 1967). Most workers have incubated amphibian cells *in vitro* at various temperatures in the range of 18° to 26°C. Uhlenhuth (1914) and Cameron (1949) proposed that the optimum temperature for growth of amphibian cells *in vitro* is around 20°C, but our trials with the frog kidney cell strain and newt lung cultures showed the growth in a limited series of cultures to be definitely retarded below 22°C and inhibited at 37°C. If frog cell cultures are gradually adjusted from 26° to 37°C, with an elevation of 1° for each passage, they can adapt to the environment and survive, but the cultures demonstrate little cell proliferation at temperatures higher than 26°C, as indicated by Danes (1949). Recent studies repeatedly showed the upper limit of the optimal temperature range from amphibian cultures to be 26°C, with few exceptions.

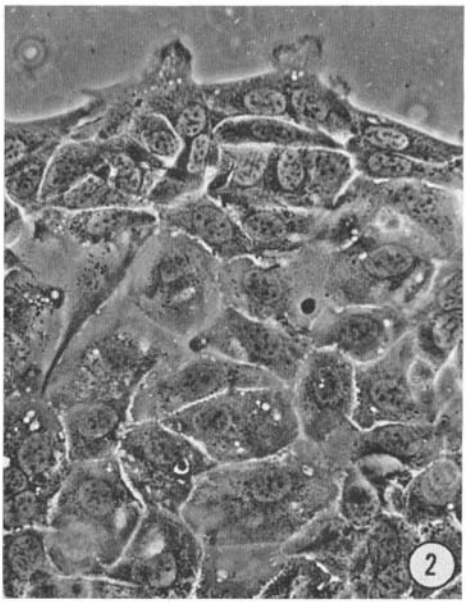
III. General Aspects of Cultured Cells *in Vitro*

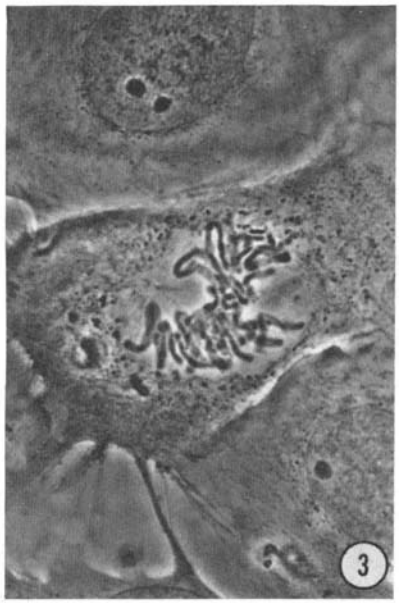
In work with the adult amphibians, frog kidney and, to a lesser degree, heart tissue may be cultivated by a variety of culture methods. For this reason, many cytologists working with amphibian tissue culture have successfully used the frog kidney (Wolf *et al.*, 1960; Auclair, 1961; Shah, 1962; and others). However, the cell size of anuran species is not as large as that of urodeles; consequently, newt tissues are recommended as a more favorable cytologist test object.

A. Urodele Pulmonary Cells

A common type of cell originating in cultures of newt lung is the epithelial-like cell. Under the dialysis membrane in the Rose chamber, the cells in the outgrowth become flattened to form a monolayer of pavement epithelium on the coverglass (Figs. 1 and 2). Within 6 or 7 days of cultivation, mitotic figures can be found with a frequency of less than 2%. The newt cell is recognized as a particularly suitable material for







FIGS. 1 to 3. Epithelial outgrowth from a fragment of pulmonary tissue of a newt (*Taricha torosa*), 15-day culture in the Rose chamber.

FIG. 1. Whole view of spreading sheet of living cells, light microscopy. × 45.

FIG. 2. A portion of Fig. 1, phase-contrast microscopy. × 200.

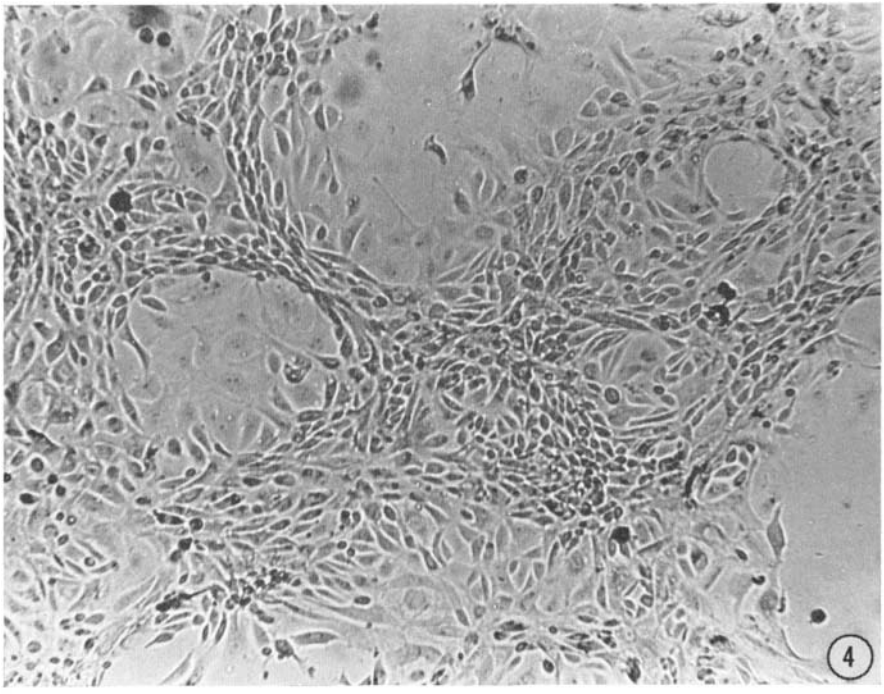
FIG. 3. Part of living culture which presents a mitotic cell. × 610.

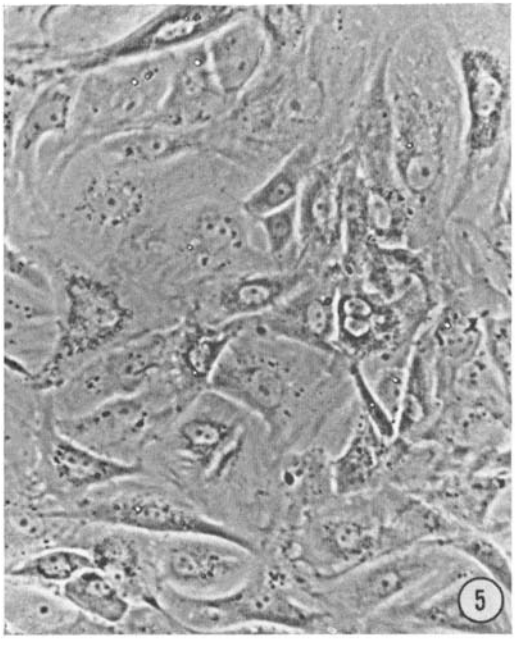
study of cell division, since the beginning of mitotic prophase is more distinguishable among interphase cells than is found in mammalian mitotic cells.

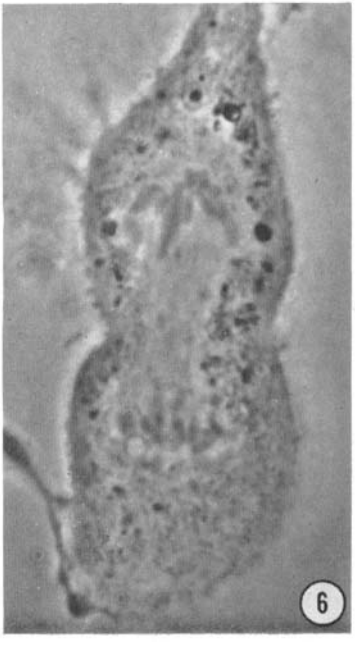
Nearly every mitotic figure can be observed with good resolution and structural detail with the phase-contract microscope due to the cellophane technique employed (Fig. 3). In cultures of lung tissue, most cells show two nucleoli, and normally binucleate or trinucleate cells appear in low numbers. Ciliated cells can be observed immediately after the cells migrate out from an explant if an appropriate region of the lung is utilized. Ciliary movements continue so long as the cell is alive under minimal culture conditions. This motion can serve as a relative indicator of physiological activity of the cell. Pinocytosis can be observed very clearly in peripheral cells of the epithelial pavement, and usually the activity increases in the older cultures. For pulmonary epithelium; the growth rate *in vitro* is rather slow, and usually primary cultures can be maintained for at least 3 weeks. If cells are left without any changes in culture conditions, they begin to gradually degenerate after a month of incubation.

B. Anuran Renal Cells

With the cold-trypsin digestion method, cells dispersed from frog kidney give rise to monolayer cultures, generally with an epithelioid morphology (Figs. 4 and 5). About one-third of the inoculated cells come into contact with the glass surface within 24 hours, and the cell population in a flask will remain constant for the initial 4 or 5 days. Mitotic figures appear on the fifth or sixth day of culture, and their frequency gradually increases up to about 2.0% at 26°C (Fig. 6). After 2 weeks, the rate of cell multiplication levels off although the persistence of a low frequency of mitotic figures and of floating, pycnotic cells suggests a continual turnover of the cell population. Subculture with trypsinization is advised after about 3 weeks, to prevent the cells from becoming too crowded in a flask. A large proportion of the cells may be injured by the enzyme (0.25% trypsin). When cells are dispersed, however, by utilizing the well-conditioned enzymatic mixture mentioned above for a minimum time and forcing the cell suspension through a fine pipette several times, it is possible to keep the cell damage at a low level. Cell population dilutions of either 1:2 or 1:3 are normally employed in each passage. During the first three passages, the cultures multiply slowly. Subsequently, if the culture is maintained in good condition, the number of cells increase progressively. It is interesting to note that if the cells in these passages are harvested with a rubber policeman when they are


4


5


6

transplanted, growth of the subcultures is markedly worse than if the trypsinization method is used.

IV. Leukocyte Culture of Amphibians

The use of the technique of leukocyte culture in mammalian species has brought about rapid advances in mammalian cytogenetics. However, the leukocytes of cold-blooded animals have been cultivated by only a few workers (Seto *et al.*, 1964; Jaylet, 1965; Volpe and Gebhardt, 1966; using amphibians, and Beçak *et al.*, 1962, Gorman, 1965, using reptilian sources). They have shown that it is definitely possible to secure metaphase mitotic figures from cultured leukocytes of poikilothermic vertebrates. For cytogenetic studies of amphibians, therefore, the leukocyte culture is a quite useful method, equal to its use in mammalian cytogenetics.

A. Culture Technique

The procedures of amphibian leukocyte culture are based fundamentally on the technique of the human leukocyte culture, which was originated in the late 1950's (Nowell *et al.*, 1958; Hungerford *et al.*, 1959; Moorhead *et al.*, 1960). However, the conventional method occasionally has the disadvantage that more blood is required than can be obtained from small animals such as salamanders and frogs. The following discussion describes techniques which were found to be successful for amphibian blood culture.

1. Inoculation of Separated Leukocytes

When relatively large-sized animals are used, such as *Ambystoma*, *Necturus* and toads, blood can be drawn from blood vessels. Large blood vessels, e.g., those of the arterial trunk and femoral artery, are exposed by careful dissection. A 27-gauge needle, having a sharp tip, is then injected into the vessel. Blood is slowly drawn into a 5-ml syringe con-

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Figs. 4 to 6. Epithelial-like cells prepared from frog kidney tissue (*Rana nigromaculata*).

Fig. 4. Living cells of 10-day culture in a T-30 flask, light microscopy. × 80.

Fig. 5. Renal cells in the third passage, phase-contrast. × 400.

Fig. 6. A mitotic anaphase cell in the third passage, phase-contrast microscopy. × 1000.

taining about 0.05 ml of heparin sodium (1000 U.S.P. units /ml). The heparinized blood is placed in a Kahn tube (7 mm diameter, 100 mm long), and allowed to stand for about 2 hours in a refrigerator (2°–4°C). The use of a small tube contributes to the ease of separation of white blood cells from small amounts of blood. Erythrocytes become agglutinated and settle to the lower part of the tube. About 1 ml of the supernatant suspension of leukocytes can be separated from the 3 ml of heparinized blood, which is aspirated into a syringe and transferred into a culture bottle. To 0.5–0.7 ml of the plasma, containing the leukocytes, 5.0 ml of culture medium and 0.2 ml of phytohemagglutinin M (PHA-M, Difco) are added. The nutrient fluid consists of 85% Eagle's basal medium made up in amphibian salt solution, with the sodium chloride at 0.65%, and enriched with 0.5% lactalbumin hydrolyzate, 10% calf serum, and 5% whole egg ultrafiltrate.

2. Inoculation of Whole Blood

In case the animal is not large enough to draw blood from a vessel, the amount of acquired blood is inadequate for the above method. Therefore, the technique which requires no separation of leukocytes from whole blood must be employed to avoid the loss of the mitotically competent cells.

By modifying the methods of Edwards (1962) and of Arakaki and Sparkes (1963), minimal amounts of whole blood from small animals can be cultivated. After animals are washed in the potassium permanganate solution and their body surface cleansed with 80% alcohol, the tip of a hind limb or tail is rapidly amputated with sterilized scissors. Blood is drained from the section into a culture bottle containing heparin (0.025 ml), phytohemagglutinin M (PHA-M) (0.2 ml), and the nutrient fluid (5 ml). An alternative source of blood is from the heart. A cardiac puncture is made by passing the needle through the upper abdominal wall and diaphragm into the heart using a 27-gauge needle attached to a 1-ml syringe wetted with heparin. However, this technique requires more experience and skill in order to insure success.

To 5 ml of the medium, about 0.1 ml to 0.2 ml of blood is directly inoculated into a T-15 flask. The blood and culture medium are mixed and placed in an incubator at 26°C. This method is less successful than the one described above, but has the advantages that: (1) very small amounts of blood are needed for each study, and (2) the procedure is simple, requiring no special handling or treatment of the blood sample prior to initiating the culture of the leukocytes. This technique is also useful in those situations where separation of the leukocytes from other

blood cells is difficult and minimizes the loss of leukocytes from the sample.

B. Mitotic Incidence in *Necturus* Leukocyte Cultures

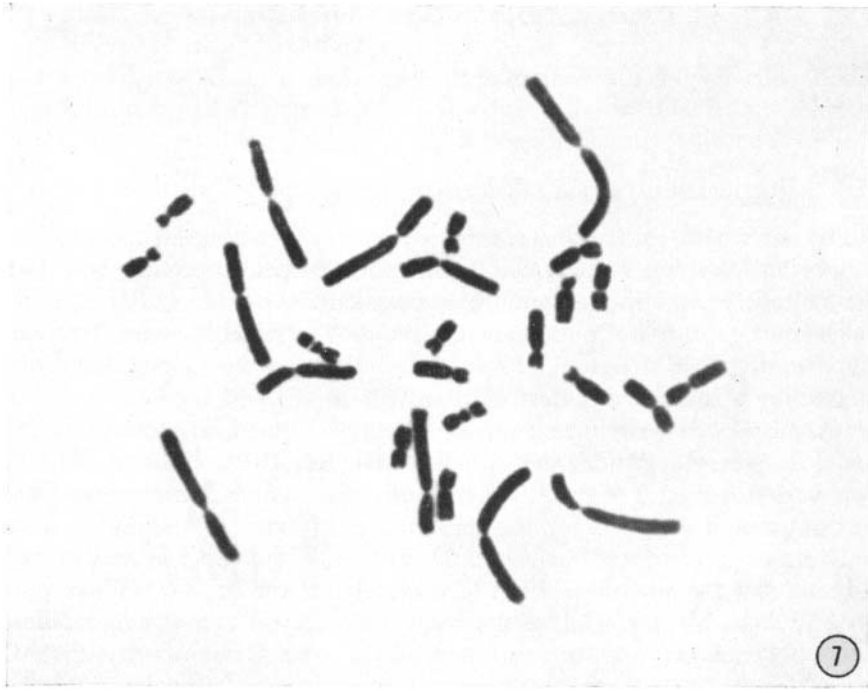
For successful cultivation, special attention must be paid to: (1) the proportionate volume of PHA-M to the total amount of medium and, (2) the culture period throughout the experiment.

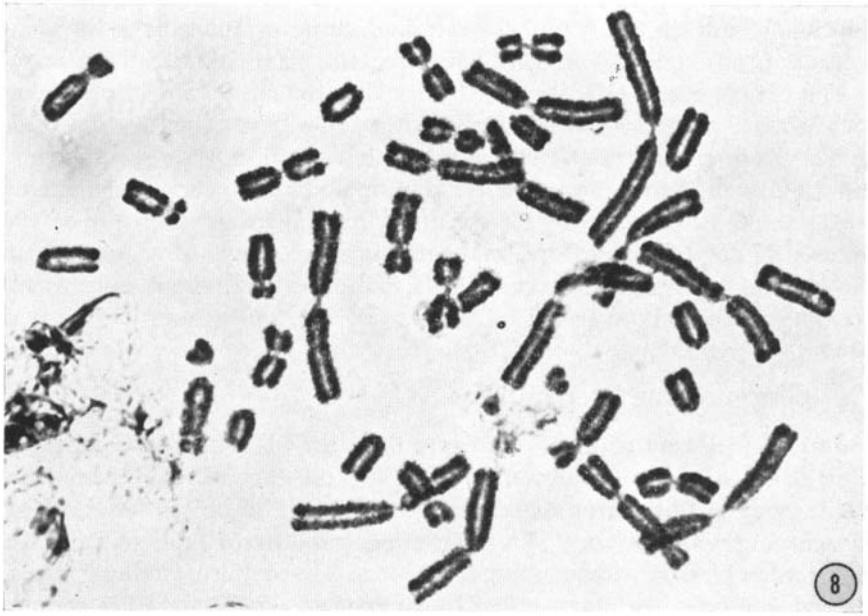
The concentration of phytohemagglutinin-M in the culture medium can significantly influence the mitotic index (Seto *et al.*, 1964). Cultures containing a lower concentration than 2.0% of PHA-M showed only few or no mitoses of leukocytes from the *Necturus* blood; 1.0 to 2.0% is the usual dose employed for mammalian leukocytes. The volume of PHA-M was varied from 1.0 to 6.0% in the total amount of medium and plasma, but satisfactory results for the production of leukocytic mitosis were only obtained at concentrations of 3.0 to 6.0%. From these figures it may be seen that the amount of PHA-M is closely related to the incubation of mitosis in leukocytes. The results suggest that there is a possible difference between mammalian and amphibian cells in sensitivity to this mitotic initiating factor. An increased concentration of colchicine was also required to produce an effect on the amphibian cell mitoses. Inhibition of mitosis at metaphase required three or four times the concentration of colchicine usually employed for mammalian cells *in vitro*.

The length of the culture period must also be considered in obtaining an adequate number of mitotic cells. Although 3 days of cultivation prior to the 24-hour treatment with colchicine is sufficient to secure some mitotic figures, 6 to 8 days of cultivation, including the time of colchicine treatment, will produce better results. Experience has shown that the mitosis of amphibian leukocytes begins within 4 days after setting up the culture at a temperature of 26°C, with the number of mitoses increasing in the following 3 or 4 days.

C. Chromosome Preparations

On the fifth day of the leukocyte culture, colchicine is added to the flask at a final concentration of 2×10^{-5} gm/ml; the cells are treated for 8 to 24 hours. The cells are then harvested from the bottom of the flask by scraping with a rubber policeman, transferred to a 10-ml conical tube, and centrifuged at 600 rpm for 5 minutes. The culture medium is discarded, the cells are resuspended in a hypotonic solution (1.12% sodium citrate solution), and allowed to stand at room temperature (20°–24°C)





FIGS. 7 and 8. Metaphase chromosomes obtained by tissue and leukocyte culture.
FIG. 7. *Rana nigromaculata* ♀, $2n = 26$, from the renal cell culture. Giemsa stain.
FIG. 8. *Necturus maculosus* ♀, $2n = 38$, from the 7-day culture of leukocytes. Acetic orcein stain.

for 30 minutes. The KCl solution (0.075 *M* in distilled water), described by Hungerford (1965), is also recommended as a suitable hypotonic solution for obtaining satisfactory spreads of chromosomes. A small amount of Carnoy's fixative (3 parts of absolute methanol and 1 part of glacial acetic acid) is added carefully to the hypotonic cell suspension so that cells are lightly fixed before centrifugation. The hypotonic solution is then replaced with a freshly made fixative. The pellet of cells must be dispersed into the fixative at once by gentle agitation with a pipette. After 30 minutes or more, the cells are centrifuged again and resuspended in fresh fixative. This is repeated twice to fix the cells completely.

Chromosome preparations are made by the air-drying method of Rothfels and Siminovitch (1958). Chromosomes are stained for 1 hour with acetic orcein (1% orcein in 50% acetic acid) or Giemsa's solution (Merck, diluted into 5% of the stock solution), dehydrated in 95% ethanol, then in two changes of absolute ethanol, and mounted in a synthetic resin (Hartman-Leddon Co.). Phase-contrast microscopy can be occasionally used for such preparations, which are lightly stained (Figs. 7 and 8). Carbol fuchsin stain (Carr and Walker, 1961) has also been favorably employed in our laboratory for the autoradiographic study of chromosomes.

If the harvested cells are fewer than the desired number, a silicone-coated tube and pipette should be used to prevent loss of the cells during the procedure of chromosome preparations. A squash technique (Makino and Nishimura, 1952) for the chromosome spreading is used on these occasions.

V. Conclusions

While amphibian tissues can be maintained with the more commonly used culture methods, the modifications summarized in this chapter have resulted in more optimal growth and cell survival. Several organ systems from anuran and urodele species have been successfully grown, but the kidney of the frog and the lung of the newt appear to be most satisfactory as starting materials for amphibian cell culture.

The relatively large cell size and the degree of resolution offered by the current technical developments in phase-contrast microscopy and photographic film permits the investigator to observe and record in satisfying detail, ciliary movement, mitochondria, and chromosomes. Under

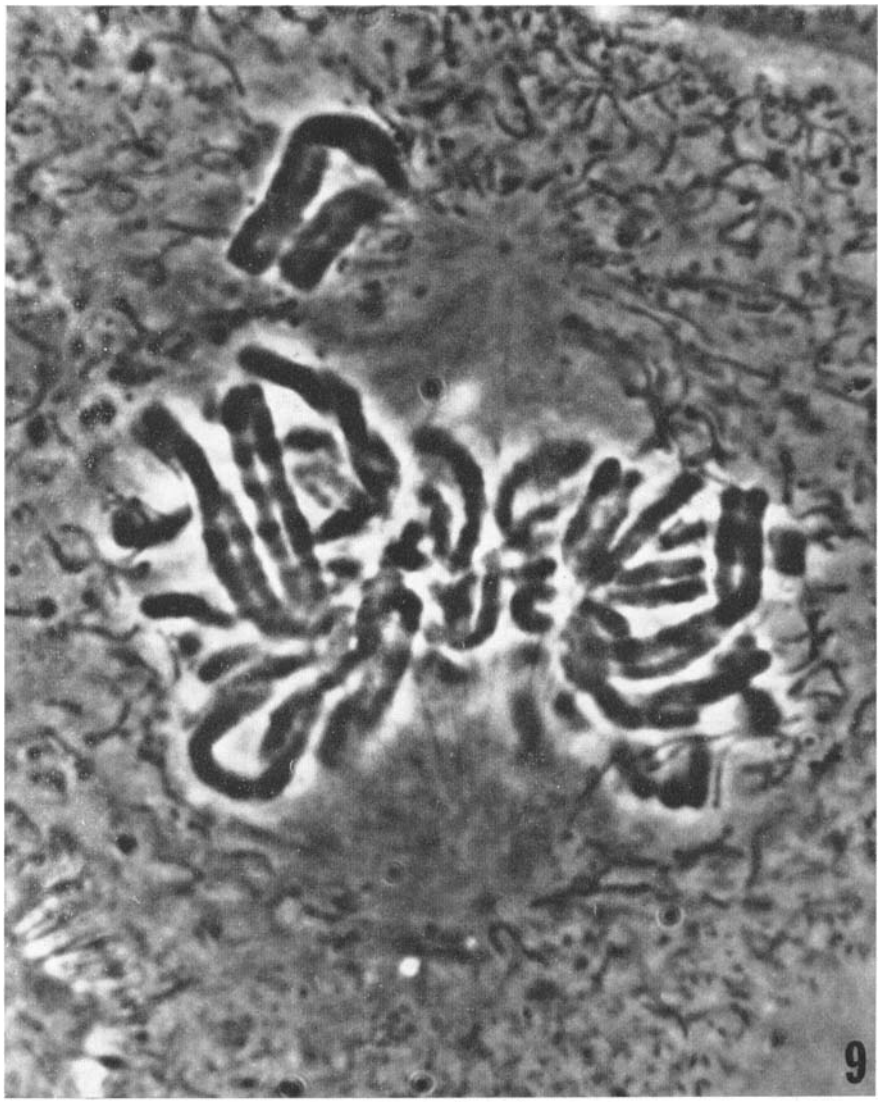


FIG. 9. A metaphase figure from a culture of the lung of the newt *Taricha granulosa*. Note the mitochondria, chromosomes, centriole, and spindle fibers. This photograph was provided through the courtesy of Dr. Y. Ohnuki, Pasadena Foundation for Medical Research, Pasadena, California.

special circumstances, the living amphibian cell can also demonstrate the centriole and spindle fibers within a mitotic figure (Fig. 9). It is hoped that, by relating these successes, more investigators will be encouraged to make use of amphibian tissues *in vitro*.

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Chapter 5

Experimental Procedures for Measuring Cell Population Kinetic Parameters in Plant Root Meristems[1]

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I. Introduction

This chapter is concerned with the determination of mitotic or cell cycle parameters with particular emphasis on cell population kinetics

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of plant root meristems. Earlier chapters have presented current concepts and models utilized in mitotic cycle work (Gelfant, 1966; Sisken, 1964; Thrasher, 1966) and they have also described autoradiography, an indispensable tool in the field (Caro, 1964; Miller *et al.*, 1964; Perry, 1964; Prescott, 1964; Prescott and Bender, 1964; Stevens, 1966; Wolff, 1964). Familiarization with these reviews should greatly enhance the reader's background and provide a sound foundation for understanding the present topic.

Even though the scope of this review has been deliberately limited to cell population kinetics of plant root meristems, application of information and experimental approach to other systems, plant or animal, that possess kinetics and cell population characteristics similar to that of a meristem are certainly feasible. The primary root meristem represents an asynchronous population of dividing cells that is evenly distributed throughout the mitotic cycle. The population remains in a quasi-steady state for a period of several days after germination until production of lateral roots has been accomplished. The primary meristem is not a bonified steady state system because the number of cells in the tissue diminishes with time after germination (Brown, 1951). The actual number of cells lost, however, is insignificant for kinetic studies if the experiments are of sufficiently short duration.

Current concepts and experiments on the mitotic cycle have their foundation in the model proposed by Howard and Pelc (1953) and the theoretical treatment of the model by Quastler and Sherman (1959) and Quastler (1963). The mitotic cycle as defined by Howard and Pelc consists of four parts: the G_1 or pre-DNA synthetic period, the S or DNA synthetic period, the G_2 or post-DNA synthetic period and mitosis. Figure 1 contains a diagram of the model.

The mitotic cycle and its interphase periods form a common reference point for many studies other than cell proliferation. Among these are studies on chromosome replication (Taylor *et al.*, 1957), chromosome aberrations (Evans, 1962; Wolff and Leoppold, 1964), and radiosensitivity (Terasima and Tolmach, 1963). Also, it is apparent that this common reference point will provide the conceptual and experimental framework for future work on cell multiplication.

Historically, the root meristem has provided both reliable and versatile cellular material for such studies. Even today these very characteristics are the reason why root tip material is still used to answer basic cytological questions.

II. Definition of Terms[2]

M	Mitosis composed of p, prophase; m, metaphase; a, anaphase; and t, telophase
S	Period of DNA synthesis
G_1	Postmitotic presynthetic period
G_2	Postsynthetic premitotic period
CT	Mitotic cycle duration ($T_m + T_{g_1} + T_s + T_{g_2}$)
T_m	Duration of mitosis
T_{g_1}	Duration of G_1
T_s	Duration of S
T_{g_2}	Duration of G_2
T_p	Duration of prophase
N_{ct}	Number of cells in the mitotic cycle; proliferative cells
N_p	Number of cells in prophase
N_{tr}	Number of cells in transition from proliferative to mature
N_f	Number of functional (mature) cells
N_t	Number of cells in the 2-mm terminal portion of the root ($N_{tr} + N_f + N_{ct}$)
$T\text{-}H^3$	Tritiated thymidine

III. Model of a Hypothetical Root Meristem

A meristem is composed of at least two large cell populations and perhaps many smaller subpopulations. The diagram in Fig. 1 considers only the two largest categories or populations—meristematic or proliferative and nonproliferative. The number of proliferative cells is represented in Fig. 1 by the rectangle with an area described by $(\overline{AB}/2)(\overline{AD})$; the nonproliferative population is described by $(\overline{AB}/2)(\overline{BC})$. The total number of cells in the meristem (N_t) is $(\overline{AB})(\overline{BC})$.

Because of population heterogeny it is necessary to estimate the relative size of the proliferative and nonproliferative populations if kinetic parameters are to be measured. The reason for this becomes apparent if one considers two hypothetical proliferative populations; one consisting of 4 cells and the other of 8 cells. Each population has a mitotic cycle duration of 4 hours. After one complete cycle the former would produce

[2] Based more or less after Quastler (1963).

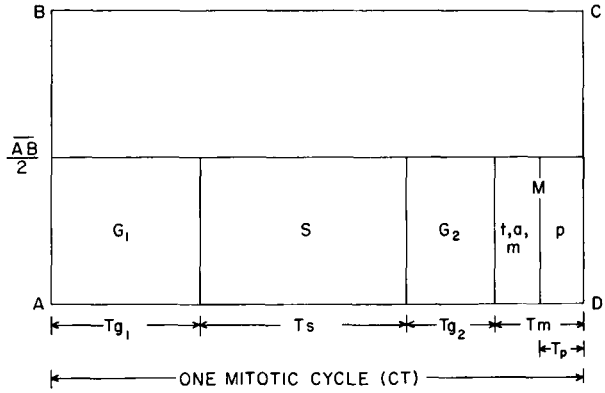


FIG. 1. A diagrammatical representation of a hypothetical root tip meristem composed of two cell populations. The entire meristem is represented by an area described by the product of lines $\overline{AB}$ and $\overline{BC}$; a nonproliferative population by $\overline{AB}/2$ and $\overline{BC}$, and a proliferative population by $\overline{AB}/2$ and $\overline{AD}$. Other symbols are identified in the definition of terms.

4 new cells—an average of one cell per hour; the latter would produce 8 new cells—an average of 2 cells per hour. The rate of the latter is twice that of the former even though the cycle durations are equivalent. This relationship can be expressed as,

$$k = N_{ct}/\mathrm{CT} \tag{1}$$

The rate of cell production (k) is a function of two parameters, the number of proliferating cells (N_{ct}), and the mitotic cycle duration (CT). Attention will be focused first on the denominator in Eq. (1)—the mitotic cycle duration.

IV. Determination of the Mitotic Cycle Duration

A. The Use of Colchicine and Induced Tetraploid Cells

In pea root meristems and in a few other species the mitotic cycle duration can be accurately measured with the use of colchicine (Van't Hof *et al.*, 1960; Van't Hof and Sparrow, 1963a; Van't Hof, 1967). The technique involves the production of a small population of tetraploid cells by the cytological action of colchicine in meristems composed of diploid cells. (Because the colchicine-induced tetraploid cells constitute

a distinguishable group among diploid cells, they are labeled not in a radioactive sense but morphologically. Therefore, in the course of the discussion a cell may be described as labeled if it is distinctive.)

When roots of seedlings are immersed in an appropriate concentration of colchicine for a short period of time, cells that enter mitosis during the time of colchicine effectiveness will not possess a functional spindle. The result is generally one in which the chromosomes clump together and remain in this configuration during kinetochore cleavage and uncoiling (Hadder and Wilson, 1958). The affected cells enter interphase in normal fashion with a minimum of delay. Examples of these events are shown in Fig. 2. The resultant nucleus has a 4C DNA content and, when chromosome replication is completed, produces a tetraploid mitotic figure at the next division. The difference between the colchicine-induced tetraploid and diploid cells in mitosis is demonstrated in Fig. 3. The time between colchicine treatment and the appearance of the tetraploid cells in the subsequent mitosis is equivalent to one mitotic cycle. Figure 4 depicts idealized data obtained from an experiment utilizing the induced tetraploid method. It is important to emphasize that success depends upon a rapid but relatively short duration of colchicine effectiveness. Therefore time devoted to determine the most satisfactory combination of concentration and treatment time is well spent for each new species used as experimental material. A good rule to remember is that clumped metaphases should be absent when the majority of tetraploid cells are in division. Investigation of various combinations of concentration and treatment times will reveal that the tetraploid population size can be regulated experimentally (Van't Hof *et al.*, 1960).

Other suggestions that may be useful are listed below.

(a) Purchase colchicine of highest purity and assay its effectiveness by obtaining a recovery curve for each concentration. The recovery curve in Fig. 4 is represented by a gradual decline of clumped metaphases with time after the maximum effect.

(b) Remember that all plant cells and all plant species do not respond to colchicine in precisely the same manner and to the same degree.

(c) Excessive colchicine concentration or treatment will cause excessive metaphase delay (Murin, 1964) and thus increase the mitotic cycle duration; the ideal case is when little or no delay occurs as in pea root meristem cells.

(d) Always use a fresh colchicine solution for each experiment.

Another colchicine method has been mentioned by Thrasher (1966). This method utilizes the rate of metaphase accumulation to estimate the cell cycle duration and has been described in detail for plant (Evans

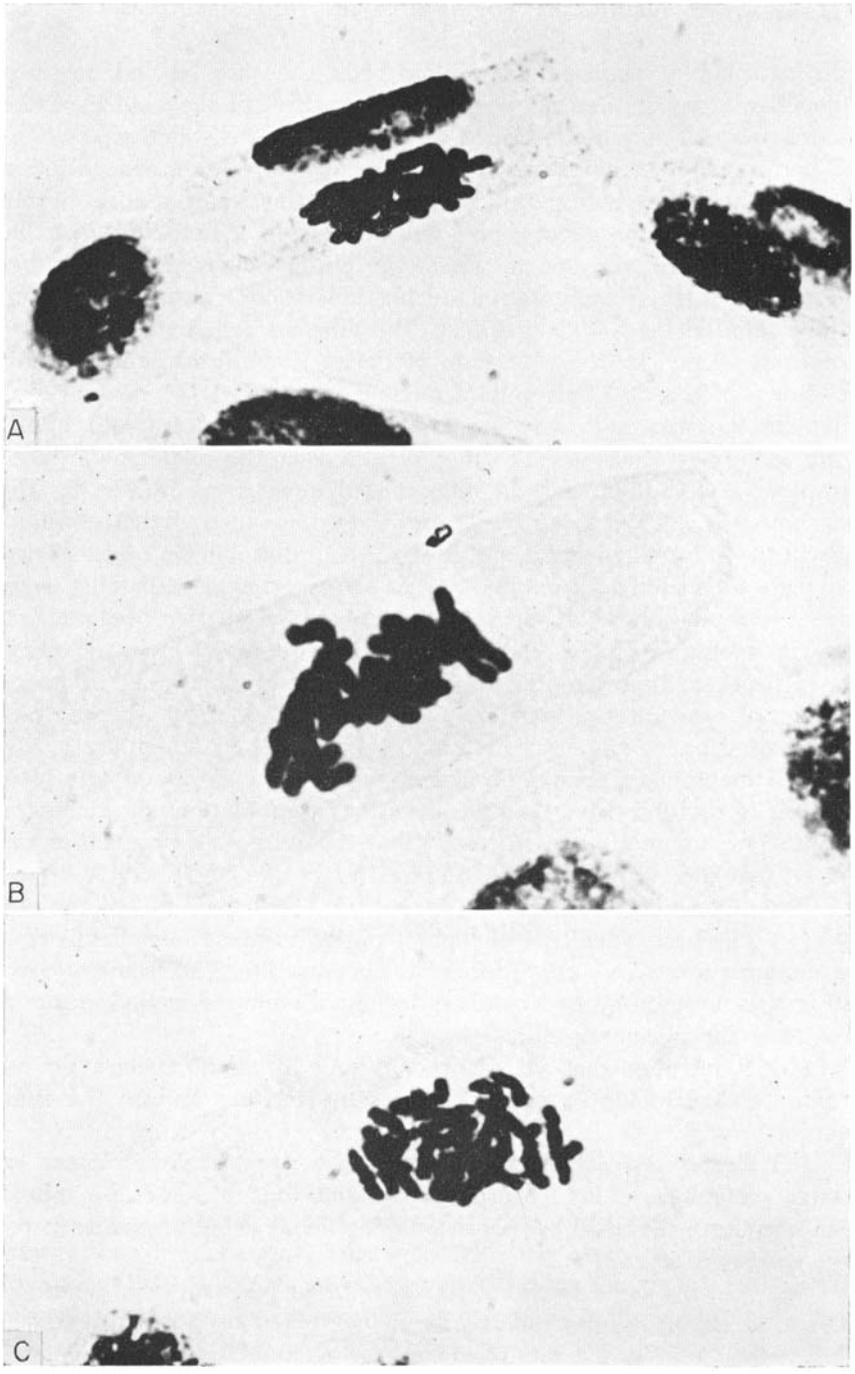


FIG. 2
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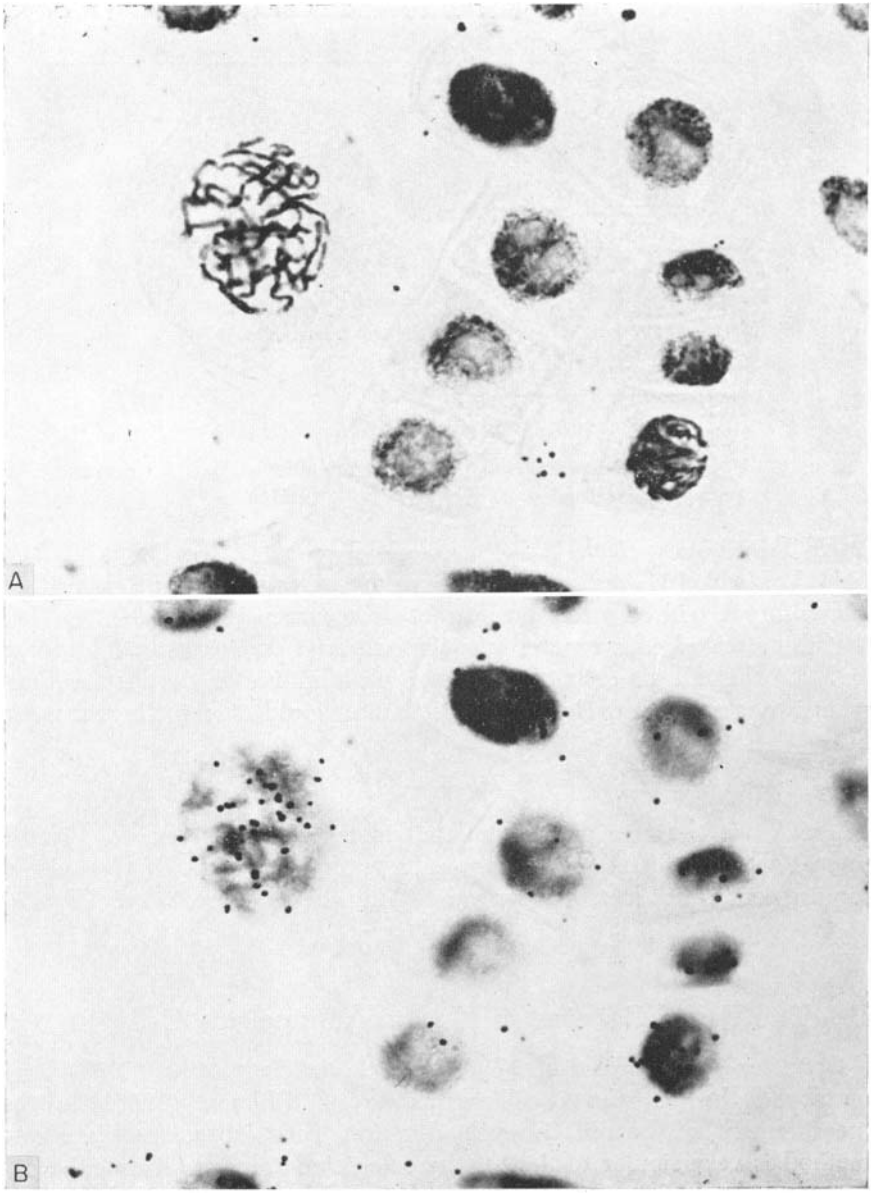


FIG. 2. (A), (B), and (C). Affected metaphase cells of *Pisum* produced by the action of colchicine. Such cells sometimes are referred to as clumped metaphases or "C" mitotic cells. (A), is the younger; (C), is the older clumped metaphase. These cells will be tetraploid the next time they divide (See Fig. 3A.)

FIG. 3. (A), A colchicine-induced tetraploid cell and a diploid prophase cell in the same meristem. (B), Tetraploid and diploid cells labeled with tritiated thymidine.

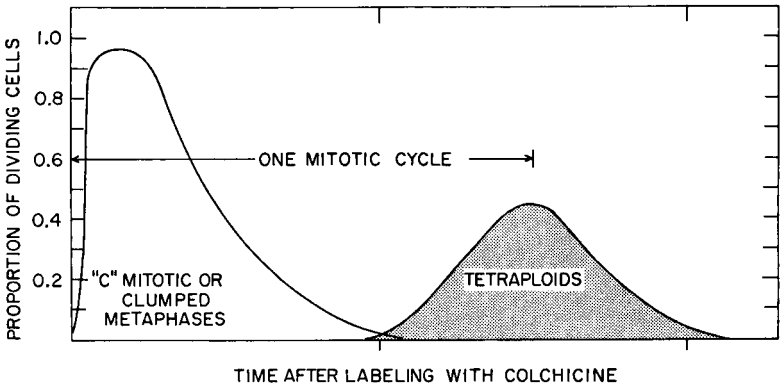


FIG. 4. Hypothetical experimental data obtained after a "pulse" treatment with colchicine. For actual data see Van't Hof and Ying (1964).

et al., 1957; Neary *et al.*, 1959) and animal cells (Leblond and Walker, 1956; Leblond, 1959). Because it has been adequately covered no further mention of it will be made except that its accuracy is dependent on (a) that the affected cells remain in metaphase and do not enter interphase and (b) that all the cells observed are proliferative. If the first requirement is not met, the cycle duration estimate would exceed the real value by

$$k_{\text{in}}/(k_{\text{in}} - k_{\text{out}})$$

where k_{in} is the rate of accumulated metaphase cells and k_{out} is the rate at which affected metaphase cells enter interphase. If the second requirement is not met, the cycle duration will be overestimated by

$$N_{\text{ct}} \text{ assumed}/N_{\text{ct}} \text{ actual}$$

B. The Use of Caffeine and Induced Binucleate Cells

Gonzalez-Fernandez *et al.* (1966) and Lopez-Saez *et al.* (1966) have employed the binucleate cell technique of Kihlman (1955) to the measurement of the mitotic cycle duration. In a sense, the binucleate method is very similar to that of the colchicine-induced tetraploid technique in that both act on cells at mitosis and both label cells by alteration of a nuclear characteristic (Figs. 3 and 5).

The general methodology is one in which an onion bulb with several roots is immersed in a 0.1% caffeine solution for 1 hour, removed, washed, and returned to tap water. At hourly intervals, samples are taken from the treated roots. Microscopic analysis of the root tips shows a phasic appearance and disappearance of binucleate cells in mitosis. The time

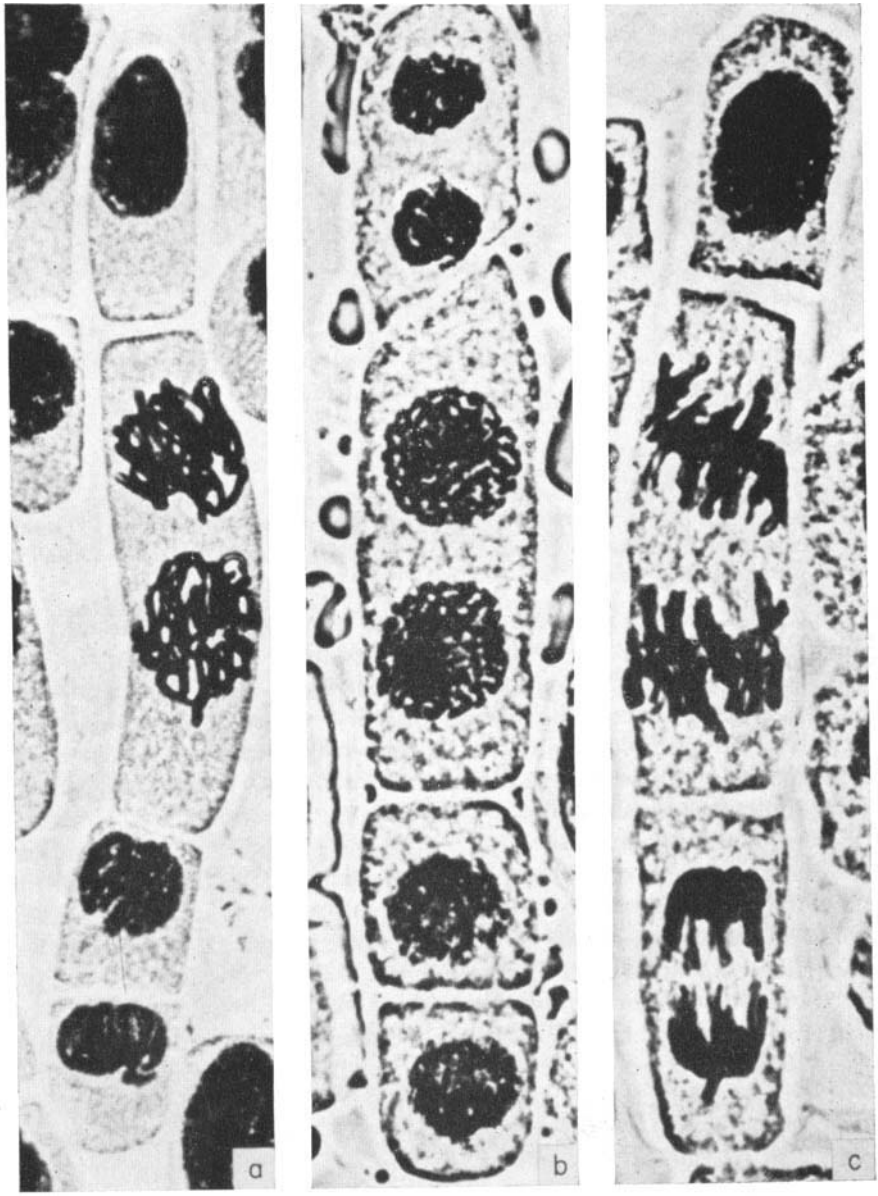


FIG. 5. Binucleate cells produced by the action of caffeine. (a), Biprophase; (b), early biprophase; (c), bimetaphase. (From Gonzalez-Fernandez *et al.*, 1966; published with permission of Academic Press, New York.)

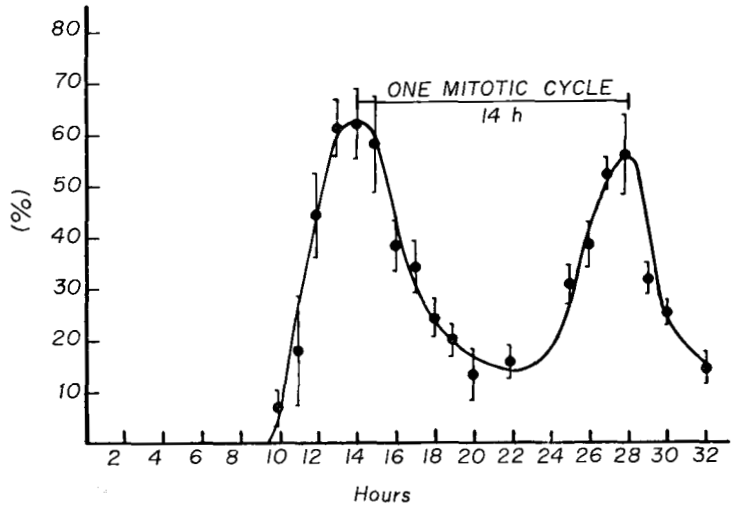


FIG. 6. The rhythmic appearance and disappearance of binucleate cells in mitosis. The time interval between successive maxima is a measurement of the average mitotic cycle duration. (From Gonzalez-Fernandez *et al.*, 1966; published with permission of Academic Press, New York.)

between the maxima of sequential mitotic appearances of marked cells provides an estimate of the mitotic cycle duration (Fig. 6). Of significance is the fact that neither the colchicine-induced tetraploid cells nor the caffeine-induced binucleate cells have a cycle duration longer than that of their diploid neighbors (Gimenez-Martin *et al.*, 1966) within a given species. Since the mitotic cycle duration is a function of cellular DNA content as demonstrated by comparative experiments with several different plant species (Van't Hof, 1965), the reliability of the tetraploid and binucleate cell techniques must be attributed to the fact that the induction of a binucleate or tetraploid cell not only produces an increase in DNA content but also a proportional increase in the chemical machinery associated with DNA synthesis and cell division (Van't Hof, 1965, 1966). To date, the binucleate method has proved useful in temperature (Lopez-Saez *et al.*, 1966) and radiomimetic drug studies (Kihlman, 1955). If it can be combined with T-H^3, its usefulness will be further enhanced, as in the case of the colchicine-induced tetraploid method (Van't Hof and Ying, 1964; Van't Hof, 1966).

C. The Use of Cell Counts

Brown and his collaborators (Brown, 1951; Brown and Broadbent, 1950; Brown and Rickless, 1949) some 19 years ago described a technique

to determine the mitotic cycle duration in plant root meristems from successive cell counts obtained at 24-hour intervals. Cell separation was accomplished by chromic acid treatment and the meristematic cells (N_{ct}) were indentified by the absence of a vacuole. The total number of cells per meristem at time zero (N_{t0}) was subtracted from the number obtained at 24 hours (N_{t24}). Then, $[(N_{t24}) - (N_{t0})]/N_{ct}$ = number of divisions per 24 hours per meristematic cell (k_{24}). If k_{24} is >1, each meristematic cell produced more than one new cell in 24 hours and the cycle is less than 24 hours; if it is <1, then, not all cells divided and the cycle duration is greater than 24 hours. Thus,

$$(1/k_{24})(24) = \mathrm{CT}$$

Brown's published CT values for *Pisum* have been substantiated by the colchicine-induced tetraploid and T-H^3 methods to a large degree (Van't Hof and Sparrow, 1963b; Van't Hof, 1966).

V. Determination of the Mitotic Cycle and Its Periods—The Use of Tritiated Thymidine

The most popular and most versatile method used to measure the mitotic cycle is that of Quastler and Sherman (1959). They described the use of tritiated thymidine for cell population kinetic experiments. This method, as with colchicine, assumes that the cells are asynchronous, equally distributed throughout the cycle, and in a steady state. When T-H^3 is made available only those cells that are actively synthesizing DNA incorporate the isotope. Such cells are those of the S period as depicted in Fig. 1. The T-H^3 labeled cells do not remain in the S period but proceed through G_2 and enter mitosis. The use of autoradiography provides a method for distinguishing these cells as they enter and exit from mitosis. Figure 7 represents a typical curve of T-H^3 labeled mitotic figures as a function of time after labeling. Such a curve is extremely useful since it provides an estimate of total cycle time, G_1, S, and $G_2 + M$ durations. Generally the mitotic cycle duration is taken to be the interval of time between two successive ascending portions of the curve as shown in Fig. 7. One-half the maximum proportion of labeled division figures intercepts of the rising and descending portions of the first wave in the curve are used to estimate $T_{g_2} + T_m$, and T_s. $T_{g_2} + T_m$ is equivalent to the interval from zero hour to the time when the ascending portion is one-half the maximum. T_s is the interval between the ascending and descending portions of the curve at the one-half maximum value.

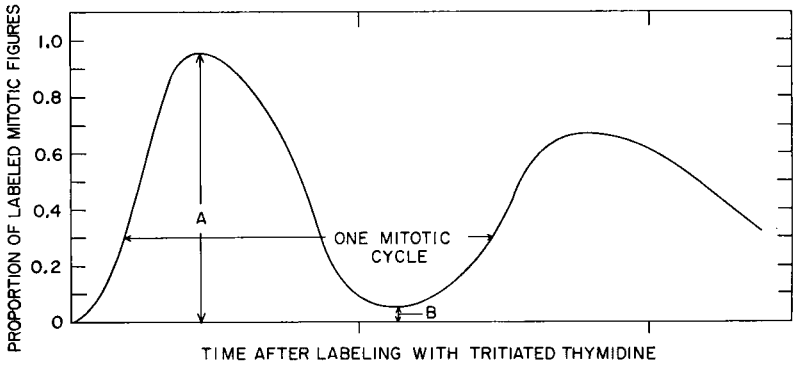


FIG. 7. A hypothetical curve representing the typical rhythmic appearance and disappearance of labeled mitotic figures following a pulse label with tritiated thymidine. In many cases, A and B are related in that with a higher A, a higher B is observed.

T_{g_1} is determined by the difference when the sum of T_{g_2}, T_m, and T_s is subtracted from CT and therefore is measured with least precision.

Measurement of T_m can be obtained when the increase in T-H^3 labeled prophase and metaphase cells is expressed as a function of time after labeling (Fig. 8). Theoretically the curves should be parallel but displaced in time if cell entry rate into prophase and metaphase are equivalent. The displacement is approximately the time required for a cell to pass through prophase (T_p in Fig. 8). If the frequency of prophase cells is proportional to the duration of prophase then the frequency of mitotic figures (all phases) is proportional to the duration of mitosis (T_m).

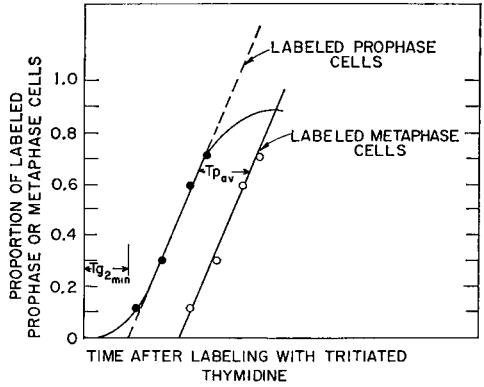


FIG. 8. The sequential appearance and increase of labeled prophase and metaphase cells after exposure to tritiated thymidine. From these data the minimum T_{g_2} and the average T_m can be determined.

The minimum of T_{g_2} can be estimated by the interval between the beginning of labeling (zero hour in Fig. 8) and the point of interception between the abscissa and the extrapolated labeled prophase curve. It is also possible to estimate the median T_{g_2} by expressing the change in slope of the curve representing the proportion of labeled prophase cells as a function of time (the smooth S-shaped curve in Fig. 8). The hypothetical curve of the change in slope as a function of time is shown in Fig. 9. The curve should reflect the distribution of T_{g_2} in time. The median T_{g_2} for G_2 cells is indicated by the maximum in the curve of Fig. 9. The maximum T_{g_2} can also be estimated for it is equivalent to the time required to obtain all labeled prophase cells.

As mentioned earlier, T_{g_1} can be estimated with T-H^3 by difference if CT, T_{g_2}, T_m, and T_s are known but this estimate lacks precision since it reflects the accumulated variances and errors of the other periods. Absolute measurement can be obtained, however, with the combined use of T-H^3 and colchicine—at least for those species with which colchicine can be utilized. This method will be discussed in the following section.

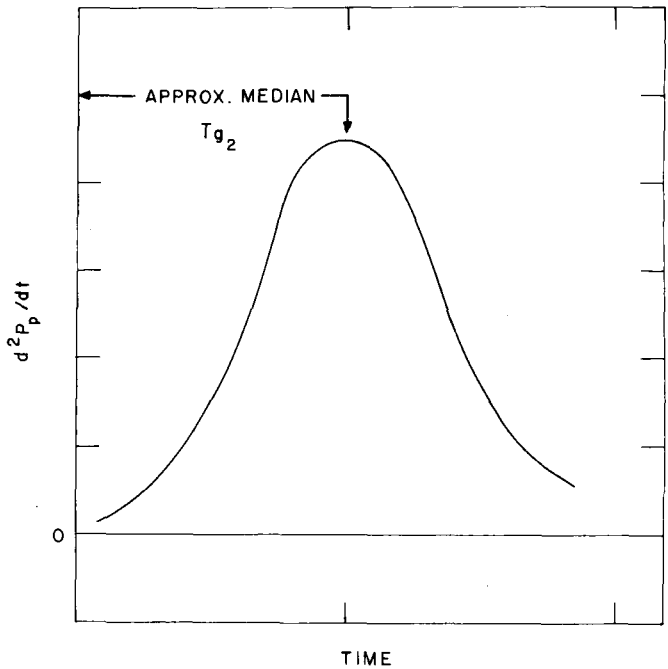


FIG. 9. A hypothetical curve representing the change in slope (rate) of the S-shaped labeled prophase curve shown in Fig. 8. The time at which the curve maximizes approximates the median T_{g_2}.

VI. The Combined Use of Colchicine and Tritiated Thymidine as Cell Markers

Because T-H^3 and colchicine selectively mark or label cells in two different periods of the mitotic cycle they can be used either simultaneously (Van't Hof and Ying, 1964) or at varying intervals (Van't Hof, 1966) for mitotic cycle studies. As shown in Fig. 10, T-H^3 marks cells that are synthesizing DNA and occupy the S period while colchicine affects cells in metaphase. The number of cells labeled by T-H^3 is dependent primarily on the number of cells in S at any given time while the number of cells marked by the action of colchicine is mostly a function of the rate of cell entry into mitosis and the duration of the colchicine effect (Van't Hof and Ying, 1964). The lower part of Fig. 10 illustrates the results of an experiment in which an asynchronous population of cells was labeled simultaneously with T-H^3 and colchicine. Observing the cells as they enter mitosis shows that the majority of the T-H^3 labeled cells divide when the colchicine-induced tetraploid cells are in interphase and vice versa. Figure 10 also demonstrates how the

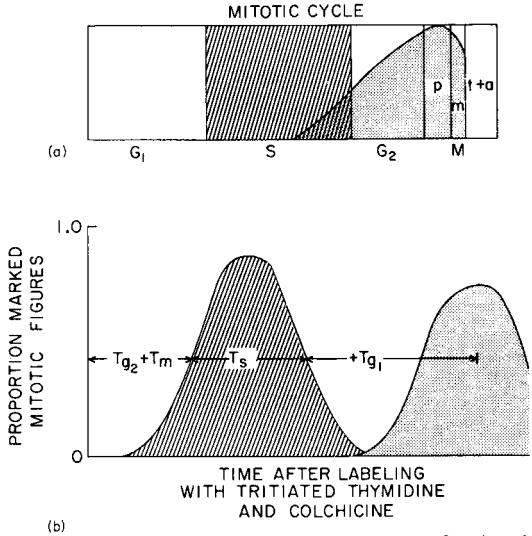


FIG. 10. (a), A diagrammatical representation of cells labeled with tritiated thymidine (hatched) and with colchicine (shaded) when labeling is simultaneous. (b), A representation of the rhythmic appearance of tritiated-labeled and colchicine-induced tetraploid cells following simultaneous labeling. See definition of terms for symbol identification.

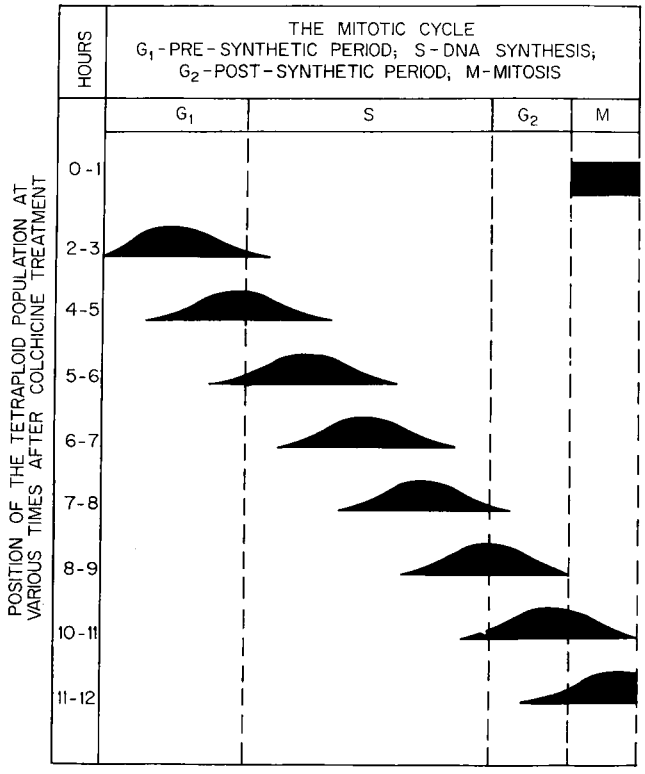


FIG. 11. A schematic drawing describing the mitotic cycle position of a colchicine-induced tetraploid population at various times after formation. (From Van't Hof, 1966; used with the permission of Academic Press, New York.)

period durations of the cycle can be obtained in such an experiment. Actual experiments of this nature have been performed with several plant varieties and species (Van't Hof, 1967).

The combined use of T-H^3 and colchicine also provides a method for measuring the minimum T_{g_1} directly (Van't Hof, 1966). This can be done by proper spacing of colchicine and T-H^3 treatments. Figure 11 describes in pictorial terms the experimental design. If colchicine treatment is followed immediately by T-H^3, no T-H^3 labeled tetraploid cells are expected because they occupy the G_1 period of the cycle. As the interval of time between colchicine treatment and exposure to T-H^3 increases the number of T-H^3 labeled tetraploids will also increase. A maximum of T-H^3 labeled tetraploid cells will be observed when T-H^3 is available at the time the tetraploid population is in the mid-S period. After the

tetraploid population has passed the mid-S period point, the number of T-H^3 labeled cells will decrease.

If it is assumed that the area under the tetraploid population curve that is located in the S period segment of the cell cycle is proportional to the number of T-H^3 labeled tetraploid cells, a plot of this area (A) versus the time interval between colchicine and T-H^3 treatment (t) should form a curve which has a maximum and which extrapolates to a time value (T_{g_1}) equivalent to the G_1 duration. The time at which A is maximal would be the mid-S period and T_{g_1} intercept would be the G_1 duration of the tetraploid cells. The latter would be an absolute value as compared to most T_{g_1} values which are obtained by difference. A hypothetical curve of A versus t is shown in Fig. 12. Because the tetraploid cells can occupy any period of the cycle, the technique provides a possible path of measuring events in G_1, S, and G_2 as well as the effects of various agents on these processes.

Thymidine labeled with H^3 or C^{14} has been used in double marking or labeling techniques (Wimber and Quastler, 1963; Pilgrim and Maurer, 1965; Pilgrim *et al.*, 1963). The method, however, has been discussed (Thrasher, 1966) and therefore only will be mentioned here.

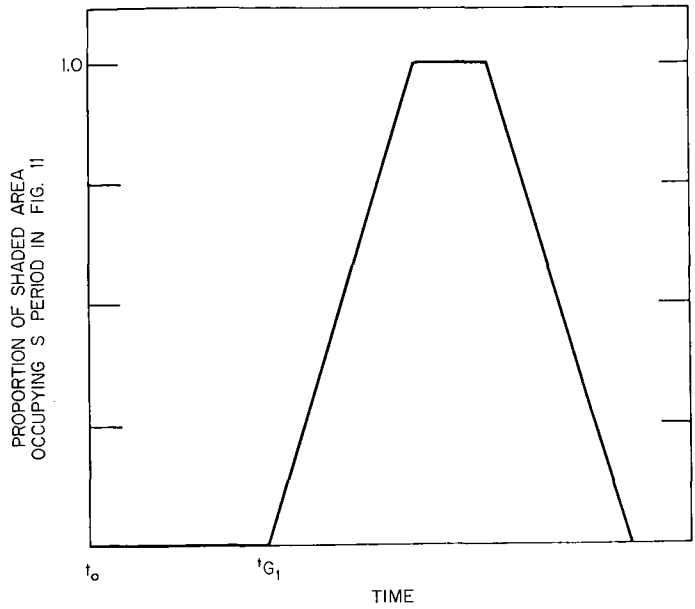


FIG. 12. A hypothetical curve representing the number of colchicine-induced tetraploid cells labeled with tritiated thymidine at various times after colchicine treatment. (From Van't Hof, 1966; used with the permission of Academic Press, New York.)

VII. Determination of the Number of Proliferating Cells

Earlier in the chapter it was stated that the rate of new cell production was inversely related to the cycle duration (CT) and directly related to the number of cells in the cycle (N_{ct}). Attention will now be focused on the latter parameter.

Perhaps one of the more difficult problems in the field of cell kinetics is obtaining a reasonably accurate estimate of the number of actively proliferating cells in a complex tissue. In this regard, the meristem is not unique since it has cells that are proliferative, quiescent (Clowes, 1964), and differentiated. However, given a number of assumptions a reasonable approximation of N_{ct} can be made. For pictorial representation of the situation Fig. 1 must be consulted again. If all the cells in a meristem are placed in two categories—those in some period of the mitotic cycle or proliferative (N_{ct}) and those not proliferative ($N_{tr} + N_f$) then a cell count of a 2-mm root tip (N_t) gives,

$$N_t = N_{ct} + N_{tr} + N_f$$

In Fig. 1,

$$N_t = (\overline{AB})\,(\overline{BC})$$
$$N_{ct} = (\overline{AB}/2)\,(\overline{AD}) \quad \text{and}$$
$$N_{tr} + N_f = (\overline{AB}/2)\,(\overline{BC})$$

Further study of Fig. 1 shows that

$$CT = \overline{AD}$$

and that the duration of prophase is T_p. Previously it was demonstrated how CT and T_p can be measured and the proportion of cells in prophase (N_p/N_t) can be obtained by microscopic analysis of the meristem. Assuming that the cells are evenly distributed throughout the mitotic cycle, the proportion of cells in the cycle can be estimated by,

$$N_{ct}/N_t = (CT/T_p)(N_p/N_t)$$

or for an estimate of the number of cells,

$$N_{ct} = (CT/T_p)(N_p/N_t)(N_t)$$

CT/T_p is the number of prophase durations in a cycle time. N_t was not cancelled out in the above equation because it was desired to present

the date in actual form as obtained from a cell kinetic experiment. N_{ct} can be obtained in the same fashion using any part of the cycle provided the proportion of the cells in a given compartment is equivalent to the duration of that period or part of the cycle. In these and similar calculations the basic assumption is that the passage of cells through any given point in the cycle proceeds at a constant rate and the cells are evenly distributed throughout the cycle.

The end result of cell proliferation within a tissue is growth and therefore an expected effect of altered proliferation is altered tissue growth. The relationships between cell and growth kinetics have been studied within and between plant species (Van't Hof, 1967) and after irradiation (Van't Hof and Sparrow, 1965). These few experiments are examples of what can be done and offer a hint of what may be done in the future when cell and growth kinetics are further combined with physiological and cytochemical studies.

VIII. Detailed Methodology

The following is a step by step description of the method used to label cells with colchicine and/or T-H^3 and is detailed to the extent that it can be a useful laboratory guide. Figure 13 shows a typical experimental set up. It consists of the following items.

(a) Germinated pea seedlings. The seeds were placed in very moist, sterile vermiculite (glass-distilled water is used exclusively) 72 hours before the photograph was taken. Germination occurred at 23°C.

(b) Germinated pea seedlings suspended in culture medium. The seedlings are suspended on ¼ inch wire mesh above a glass container filled with full strength Hoagland's nutrient medium with a pH of approximately 5.2. The formula for the nutrient solution is listed in Table I. The wire mesh has been plastic coated to prevent heavy metal poisoning.

(c) Source of aeration. In this case a small vibration air pump, air manifold, and several jets are pictured. Serum tubing connects the jet and bubbler (an extended piece of glass tubing) which is immersed in the nutrient solution. Aeration is never vigorous, just continuous and gentle.

(d) A container with T-H^3 and colchicine (100–250 mg/liter for *Pisum*) dissolved in nutrient solution.

(e) A container with nutrient solution to be used as a wash after labeling.

After equilibrating the seedlings in nutrient solution overnight at a

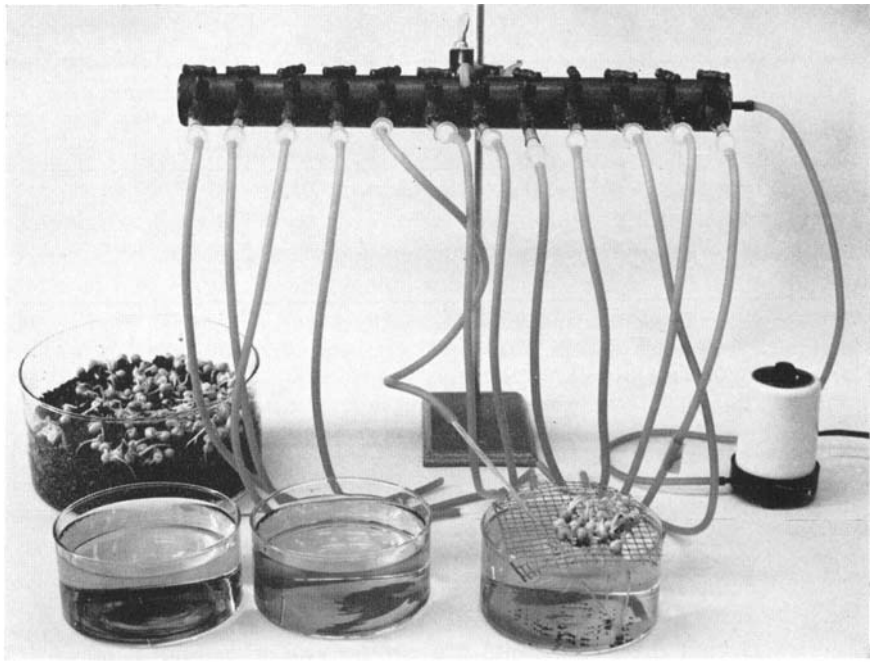


FIG. 13. A typical set up for an intact pea root experiment. Liquids in foreground from left to right, nutrient solution with either tritiated thymidine or colchicine or a mixture of each, a nutrient solution wash, and nutrient solution in which the seedlings are grown. For other information see text.

given temperature, they are removed, in total, by lifting the wire mesh and placing it on the container with T-H^3, colchicine mixture. After 30 minutes, the seedlings are again removed, in total, and placed in the wash solution for a few minutes and then returned to the original container with nutrient solution. Rapid and efficient movement of seedlings is obviously the result of being suspended on the wire mesh. Following each transfer, the air bubbler is also shifted to maintain constant aeration. Three to six seedlings are removed per time interval for sampling purposes. Samples usually consist of the terminal 1 cm of the root. The additional tissue allows handling of the root tip without touching it. After removal of the root tip from the seedling, the material is fixed in 3 parts absolute ethanol and 1 part glacial acetic acid. The roots are fixed at room temperature within 30 minutes to an hour; if they are to remain in the fixative for a longer period of time, place them in a refrigerator (about 4°C). If the meristem cells are going to be stained with Schiff's reagent (Feulgen stain) the following procedure may be used:

TABLE I
HOAGLAND'S NUTRIENT CULTURE SOLUTION NO. 2[a-c]

Stock solution (grams/liter)[d]	Stock solution (ml) used/liter of water (ml/liter)
$NH_4H_2PO_4$ 115.1	1
KNO_3 101.1	6
$Ca(NO_3)_2$ 164.1	4
$MgSO_4$ 120.4	2

[a] Minor elements solution: use 1 ml/liter of final nutrient solution. 2.86 gm/liter H_3BO_3; 1.81 gm/liter $MnCl_2 \cdot 4H_2O$; 0.22 gm/liter $ZnSO_4 \cdot 7H_2O$; 0.08 gm/liter $CuSO_4 \cdot 5H_2O$; 0.02 gm/liter $H_2MoO_4 \cdot H_2O$.

Iron salt: 0.5% iron tartrate, use 1 ml/liter once or twice a week (=0.0087 ppm Fe in nutrient solution); or, use iron versenates sold in garden shops.

The final nutrient solution pH should be approximately 5.2 for *Pisum*.

[b] D. R. Hoagland and D. I. Arnon. The water-culture method for growing plants without soil. College of Agriculture, University of California, Berkeley, California, Circular No. 347, revised January, 1950.

[c] From Conger (1964).

[d] Add to nutrient solution in the order given.

(a) Decant the fixative into a waste jar and add about 5 ml of 1 *N* HCl which has been warmed to 60°C. Hydrolyze the roots in the acid for 15–30 minutes, taking care that the temperature remains relatively constant. (Usually an oven pre-set at 60°C is used for hydrolysis.)

(b) Decant the HCl into the waste jar and add about 5 ml of Schiff's reagent. Allow the roots to remain in Schiff's reagent at least 20 minutes; they may remain in this solution for 6 hours. (See the simple method for preparing Schiff's reagent described in Section VIII,A,3.)

(c) When the meristem is stained a deep purple, remove one root with a forceps, blot it gently on paper toweling to remove excess Schiff's reagent, and place the root on a microscope slide. Remove about 2 mm of the root tip, moisten slightly with a very small drop of 45% acetic acid, and macerate with a blunted glass rod. When the cells are separated, moisten again with a little drop of 45% acetic acid, spread the cells over an area of about 1 square cm, and place a cover glass over the separated cells taking care that about 2 mm of the cover glass protrudes over the edge of the microscope slide. Place the slide between absorbent toweling and press firmly on the cover glass with the thumb.

(d) Examine the preparation with the microscope using the dry objectives.

(e) If preparations are to be used for autoradiography, see (g) below, otherwise place the slide and cover glass in a staining dish which

contains 90% tertiary butyl alcohol and 10% ethanol. Take care that the preparation is completely immersed in the alcohol. Allow the dehydration to take place overnight.

(f) To make the preparation permanent, remove the slide from the alcohol solution (the cover glass should still be attached) and remove the cover glass by gently grasping the 2-mm "lip" that protrudes over the edge of the slide with one forceps and holding the slide with another. Remove the cover glass, taking precaution not to slide it off. Immediately place a few drops of an alcohol-soluble permanent mounting medium directly on the cells. Place cover glass on the slide directly above the mounting medium; this time do not leave a "lip." Remove the excess mounting medium by placing the slide between absorbent toweling and press firmly but gently on the cover glass. Check preparation with the microscope and allow it to air dry for at least a day.

(g) If preparations are to be used for autoradiography, place slide on the flat surface of a piece of dry ice. Allow the slide to remain on the dry ice until it is thoroughly frozen.

(h) When frozen, remove the cover glass by prying a razor blade under a corner of the cover glass. The cover glass should snap off the slide.

(i) Immerse the slide in 100% ethanol for several minutes, and then hydrate to water by means of a graduated ethanol series. When the squash preparations are hydrated, they are ready for the application of emulsion.

A. Solutions

1. Carnoy's fixative—3 parts ethanol and 1 part glacial acetic acid; made fresh before each use.

2. 1 *N* HCl (approximately)—83 ml of concentrated HCl added to 500 ml of distilled water and diluted to 1000 ml with more distilled water.

3. Schiff's reagent—This may be made simply by adding 1 gm of basic fuchsin and 1.9 gm of sodium or potassium metabisulfite to each 100 ml of 0.15 *N* HCl. Allow the mixture to stand for 24 hours and then decolorize with activated charcoal, a heaping teaspoon for each 100 ml of mixture. Filter the mixture with a Büchner funnel, taking care to moisten the filter paper with a few drops of 1 *N* HCl. The product should be almost colorless.

4. 0.15 *N* HCl (approximately)—150 ml of 1 *N* HCl diluted to 1 liter with distilled water.

5. 45% Acetic acid—45 ml glacial acetic acid diluted to 100 ml with distilled water.

B. Materials

1. Glass containers suitable for seed germination
2. Paper toweling or some other absorbent paper
3. Pint size jar for liquid waste
4. Shell vials with corks
5. Blunted glass rod
6. Two forceps
7. Microscope slides and cover glasses

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Chapter 6

Induction of Synchrony in Chlamydomonas moewusii as a Tool for the Study of Cell Division

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I. Introduction

The first published report of an experimentally induced synchronous culture appeared in 1953 (Tamiya *et al.*). It was concerned with the

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unicellular green alga, *Chlorella*. A rather large body of literature, both on synchrony in general and on *Chlorella* and other green algae in particular, has accumulated since that date. Since the purpose of this chapter is not to review the literature, but rather to describe the techniques employed with a single cell, the reader is referred to the following references: Barner and Cohen (1955), Cameron and Padilla (1966), Hotchkiss (1954), Maaløe and Lark (1954), Pirson and Lorenzen (1966), Zeuthen (1964), and Zeuthen and Scherbaum (1954).

We chose to work with *Chlamydomonas moewusii* and to attempt to synchronize it rather than *Chlorella* or several other green algae for a very important reason: *C. moewusii* is an obligate photoautotroph. Our main interest has been the control of cell division. The origins of this process in the history of any cell cycle have been extremely difficult to trace. Mitosis and cytokinesis can be seen to occur by conventional cytological techniques. One can even quantitatively describe processes such as the replication of DNA as occurring during interphase, processes which we deduce must be completed before mitosis can occur. However, no one has been able to demonstrate with certainty a direct cause and effect relationship between any of these interphase processes and division. The completion of DNA synthesis (e.g., Howard and Pelc, 1953) or the cessation of growth (e.g., Prescott, 1955; Bernstein, 1964) do not by themselves lead to division. It has been proposed that one or more intervening reactions must take place.

One of the difficulties in demonstrating these hypothetical reactions is the inability to discriminate between what may appear at first glance to be very similar biochemical processes both during interphase and part of the mitotic period. A particular type of protein, for example, may be needed before mitosis can occur. Yet since in all probability it would be synthesized concurrently with a host of other proteins not directly involved in division, it could be overlooked. Furthermore, even though the term "intervening reactions" was used, it should not be assumed that the cessation of one process signals the beginning of another. Considerable overlapping may occur, so that the intervening reactions, although on a direct line to division, may begin very early in the cell cycle. This is not a refutation of either the existence of a G_2 period or of some of the excellent data produced to support it. It is merely a suggestion that part of the G_2 might exist side by side with the S period and part by itself after the completion of the S period.

To add to the confusion for the purpose of emphasizing the complexity of the picture, the overlapping of G_2 need not be confined to the S period. There is no good evidence to rule out investigations probing for the beginnings of G_2 in G_1 or even at the end of M in the previous cell

cycle. In fact, the demonstration of the replication of the centriole during M in preparation for the next division cycle may prove to have important biochemical significance. The search for "division triggers" may continue to be frustrated, in spite of the recent work of some who have discovered possible division proteins (e.g., Watanabe and Ikeda, 1965), if the Howard and Pelc (1953) model is taken too literally.

Thus, DNA replication might be both an absolute requirement for cell division and not a trigger for division. However, just because the end of DNA synthesis does not signal the cell to divide (see the excellent review of this subject by Prescott, 1964) does not prove *a priori* that a new series of reactions is begun and completed between the end of S and the beginning of M. One can immediately see how many models can be proposed as a basis for experimentation.

A reasonable approach is to work with a cell with a very strict, yet definable and controllable, exogenous requirement needed to complete a single cycle. The expectation would be to use this requirement as a tool for sorting out those processes dependent upon the requirement from those which are not. Hopefully, those reactions directly related to the control and the initiation of division would fall into one or the other category, and thus would be made easier to investigate as entities separate from those which are irrelevant to the problem. The possession of such an obligate requirement then, is the reason for working with synchronous cultures of *C. moewusii* rather than other cells at our disposal. Its characteristics are described in the next section.

II. Description of the Organism

A. General Remarks

Unlike almost all of the strains of *Chlorella* (Kuhl and Lorenzen, 1964) and many of *Chlamydomonas, C. moewusii* is an obligate photoautotroph. It has defied every attempt to culture it heterotrophically in the dark (e.g., Hutner and Provasoli, 1951).

Taxonomically, *C. moewusii* is placed either with other algae in the botanical class Chlorophyceae, or with the flagellated protozoa in the zoological class Phytomastigina. It reproduces either asexually by the mitotic division of its haploid vegetative cells or sexually by fusion of morphologically similar (isogametic) but physiologically distinguishable (heterothallic) haploid cells with subsequent meiotic division. The two mating types are designated plus and minus strains. This chapter is

concerned only with cultures of asexually reproducing members of the minus mating type.

Physiologically, the genus belongs to a group of organisms which because of its propensity for acetic acid has been called the "acetate flagellates" (Pringsheim, 1921). Members of this taxonomically diverse group not only tolerate, but thrive on concentrations of acetate which would be toxic to many other organisms. Many chlamydomonad species obey the rules of the group, growing and dividing in the absence of light on acetate as a sole source of carbon (Lucksch, 1932). It is because *C. moewusii* so closely resembles other species of the genus (e.g., *C. reinhardii*) and group, except for its inability to reproduce in the dark on any organic carbon source, that it was thought to meet the criteria considered desirable for our work.

B. The Obligate Requirement for Light

Since we have felt it desirable to work with a cell with a single, nonsubstitutable requirement to complete a cycle rather than one which has more than one pathway at its disposal, it is necessary in a book of this sort to define and discuss the requirement briefly. The subject of autotrophy versus heterotrophy as it applies to protistan cells has been reviewed (Danforth, 1962).

Where the "dark block" lies is still incompletely understood. No universal explanation is warranted from the work done on species of *Chlamydomonas, Gonyaulax, Monodus,* or others. Different causes for the block appear to exist for each of these different organisms. From our own and other's work, it would appear that *C. moewusii* does have the ability to oxidize acetate and other carboxylic acids in the light and probably in the dark (Lewin, 1950; E. Bernstein, unpublished data). The organism however appears to lack the ability to assimilate anything other than CO_2 either in the dark or the light. (Danforth, 1962, without citing his source, briefly mentions that *C. moewusii,* though unable to grow heterotrophically, was found to assimilate acetate in the dark.) In heretofore unpublished experiments, we showed that cells cultured in the light, in an atmosphere continuously being purged of CO_2, on media containing either acetate, glucose, succinate, fumarate, or citrate did not reproduce. Carbon dioxide must be present if the cells are to grow and divide. Furthermore, in an atmosphere where CO_2 was not removed, the above-named substrates did not stimulate the growth rate in the light.

That the obligate photoautotrophy in regard to assimilation but not to oxidation is complete was further demonstrated by the use of acetate-C^{14}.

Labeled CO_2 was recovered within 10 minutes of the time of introduction of acetate-C^{14} in the light or dark. However, only in light-cultured cells were the extracted carbohydrate, protein, lipid, and nucleic acid fractions labeled. No activity could be demonstrated in these fractions following dark incubation. The data seemed to indicate that assimilation of carbon sources other than CO_2 cannot occur. Any synthetic products which seem to owe their origin to other substrates arise through the photosynthetic reduction of the CO_2 derived from the oxidation of those substrates. In the dark, therefore, any CO_2 resulting from the oxidation of the acetate would not be utilized.

Having established that *C. moewusii* displays no facultative tendencies, one can then ask whether the cell requires light throughout its life cycle or for only a portion of it. Having converted the light into chemical energy for the reduction of CO_2 into certain compounds, does the cell continue to need light for its many functions (growth, movement, differentiation, mitosis, cytokinesis, etc.), or can it now use the compounds in the dark? Do some of the functions depend upon the light requirement more than others? The sorting-out procedure referred to in Section I can be attempted as a function of light dependency. The first step in this procedure has been to use the light requirement to produce synchronous cultures, and the second is to use these cultures for the sorting out of the processes. The use of synchronous cultures is particularly advantageous with this species because they are produced as a consequence of their own biological need. Thus, in a sense to study the mechanisms underlying cell synchrony may be the same as studying the control of cell processes including cell division.

III. Method for Maintaining and Culturing Cells

A. Preparation and Dispensation of Media

Chlamydomonas moewusii will grow and reproduce in a simple, defined, inorganic salt medium, the composition of which is described in Table I. The salts are dissolved in the order shown, the chelating agent (sodium citrate) being added first to prevent precipitation of the other ions when the medium is made up in much higher concentrations for storage purposes. Usually, 2–4 liters at 8 times the concentration are made at a time. This solution is divided into 200-ml aliquots in polyethylene bottles and frozen before the pH is adjusted and without benefit of

TABLE I
CULTURE MEDIUM FOR *C. moewusii*[a]

Salt	Concentration (mg/liter)	Molarity
Sodium citrate · $2H_2O$	500.000	0.00170
$CaCl_2 \cdot 2H_2O$	52.900	0.00036
$MgSO_4 \cdot 7H_2O$	300.000	0.00120
NH_4Cl	200.000	0.00370
$FeSO_4 \cdot 7H_2O$	8.000	0.00003
KH_2PO_4	1360.900	0.01000
H_3BO_3	1.000	0.00001
$ZnSO_4 \cdot 7H_2O$	1.000	0.000003
$MnSO_4 \cdot H_2O$	0.300	0.000002
$CoCl_2 \cdot 6H_2O$	0.200	0.0000008
$Na_2MoO_4 \cdot 2H_2O$	0.200	0.0000008
$CuSO_4$	0.025	0.0000001

[a] From Bernstein (1964).

sterilization. When needed a bottle at a time is thawed, shaken well, diluted to the concentration shown in Table I, and prepared in the manner described below.

If distilled water from a central tin-lined still is available, it is passed through a deionizing column first. Otherwise, singly glass-distilled water (e.g., from a "Bellco" still) is used. The first 6 salts are weighed on a torsion balance with sufficient accuracy. The last 6 trace elements are weighed on an analytical balance. Each salt is added to a 2-liter volumetric flask containing 1 liter of distilled water and completely dissolved before the next salt is added. After the addition of the last salt, the balance of the water is put in the flask. As stated above, this is usually a concentrated solution which is frozen. After thawing a particular aliquot, the medium is measured out into an appropriate sized volumetric flask again and diluted.

The entire contents of the flask are transferred to a beaker so that the pH can be adjusted to 6.8 in a single operation. The KH_2PO_4 acts as a suitable buffer to maintain the pH at a proper level during most of the life of the culture being studied.

Following the adjustment of the pH, the medium is dispensed into borosilicate bottles or Erlenmeyer flasks of 2 to 3 times the volume of the medium. Each vessel is capped with a neoprene stopper into which has been inserted 2 pieces of glass tubing for gassing the culture. The inlet tube extends to almost the bottom of the vessel, the outlet tube only to just below the stopper. Loosely packed cotton is used to plug each glass tube. A Teflon-coated magnet is placed at the bottom of each vessel.

In addition to the vessels described, medium is also dispensed into screw-capped borosilicate tubes both as the liquid and as a 1.5% w/v agar slant. These are used to maintain stock cultures.

Sterilization is accomplished by autoclaving the filled tubes and vessels at 121°C for 20 minutes. These are allowed to cool to about 80°C before being removed from the autoclave. After the vessels are removed from the autoclave, the caps are tightened, the medium cooled to room temperature, and finally stored at 10°C.

B. Other Culture Conditions

1. Temperature

All of the experimental work described here was conducted at 25°C. All of the stock cultures are maintained at the same temperature. The proper temperature is maintained by the use of refrigerated B.O.D.-type incubators, designed so that the heating unit controlled by its own thermostat as well as the compressor can be regulated. The temperature fluctuates no more than ±0.05°C in any one spot or more than ±0.1°C from one shelf to another. More sensitive temperature control has not been found necessary for this photosynthetic organism.

2. Lighting

Light is provided by a series of either 15-watt or 30-watt (depending upon the size of the incubator) cool white fluorescent tubes at an intensity of 1500 ft-c which is equivalent to 16,146 lux. The intensity is measured with either a Weston Illumination meter (model 756) or a General Electric exposure meter (model PR-3) used with its incident attachment. The reading is taken from the outside surface of the vessel facing the lights, and therefore must be considered more than the amount received by the cells. To minimize the heat produced within the incubator, the ballasts for the fluorescent lights are mounted outside of the incubator.

For the induction of synchronous cultures, where light–dark cycling had to be used, the bank of lights is connected to a time switch (Zenith P-815 Program Timer) which was adapted to turn the lights on or off every 15 minutes if required. This same clock has a second circuit which is independently connected to a switch activated by an adjustable cam which is used to regulate the stirring time (Section III,B,4).

3. Aeration

Experimental cultures, but not tubed stocks, are aerated continuously with a mixture of 95% air and 5% CO_2. The mixture may be commercially

obtained in a single cylinder, or prepared and measured in the laboratory. The gas mixture is first bubbled through a flask containing water to decrease the evaporation rate in the culture. Plastic or rubber tubing is used for the hose connections between the cylinder and the vessels. Several vessels are connected in series to a single tank.

4. Agitation of Cultures

Routinely, unless otherwise stated, experimental cultures are intermittently agitated. Continuous agitation has been found to raise the temperature in the vessel unless special precautions are taken. Intermittent stirring is accomplished automatically by connecting magnetic stirrers to the time switch described in Section III,B,2. The Teflon-coated magnetic bars stir the cultures at 10-minute intervals for either 30 or 90 seconds depending upon the volume of the culture. This agitation is essential, unless one bubbles the gas into the flask at a furious rate, because the cells become quite sticky during the second half of their life cycle and tend to form clumps. Clumps increase the mutual shading of cells, preventing each cell from receiving sufficient light. Thus, the stirring helps to keep the cells in suspension, assuring more uniform light exposure.

C. Stock Cultures

Routinely, duplicate tubes of liquid medium are inoculated, using 0.2 ml sterile pipettes, every 2–3 weeks from only one of the previously inoculated pair of tubes. The other tube is kept sealed as a precautionary sterility measure, and may not be discarded for a couple of months. These tubes are kept in separate incubators as they accumulate, as well as in a cool room, to preclude the possible loss of all of the cultures as a result of mechanical failures in any one incubator. About every 3 months, 5-ml agar slants of the medium in Table I are streaked from one of the liquid stocks. The agar cultures may last over a year.

Bacterial nutrient agar plates are streaked from the stocks at regular intervals to determine the presence of contaminating organisms.

D. Handling Experimental Cultures and Some Characteristics of Their Growth

Although stock cultures are always kept in reserve, they are never used as an immediate source of experimental material. Several days before an experiment, an aliquot is taken from a liquid stock and inoculated into a vessel of the type to be used in the experiment. This

culture is placed in continuous light and allowed to grow into middle or late log phase, and then a precise number of cells is transferred into the experimental vessel. The experimental vessel may be kept in continuous light and used to culture more logarithmic cells for certain types of experiments, or placed on light–dark cycles to culture synchronously reproducing cells (Section IV,A). This first synchronous culture usually is not used in an experiment, as will be explained below, but serves as a source for future synchronous cultures.

This procedure of using cells cultured in a flask or bottle rather than those coming directly from a tube is followed to avoid the use of suboptimally cultivated cells, which is what one finds in cultures which have not been aerated or agitated. The characteristics of optimally versus suboptimally cultured cells have been described in detail (Bernstein, 1964) and will not be reported on again here. What is important in the context of the present article is the emphasis on not working with deprived cells (i.e., light-starved, CO_2-deficient, overcrowded, etc.) if one is to derive meaningful data from physiological and biochemical experiments. This is particularly true if one is to work with synchronous cultures. Since the induction of synchrony in any species involves, even under the most natural conditions, a shock or temporary deprivation for the cell, one should not work with cells already deprived from other causes. The conditions become uncontrolled, and the presence of a single definable variable (i.e., the exogenous shock used to induce synchrony) no longer is true. In fact, if one does not have optimal conditions for logarithmic (i.e., asynchronous) growth, one will not obtain the highest degree of synchrony.

One way to determine whether the cells are in their healthiest condition before either synchronizing them or subjecting them to further analysis is to see whether they are reproducing with the shortest possible generation time and whether the culture is growing to the greatest possible concentration while still in the log phase. If one follows this practice, it will be found that the density of the culture obtained under synchronizing conditions will be the same as that obtained for a continuously illuminated, randomly reproducing, logarithmic culture. Presumably, this may also reflect a high degree of healthiness for the synchronized cell, a fact sometimes disputed by those critical of the use of artificially-induced synchronous cultures.

The shortest possible generation time yet obtained for *C. moewusii* is about 4 hours. The highest density during the log phase is 800,000–1,000,000 cells/ml (Fig. 1). Much higher densities are obtained (several million per milliliter) if the culture is kept longer, but this growth takes plate during the relatively long retardation phase characteristic of this

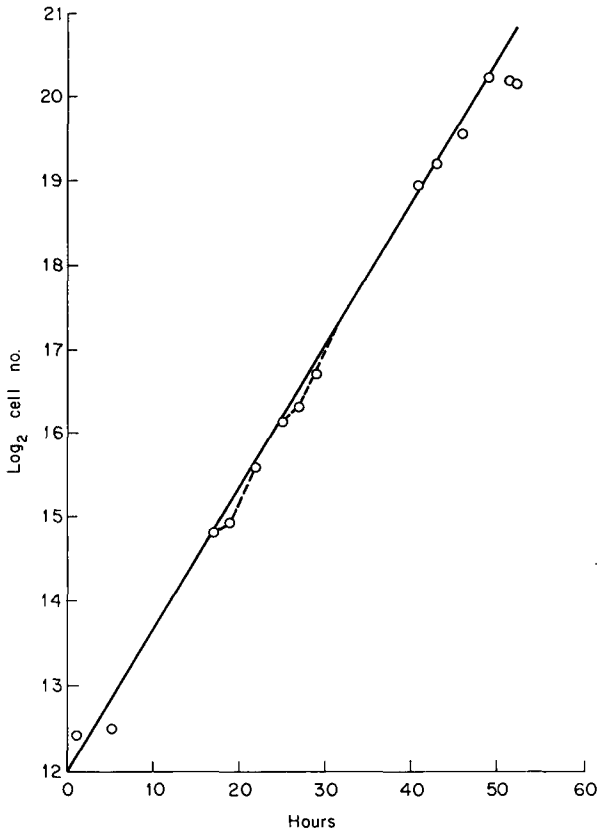


FIG. 1. Growth of a population of *Chlamydomonas moewusii* in continuous illumination. The culture was continuously aerated and intermittently stirred. It was begun with actively dividing cells from a logarithmic culture. The solid line represents the logarithmic slope extrapolated to the y-axis; the broken line represents the deviations from this slope which may indicate a small degree of synchronous division. (From Bernstein, 1964.)

organism. During this phase, the generation time far exceeds the minimal 4 hours. Cells taken from this period are physiologically different from those taken from the true log phase (Bernstein, 1964).

IV. Induction of Synchrony

A. Method of Induction

Healthy logarithmically reproducing cells are transferred to fresh medium and the culture subjected to 12 hours of light followed by 12

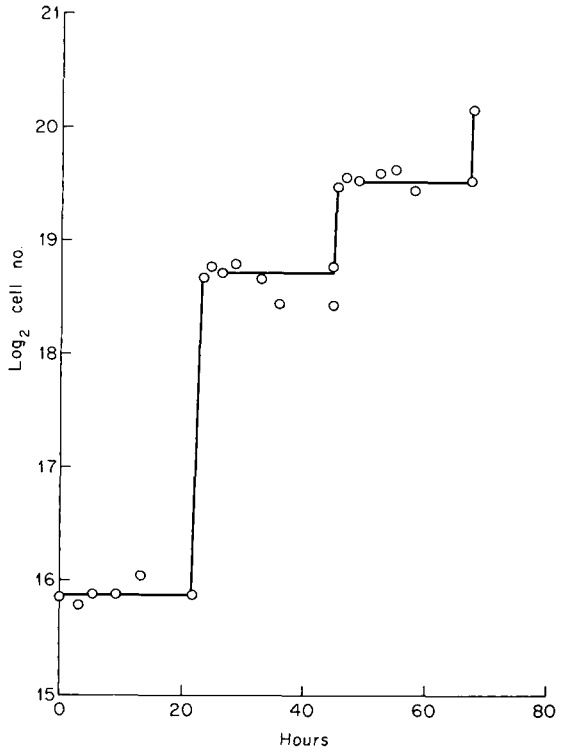


FIG. 2. Growth curve of *Chlamydomonas moewusii* showing synchronous division as a function of a light-dark cycle. (From E. Bernstein, *Science* **131**, 1528. Copyright 1960 by the Am. Assoc. Advan. Sci.)

hours of dark. The cycle is repeated indefinitely. Except for the first 24-hour cycle, during which the cells may have to adjust from a continuous light environment to a light–dark one, the cells thereafter reproduce synchronously every 24 hours. The type of synchrony obtained is shown in Fig. 2. Only the last portion of the growth curve is drawn.

It can be seen from this log to the base 2 plot that the curve begins with a culture which had synchronously reached a level of 50,000–60,000 cells/ml (0 hours). During the next 23 hours, no further increase in cell number occurs. Between the 23rd and 24th hours, the population increases almost 8 times (i.e., almost 3 log units). Sometimes, the synchronous burst occurs until about the 25th hour, or it may begin between the 22nd and 23rd hours. This was first described in detail by Bernstein in 1960. In any case, the burst always occurs at the end of the 12-hour dark period, and always in multiples of 8, providing that the population density prior to the burst does not exceed $\frac{1}{8}$ of the maximum density

(ca. 800,000–1,000,000/ml) attainable under the culture conditions described in the previous section. This last provision explains why the second and third bursts shown in the curve are even less than complete doublings; they are more or less comparable to what occurs during the retardation phase of a log culture.

The reason why the population increases in increments of 8 whether 10 or 100,000 cells/ml are present is to be found in the cell's cycle. Although described in detail elsewhere (Bernstein, 1964), a brief descrip-

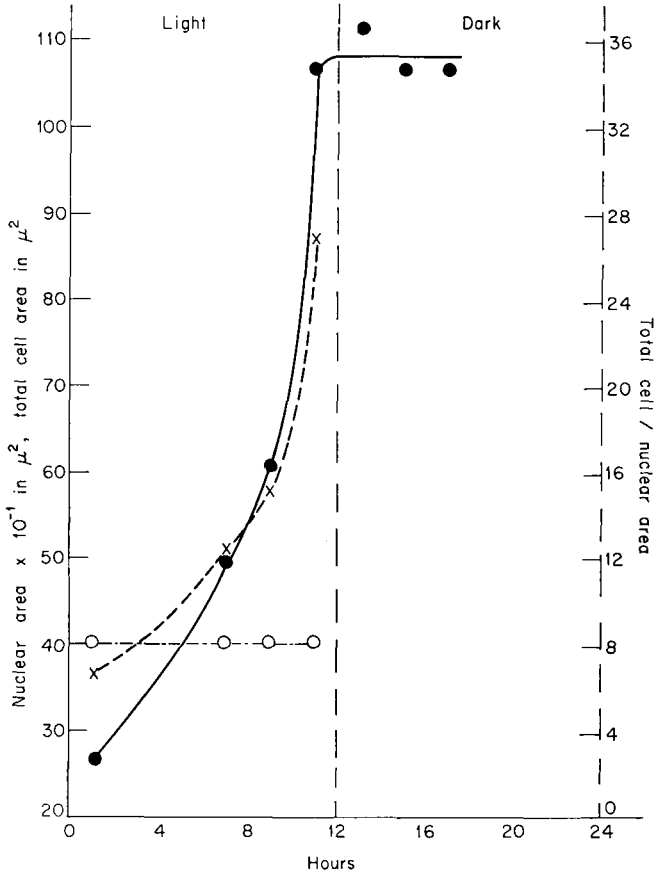


FIG. 3. Growth of cells during a synchronized light-dark cycle. ●, Total cell growth; ○, nuclear growth; X, ratio of total cell to nuclear area. No change in nuclear area was observed during the light period when total cell growth was occurring at a rapid rate. As a result, the cytoplasmic to nuclear ratio increases at the same rate as cellular growth. Division begins soon after growth ceases in the dark. (From Bernstein, 1964.)

FIG. 4. (a–c), 1st hour of synchronized cycle; (d–g), 7th hour cells; (h), 9th hour cells; (a), (d), and (f), phase contrast. Extranuclear Feulgen-positive particles focused on in figures. (a) and (h), largest dark sphere in each photograph is the nucleus; largest light sphere is the pyrenoid. (From Bernstein, 1964.)

FIG. 5. (a–c), 9th hour cells; (d–f), 11th hour cells; (b), and (d)–(f), phase contrast. Gradual loss of staining intensity of nucleus seen in (d)–(f). (From Bernstein, 1964.)

FIG. 6. (a) and (b), 11th hour cells; (c) and (d), 13th hour cells; (d), phase contrast. Feulgen bodies resembling a prophase and telophase seen in (c) and (d), respectively. (From Bernstein, 1964.)

FIG. 7. (a–d), 13th hour cells; (b) and (d), phase contrast. Cleavage line seen in (b); two dividing nuclei in (a) and (c). Four nuclei seen in (b) and (d). (From Bernstein, 1964.)

FIG. 8. (a–d), 15th hour cells; (a), phase contrast. Four "diffuse" nuclei formed in (a), (b), and (d); six of the eight nuclei seen in (c). (From Bernstein, 1964.)

FIG. 9. (a)–(d), 17th hour cells; (b)–(d), phase contrast. Eight nuclei stages. The nuclei become progressively more deeply stained and "compact." Daughter cell formation occurring in (d). (From Bernstein, 1964.)

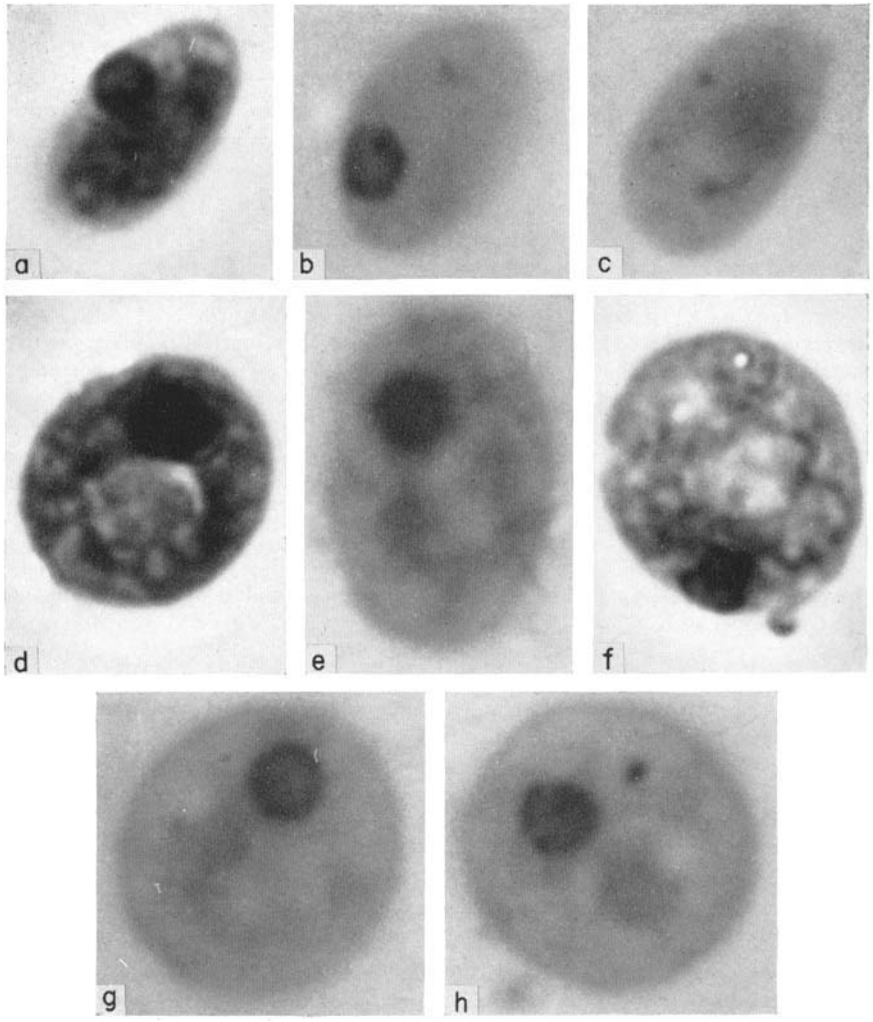


FIG. 4. For legend see p. 131.

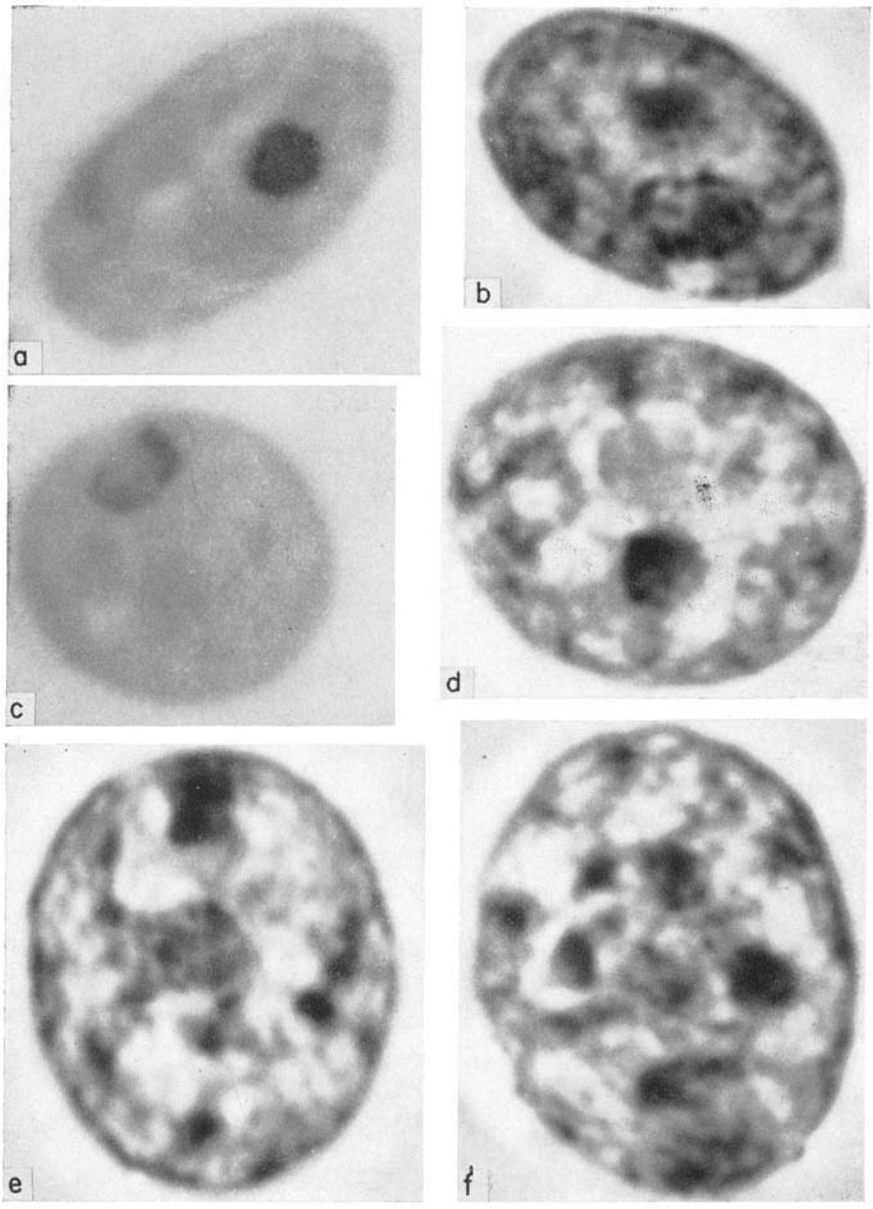


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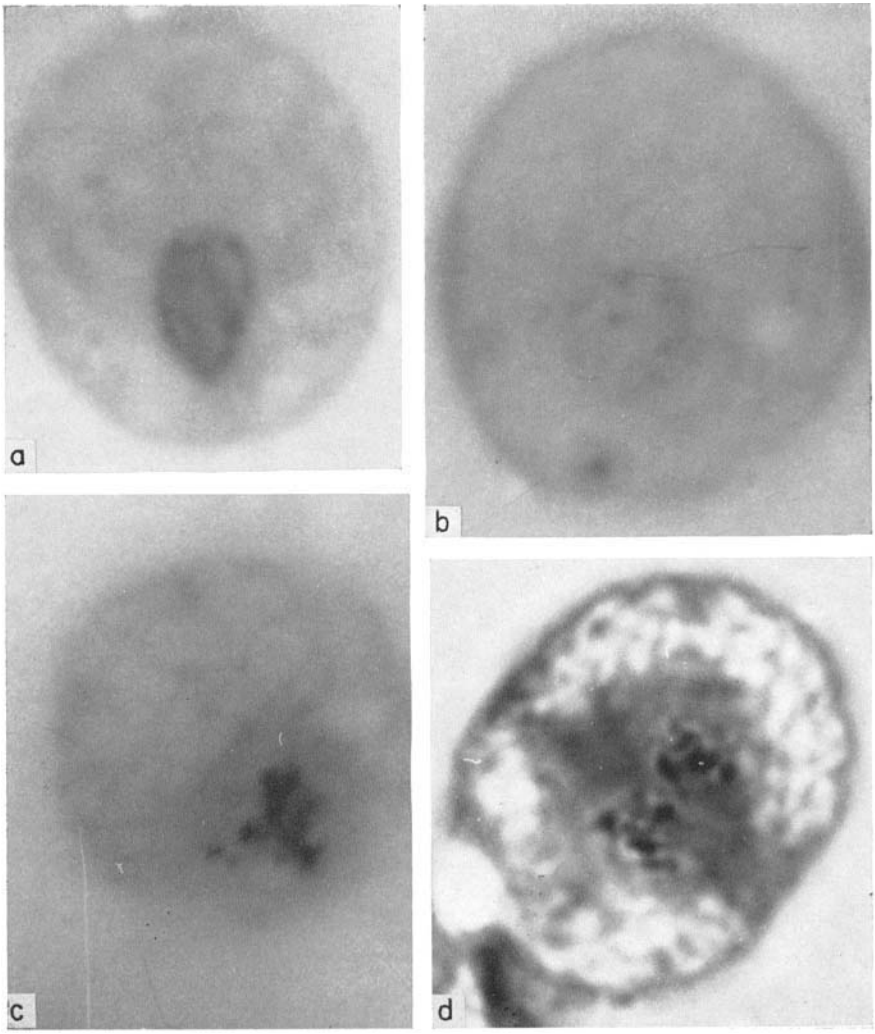


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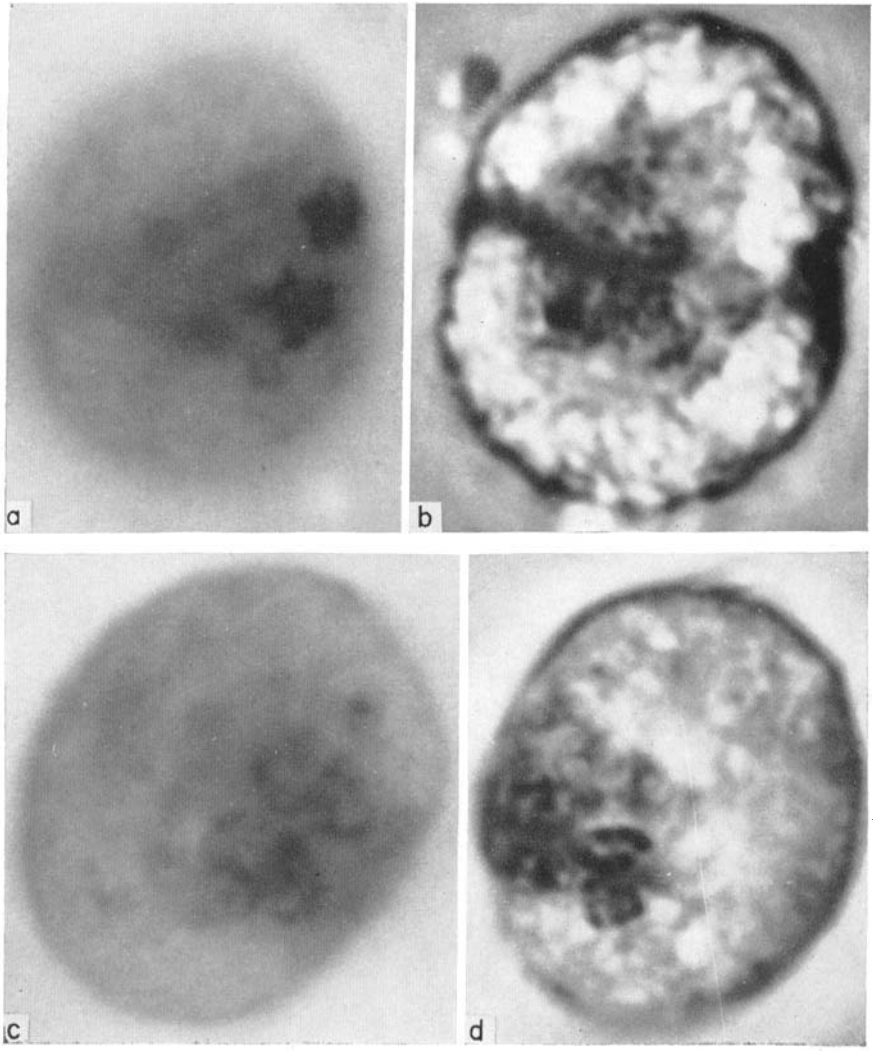


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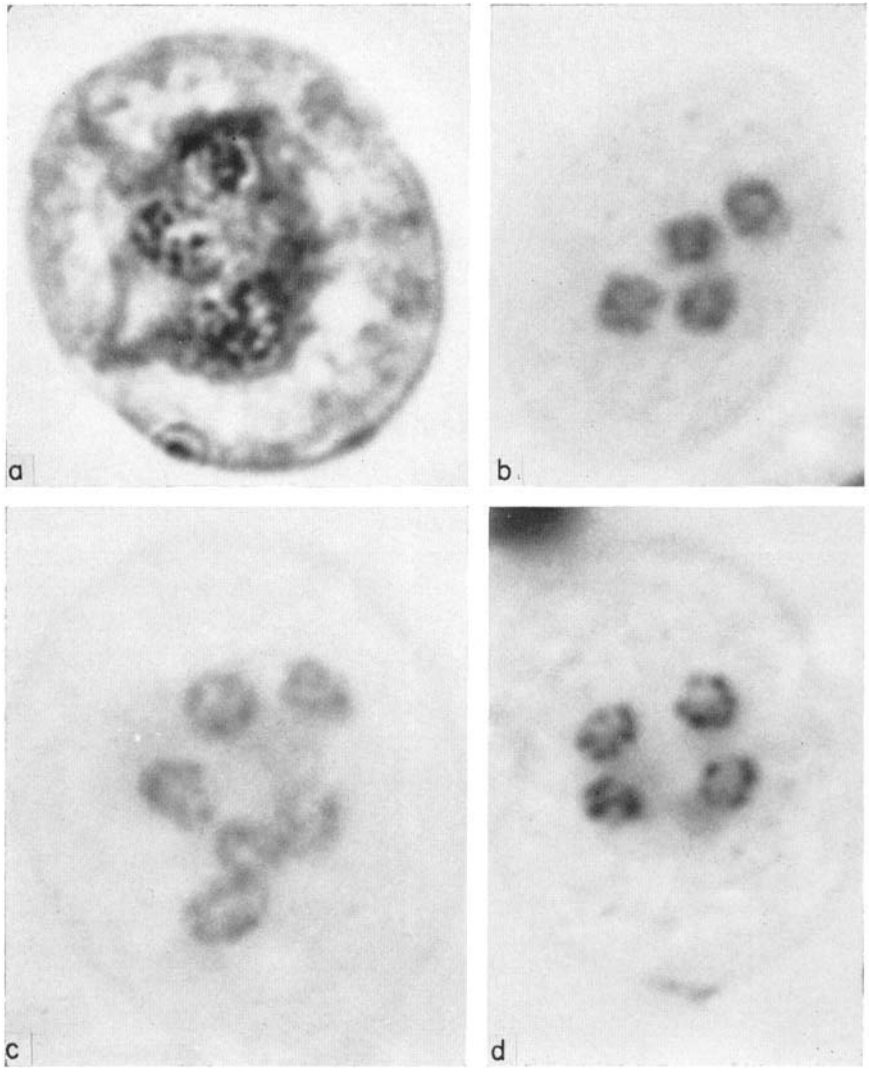


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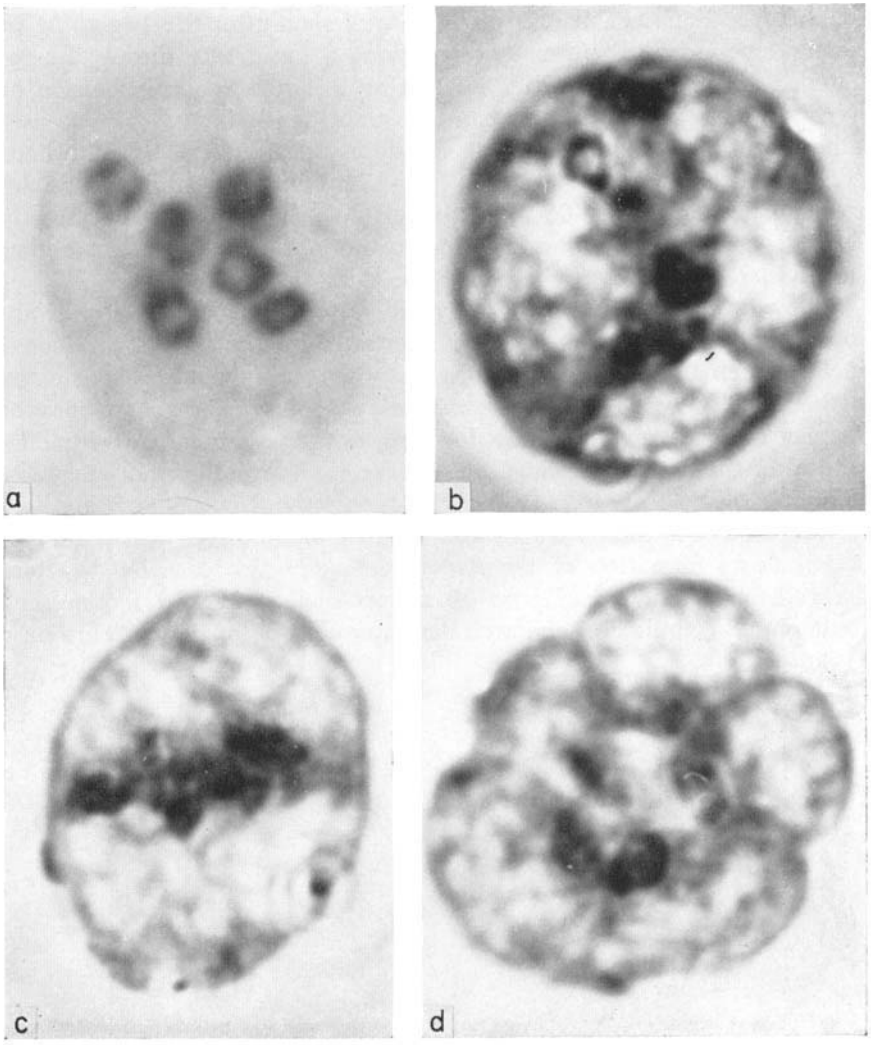


FIG. 9. For legend see p. 131.

tion follows. Each cell begins growing very soon after the beginning of the light period and continues to grow at a rapid rate during the remainder of the 12 hours (Fig. 3). During this time, no cell division or even positively defined mitosis can be seen (Figs. 4–6b). Growth ceases rather abruptly as soon as the cells are deprived of light at the 12th hour (Fig. 3). At about the same time (11th–13th hours), the first mitotic figures can be seen (Figs. 6c–7). Although growth appears to be directly dependent upon light, our experiments do not seem to indicate that the division process is triggered by the dark.

Following the first mitosis, 2 independently existing daughter cells are not produced. Rather, the 2 nuclei soon after dividing again produce 4 nuclei, which then undergo a third mitosis, resulting in an 8-nucleated mother cell. Between any of the mitoses, cytoplasmic cleavages may or may not occur. Even when they do occur, cytokinesis is rarely completed until after the end of the third mitosis. Very often one cannot even discern any cleavages (Figs. 7–9). Following the last nuclear division, cytokinesis is completed within the original mother cell wall. Differentiation (e.g., formation of the flagella, etc.) is also completed before the cells are liberated. Figure 10 shows such a packed mother cell containing 8 completely separated daughter cells (3 of the cells are on a

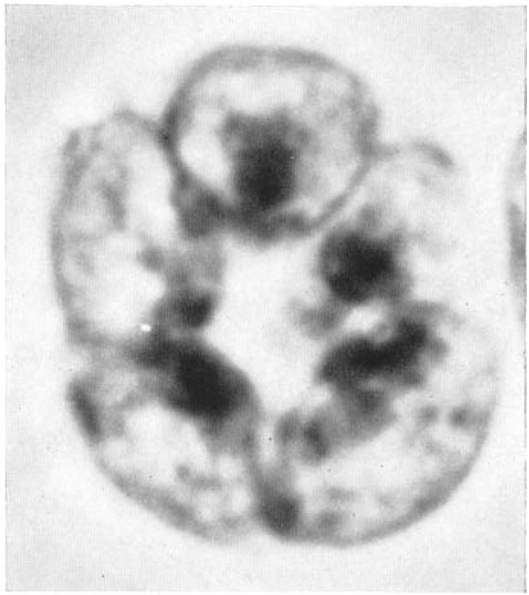


FIG. 10. Twenty-first hour mother cell containing 8 completely separated daughter cells. The nuclei are very compact and extranuclear Feulgen particles can be seen. (From Bernstein, 1964.)

TABLE II

DEGREE OF SYNCHRONIZATION OF PHASES OF CELL CYCLE[a]

Hour of cycle	Population based on nuclear counts (%)					Population based on cytoplasmic and chloroplast appearance (%)							
						No cleavage stages	Incomplete cleavage stages			Complete cleavage stages (true daughter cells)			
	Single compact nucleus (1)	Single diffuse nucleus (2)	2-nuclei (3)	4-nuclei (4)	8-nuclei (5)	Multiple nuclei (6)	2-Cell (7)	4-Cell (8)	8-Cell (9)	2-Cell (10)	4-Cell (11)	8-Cell (12)	1-Cell (free cells) (13)
0	100	0											
1	100	0											
7	100	0											
9	90	10											
11	60	40											
12	59	35	6	0	0	100	0	0	0				
13	1	51	37	9	2	91	7	2	0				
15	1	26	54	12	7	87	11	2	0				
17	0	1	10	35	54	25	27	34	12	0	0	2	0
19	0	0	1	10	89	7	10	8	12	0	13	50	0
21	0	0	0	1	99	0	0	1	1	0	10	88	0
23	95	0	0	0.5	4.5	0	0	0	0	0	0.5	4.5	95
24	98	0	0	0	2	0	0	0	0	0	0	2	98
25	99+	0	0	0	1−	0	0	0	0	0	0	1−	99+

[a] From Bernstein (1964).

different focal plane). Finally, at about the 23rd hour, these daughters leave the mother (the mechanism is incompletely understood), swimming as independent, fully formed cells.

A summary of the cytological events occurring during both the light and dark halves of the cycle are shown in Table II. Growth occurs only during the first half, while mitosis, division, differentiation, and liberation of new cells occurs in the second half of the cycle.

The experimenter may wish to know for practical reasons how long it takes a log culture to become synchronized and a synchronized culture to become randomized again. The answers to these questions have theoretical importance also when looking into the mechanisms underlying synchronous and normal division, but a discussion of this aspect is beyond the scope of this paper (Bernstein, 1964). Rather than inoculating fresh medium with healthy log cells, one can remove a log culture from continuous light, and subject it to the light–dark regimen. The results are shown in Fig. 11. Almost as soon as the culture is placed in the dark, the slope of the log curve declines (the generation time increases from 4 to 8 hours), and soon thereafter reproduction ceases completely. None

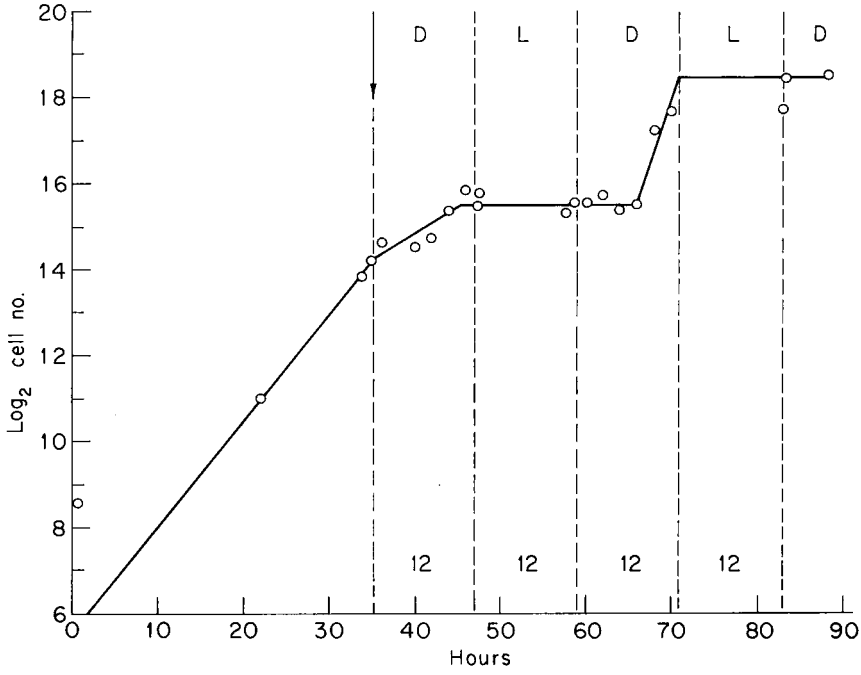


FIG. 11. Response of a continuously illuminated exponential culture to light-dark cycling. The arrow at the 35th hour indicates the beginning of the first dark period. (From Bernstein, 1964.)

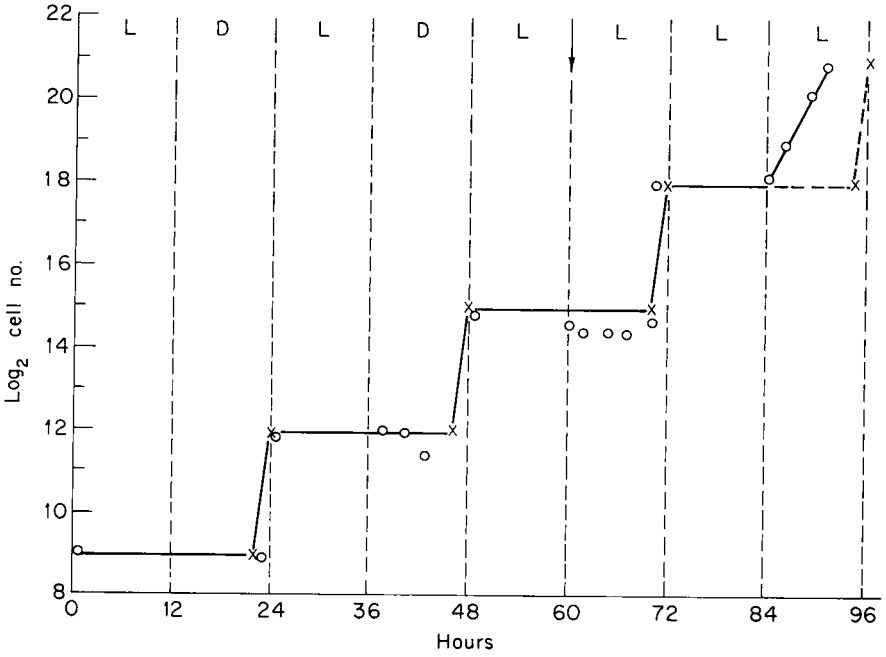


FIG. 12. Response of light-dark synchronized cells to continuous illumination. The arrow at the 60th hour indicates when the dark period normally would have begun. X, Calculated cell numbers; ○, actual cell numbers. (From Bernstein, 1964.)

takes place even during the first 12 hours of light, so that some measure of synchrony already is induced. A burst then takes place during the next dark period. It is an 8-fold increase, but occurs over a longer period of time (4–5 hours) than when the culture has been synchronized for 2 or more cycles.

A culture which has been growing synchronously for many generations and has been transferred many times when placed in continuous light behaves as is shown in Fig. 12. It appears to ignore the light during the first 12 hours when it would have been in the dark. It does not reproduce until the exact time it would have in the dark. When it does burst, it does so identically to a cycled culture—an 8-fold increase in 1–2 hours. A lag phase then ensues during the next 12 hours, at which time true logarithmic reproduction begins with a generation time of 4 hours.

B. Control of Division and the Induction Technique

OTHER LIGHT-DARK CYCLES

It can be shown that the production of 8 cells per mother is not completely genetically determined. Environmental influences are impor-

tant and can be used to regulate division as shown in the previous section. The same method suggests other experiments to study the control of division. For reasons not completely understood, but which were explored in depth previously (Bernstein, 1964, 1966), the length of the light period required to induce synchrony is related to the generation time of a continuously illuminated, log culture. Eight cells are produced for every 12 hours of light provided to a cell. It would appear that 4

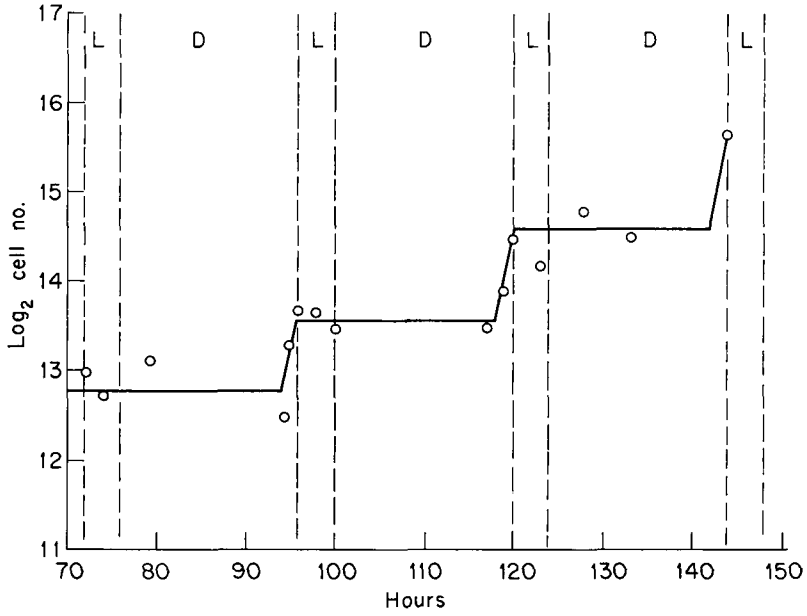


FIG. 13. Growth of a population of *C. moewusii* in response to 4-hour light-20-hour dark cycles. (From Bernstein, 1966.)

hours of light are required for each of the 3 mitoses. Four hours is also the generation time of a log cell. Thus, even when a cell is not deprived of light, it can divide on an average of only once every 4 hours.

Cultures can be subjected to varying amounts of light and darkness using the same culture techniques described. Figures 13 and 14 show the results of experiments in which cultures are exposed to 4 and 8 hours of light, respectively, during each 24-hour period. The population doubles on a 4-hour light, 20-hour dark cycle and quadruples on an 8-hour light, 16-hour dark cycle. One, two, or three mitoses can be produced and studied under controlled conditions by varying the cell's one obligate requirement, light.

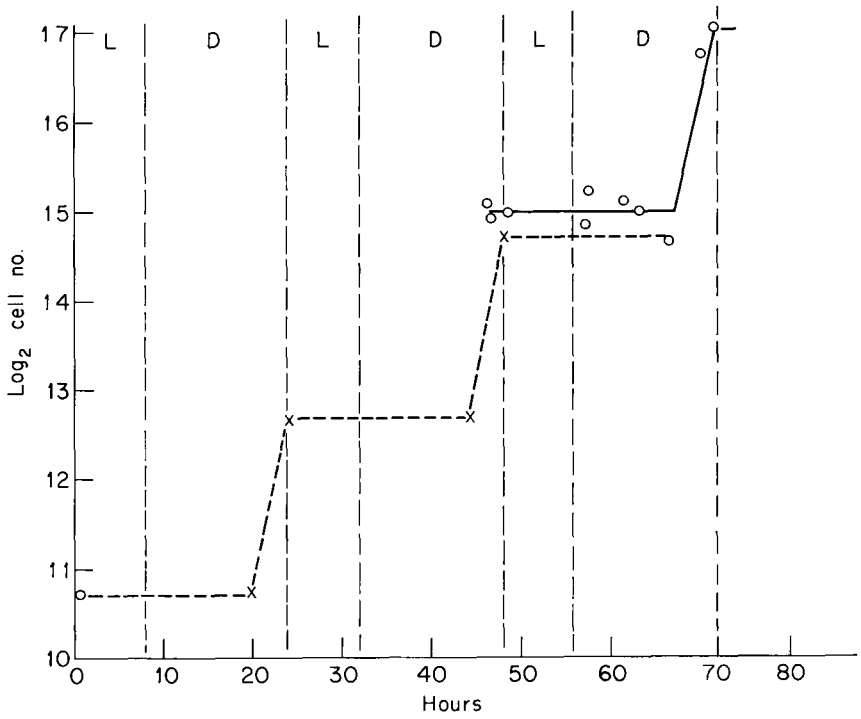


FIG. 14. Growth of a population of *C. moewusii* in response to 8-hour light–16-hour dark cycles. ○, Represent actual cell counts and are joined by the solid line. X, Joined by the broken curve represent the predicted division pattern if every cell were to divide synchronously into 4 daughter cells every 24 hours in the dark. (From Bernstein, 1966.)

C. Concluding Remarks

Although the experiments just described demonstrate a direct relationship between the length of the light period and the number of divisions a cell can undergo, one cannot ignore the important fact that mitosis and the division processes themselves are light independent. The obligate nature of the cell's photoautotrophy lies not in the observable events of mitosis and cytokinesis, but in those processes upon which division depends. These can be collectively referred to as growth; it is that process which remains to be sorted out into those events which directly and *which indirectly control division. What in the growth process,* other than the increase in volume (Fig. 3), requires light and perhaps determines the future course of division?

Synchronized cultures lend themselves nicely to an analysis of biochem-

ical and other events in the cell cycle. Large populations can be grown which theoretically should yield the same information as a single cell except in larger, more analyzable amounts. A great many studies of this sort have been conducted through the years and need not be reviewed here again. In the context of this paper, it should be mentioned that an analysis of the rate of synthesis of DNA, RNA, and protein as a function of the various phases of the cell cycle has been done and is mentioned here as an example of how both the technique itself of inducing synchrony as well as synchronized cultures can serve as tools for the study of the control of cell division (Bernstein, 1963). Of all of the synthetic processes comprising growth which have been investigated, only the rate at which RNA is made seems to change when the cells are placed in the dark during the synchronization procedure (Fig. 15). RNA and protein synthesis occur before DNA synthesis at the beginning of the light part of the cycle. However, once begun, DNA synthesis continues uninterrupted even if the cells are deprived of light. Tentative evidence seems to indicate that at the completion of the dark part of the synchronizing cycle neither RNA nor DNA synthesis would begin unless the cells are again exposed to light, the light being required for the synthesis of

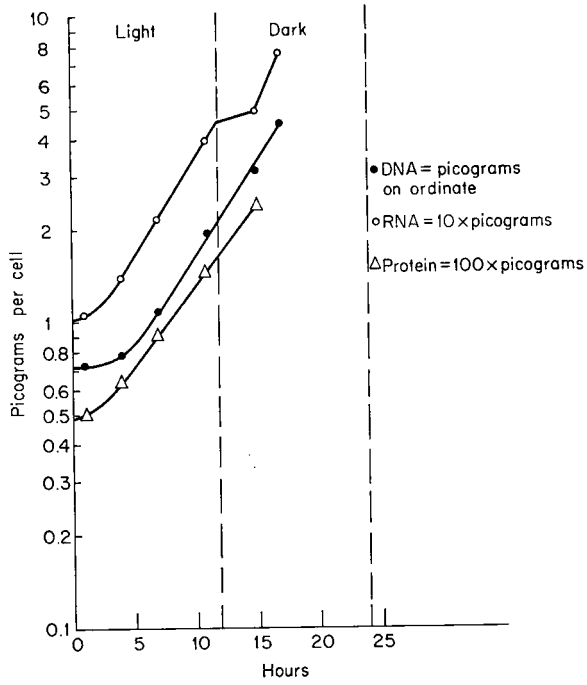


FIG. 15. Nucleic acid and protein synthesis as a function of the light-dark synchronizing cycle. (From Bernstein, 1963.)

some RNA fraction needed for the initiation of DNA synthesis (possibly through the intermediary of an RNA directed protein).

Whatever the exact mechanism may be, evidence from studies with synchronized *C. moewusii* has been corroborated by a recent study with heat-synchronized cultures of *Tetrahymena* (Bernstein and Zeuthen, 1966). Whereas deprivation of light is used in the synchronization procedure for *C. moewusii,* high temperatures are used for *Tetrahymena.* If the interference with RNA synthesis by darkness is indicative of a more universal phenomenon, then it was hoped that heat would produce similar effects; it did. RNA may be the primary target sensitive to environmental shocks (darkness or heat) in a sequence of reactions leading to synchronous division. Thus some type of RNA might play a very important role in the control of normal cell division.

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Chapter 7

Staging of the Cell Cycle with Time-Lapse Photography

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I. Introduction

Time-lapse photomicrography stages cells during the cell cycle without disturbing cellular metabolism. Although the number of cells examined is small, a valuable comparison can be made with large populations of cells synchronized artificially by physical or chemical treatment. The difficulties of tracing rapidly growing cells or the tedium of direct observation over long periods is reduced. This chapter describes the use of cinephotomicrography to delineate the sequence of morphologic and metabolic changes occurring during interphase and division. In some instances the experimental steps are outlined, while in others, applications with different cell types are cited as a guide to the procedures used.

II. General Methods

A. Equipment

Complete time-lapse units for cinephotomicrography are commercially available. The units include a 16- or 35-mm movie camera, time-lapse and normal speed drives, exposure controls, and tungsten and electronic flash light sources. Incubators enclosing the microscope, or air curtain heating systems, provide precise temperature control for the living preparations on the microscope stage. Various aspects of design and instrumentation are detailed by Leferer, Earle, Brice, and others in *Cinemicrography in Cell Biology* (Rose, 1963) and by Michaelis (1955).

Different optical microscopes are selected to observe particular aspects of the cell cycle. Phase contrast systems facilitate the viewing of thin transparent living cells. Bright field optics are suitable for studies such as the early cleavage of egg cells (Borghese and Cassini, 1963). In early applications of cinephotomicrography, fine photographic records were made through light microscopes (e.g., Lewis, 1931). Refined polarizing microscopes (Inoue, 1961) serve for the study of birefringence in contractile elements in living cells. Interference microscopy permits measurement of the mass of a specimen and the thickness of the optical path (Ambrose, 1963). The Nomarski interference phase contrast system (Nomarski, 1955; Bajer and Allen, 1966) reveals striking detail in chromosomes. Ultraviolet-microbeam irradiation can be combined with visible light phase contrast microscopy and television (Montgomery and Bonner, 1963).

The magnification chosen for observation varies according to the cellular events that are to be followed. In studies of the duration of the cell cycle and the intermediate G_1, S, and G_2 phases, the lowest magnification permitting adequate recognition of cell division is used. In this way a large number of cells can be observed and few cells migrate from the field. A $10\times$ objective serves well for generation time studies of cultured mammalian cells, although certain cell types may be suited to the use of still lower magnifications. The detailed analysis of events, such as mitosis or the tracing of small, rapidly growing bacteria, require the high magnifications obtained with oil immersion objectives. Long-working distance condensers are needed to accommodate certain culture chambers. Heat-absorbing and color filters (Frederic, 1954) inserted in the light path reduce damage to the cells.

The culture chamber should be thin enough to permit adequate mag-

nification of the cellular activities being studied and provide optimum conditions for exponential growth of the cells during the experiment. A great variety of chambers has been devised. Bacteria can be followed on simple slide mounts. Kubitschek (1962a) grew microorganisms on a thin agar square in a specially constructed depression slide sealed with a coverslip. Powell (1956a) used a culture chamber in which the medium circulated. A chamber on a microscope slide has been used to follow tobacco cells in culture (Jones *et al.*, 1960). The Sykes and Moore (1960) and Rose (1954) chambers, which can be purchased, are designed for mammalian cells. Wider fields can be examined by increasing the diameter of the chamber. Thin gaskets allow high magnifications with microscopes that are not equipped with long-working distance condensers. The nutrient medium can be renewed by connecting the chamber to a perfusion system. Specially designed plastic petri dishes with a recess in the cover permit the use of low and medium power objectives (Cooper, 1961). The dishes, after sealing with silicone grease, provide an adequate air and medium supply for growth of mammalian tissue culture cells for several days. The cells grow either on the plastic bottom of the dish or on a coverslip placed within the dish.

B. Cultures

In time-lapse studies, cellular activities are tabulated after the completion of an experiment by examining a film record. Thus, in addition to the considerations ordinarily encountered in selecting material for microscopic study, the growth characteristics must allow the cells to be traced on the film. Cells moving over one another are difficult to follow. Other cells can migrate from the field. With mammalian tissue cultures it is sometimes possible to select a more suitable clone or to vary the composition of the medium (McQuilkin and Earle, 1962).

C. Photography

Familiarity with the growth characteristics of the cells aids in selecting a field for photography. For generation time studies an isolated single cell or a small group of cells is located. Several generations can then be followed before maximum cell density is reached and division inhibited (Levine *et al.*, 1965). When only the age of the cells is to be scored, a field with numerous scattered cells is selected. If the microscopic field is to be relocated for staining or autoradiographic analysis, the coverslip can be circled with a diamond objective marker or a set of coordinates obtained. When coverslips are used inside a petri dish, a number of marks

are scratched on the coverslip surface beforehand, and a suitable field chosen in proximity to a mark.

The ease of tracing individual events of interest determines the frequency needed for the time-lapse sequences. Highly migratory populations require frequent exposures, wide fields, and, consequently, greater footage of film to obtain appreciable data. Rapidly occurring events such as division of bacteria are photographed several times a minute. Intracellular parasites are followed at intervals of a few seconds. One frame a minute adequately records generation times of mammalian tissue culture cells.

Direct positive films, such as Plus X Reversal, are convenient for ordinary records. If frames are to be enlarged or copies of the film made, a negative-type film may be preferred. After processing, the film can be edited with a film projector equipped with a frame counter. A filter protects the film from heat, and a wide-angle projection lens facilitates viewing.

III. Staging and Analysis of the Cell Cycle

A. Generation Time

Mitotic histories are filmed over a period of 3 to 4 average doubling times or, if the material permits, for longer intervals. When the cells are traced back in time on the film from division to division, the number of frames indicates the interval of time elapsed. Records of pedigree, generation times of mother and daughter cells, viability, and failure to divide are obtained. The mean individual generation time for the population is calculated from the individual generation times.

Prerequisites for generation time studies are that the cells grow under conditions of exponential growth with all cells dividing and viable (Powell, 1955, 1956b). Such growing populations show a characteristic age distribution with a higher proportion of young cells (Walker, 1954; Powell, 1956b). The bias introduced by the greater number of young cells can be avoided by measuring the same number of generations in every line of descent from the original ancestors.

Several findings regarding generation time are pertinent to the staging and further analysis of the cell cycle. Individual generation times, such as those illustrated in Fig. 1, differ considerably. An excess of long-lived cells, noted in many species, skews the distribution to the right (for example, Powell, 1956b; Hsu, 1960; Kubitschek, 1962b; Cook and Cook,

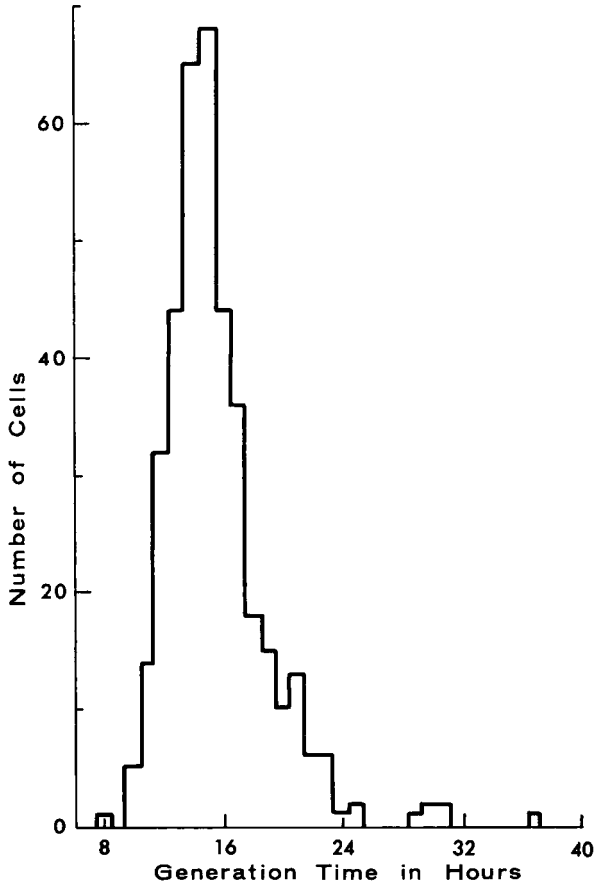


FIG. 1. Histogram showing frequency distribution of individual generation times of green monkey kidney cells.

1962; Schaechter *et al.*, 1962). The coefficients of variation of generation times in a number of mammalian cells exceed 10% (Dawson *et al.*, 1965). Consequently, the designation of cycle phase by cell age is approximate. It is not surprising that a synchronous population soon returns to asynchrony as the direct observations of individual cells by Prescott (1959) and Kubitschek (1962a) illustrate. In efforts to find less variation in generation time closely related cells have been examined and in some instances correlations appear. Time-lapse photographic studies of mammalian cells reveal both mother-daughter correlation (Sisken, 1963; Dawson *et al.*, 1965) and independence (Froese, 1964). Sister-sister correlation in generation time occurs (for example, Mc-

Quilkin and Earle, 1962; Dawson *et al.*, 1965; Killander and Zetterberg, 1965). In a hamster cell line the degree of correlation differed with the proximity of the sister cells (Froese, 1966).

B. Cell Division

The duration of cell division is measured from the first and last observable division events on a film record. In bacteria, the process of furrowing, cross wall formation, and cell separation serve as criteria for division. Mason and Powelson (1956) employed a high refraction index medium to observe separation of daughter chromosomes in bacteria.

Mitotic division in mammalian cells can be followed from nucleolar disappearance until the separation of daughter cells. Increased precision is obtained with higher magnification since early condensation of the chromatin and other cytological changes can be recognized. Division times as short as a few minutes were followed in bacteria (Hoffman and Frank, 1965). *Plasmodium gallinacium,* multiplying within embryonic chick cells, completed nuclear division within 24 minutes (Huff and Weathersby, 1963). Nuclear division in intracellular *Toxoplasma gondii* was completed within 15 minutes, and individual parasites within a clone showed closely synchronous divisions (Lunde *et al.*, 1961). In contrast, mitosis in cells from the tobacco plant required 2.5–5 hours (Das *et al.*, 1966). Where a sufficient number of measurements have been made, it is evident that the time spent in division varies from cell to cell. Variation in the mitotic time of mammalian cells is clearly shown in the human amnion cultures examined by Sisken and Wilkes (1967).

Detailed analyses of division have been made in a number of species. Bajer (1966) utilized endosperm of young seeds of *Haemanthus* to reveal morphological structure and movements of the chromosomes. Inoue (1964) followed changes in the organization of the mitotic spindle in cell types as varied as the jelly fish, *Halistuara cellularia,* the grasshopper, *Dissosteira caoline,* and the plant, *Lilium longiflorum.* Michel (1943) recorded the meiotic process in the grasshopper. Many earlier studies of cell division are included in the review by Michaelis (1955).

C. Determination of G_1, S, and G_2 Phases

The times since mitosis, for a group of cells in exponential growth, are recorded by cinephotomicrography. The microscopic field is marked. Tritiated thymidine (1 μc/ml, specific activity 5 c/mmole) is added to the culture chamber for 10 minutes, the cells fixed, and autoradiographs pre-

pared as described by Prescott (1964). After an appropriate exposure time, the autoradiographs are processed and stained. A photographic enlargement is made of the final field, from the slide mount or the last frame of the film. The cells within the field are scored for the presence or absence of silver grains over the nuclei. The age (time since mitosis) of the thymidine-labeled and the unlabeled cells in the last frame is determined by editing the film. The percentage of cells in the DNA synthesis period, indicated by the presence of silver grains over nuclei, is plotted against the cell age at the time of pulse labeling.

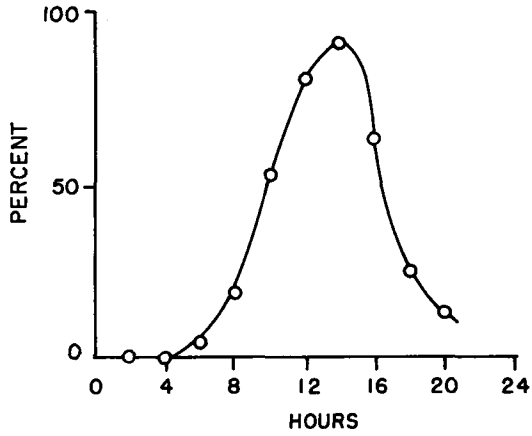


FIG. 2. Distribution of the percentage of cells in various age classes that incorporate tritiated thymidine during a short labeling period. (Sisken and Morasca, 1965.)

From the curve, estimates of the length of G_1 and S are obtained. The slope of the ascending portion of the curve reflects the variation in time required for individual cells to get from mitosis through G_1 into S. Often only one cell of a sister pair is labeled (Sisken, 1963) due to the variable length of G_1. The 50% level is taken as the average G_1 time. The width of the peak at the 50% level is an indication of the average length of S. The highest point in the curve may be less than 100% when a portion of the cells complete the S period before others begin. The presence of stationary cells in the population can also contribute to the number of unlabeled cells. Data illustrating this type of study (Fig. 2) are from the work of Sisken and Morasca (1965). G_2 is calculated by subtracting values for mitosis, G_1, and S from the mean individual generation time. These methods are discussed in greater detail by Sisken (1964) in a previous volume of this treatise.

D. Viability

Accurate timing of cell death is possible with time-lapse photomicrography. Generally, only a small proportion of the cell population is nonviable. Powell (1958) estimated that less than 1% of newly formed bacteria fail to survive. A similar situation is suggested by the close agreement between the mean individual generation time and the average doubling time in the mammalian cell line examined by Dawson *et al.* (1965). As a consequence, few tabulations by time-lapse methods have been made of the death of individual cells of known pedigree. Viability data provide a basis against which the effects of detrimental conditions, such as radiation (Froese, 1966; Marin and Bender, 1966), can be measured. The loss in plating efficiency with time, as certain tissues are subcultivated *in vitro* (Hayflick, 1965; Todaro and Green, 1963), can also be examined. Studies with mass populations may obscure data clearly shown by time-lapse experiments. The film records correlations within families of cells, changes in individual generation times, and the timing of death in relation to the cell cycle. For example, a population of green monkey kidney cells at a high passage level consists not only of rapidly dividing cells, but also of cells with extended generation times, and an increasing proportion of cells that die long after division.

E. Cellular Entities

Although many fine cine records have been made of nuclear and cytoplasmic components, few, except studies of mitosis, are related to a specific time in the cell cycle. An even smaller number of investigations utilize the techniques of autoradiography in conjunction with time-lapse photography. Of the cytoplasmic organelles, mitochondria and plastids have been extensively filmed. Centrioles were observed during mitosis in *Acanthamoeba* (Comandon and de Fonburne, 1939). In the majority of cell types mitochondria are too numerous and mobile to follow individually through the cell cycle. Frederic and Chevremont (1951; and Chevremont and Frederic, 1952) contrasted mitochondria in mammalian cells during a part of interphase and during mitosis. Movement, division, fusion, and pleomorphism of mitochondria were recorded. Plant mitochondria and plastids were graphically illustrated by Guilliermond (1941) and more recently by Jones *et al.* (1960) and Honda *et al.* (1964). Large, modified mitochondria, present during spermatogenesis in the grasshopper, have been recorded (Michel, 1943). Time-lapse methods to study the timing of mitochondrial DNA replication in the cell life cycle warrant exploration. Fixation procedures should preserve the organelles.

Other cellular components, such as heterochromatin and the nucleolus,

can be more easily followed. Distinct sex chromatin appears in rapidly proliferating human fibroblasts in the G_2 period (Demars, 1962). The nucleolus disappears during mitosis and is reconstituted in early G_1. Miles and O'Neille (1967) and Gonzalez and Nardone (1965) did not observe gross changes in nucleolar morphology as interphase progressed. To distinguish between synthesis of RNA in the nucleolus from synthesis in the chromatin, a cell type with few and prominent nucleoli was selected. Incorporation of RNA into the nucleolus reached a maximum early in G_1 and then appeared constant during interphase (Showacre *et al.*, 1967).

Time-lapse analysis is also suitable for the study of cellular components through several generations. For example, the segregation of sister chromatids in the divisions after labeling the DNA or the conservation of organelle DNA can be examined. Although not directly related to cell cycle analysis, time-lapse methods are valuable in determining the frequency of cell fusion and in following nuclear and cytoplasmic interactions when a hybrid cell is formed (Barski and Belehradek, 1963).

F. Metabolic Steps

In analyzing the cell cycle, numerous cells, at known stages, facilitate the search for the specific metabolic sequences involved. Time-lapse studies are based on division-synchronized cells. The starting point for the time measurement is cell division, recorded either as the time elapsed following division or the time elapsed prior to division. The position of the cell within the cycle is estimated from these age determinations. To lessen the effect of the variation in cycle time on the staging of the cells, selected cell types can be compared. In cells from a number of mammalian tissues the most variable phase is G_1 (Sisken and Kinosita, 1961; Defendi and Manson, 1963). In contrast, cells from other mammalian tissues lack the G_1 phase (Baserga, 1963; Starkey, 1963; Lala and Patt, 1966; Robbins and Scharff, 1967). Comparisons of such cell types, differing in the phasing of DNA synthesis, help to separate events associated with the S and G_1 phases. In addition, S and G_2 are less variable in length, and for these phases estimates based on the times until mitoses are more accurate. Different stages of synchrony (for example, division, S, or G_2), can thus be approached by time-lapse cinematography without disturbing the cells. Cycle metabolism can be surveyed in a general way or certain more precise events can be timed. In the following paragraphs, methods for examining trends in RNA and protein synthesis and studies directed toward the timing of specific events related to cell division are discussed.

The rate of synthesis of rapidly labeled nuclear RNA can be examined

in the following manner. The ages of a number of cells in logarithmic growth are recorded on film for a period greater than the mean generation time. The cells are then pulse labeled (e.g., uridine-5-H^3, 1 μc/ml, specific activity 16 c/mmole for 10 minutes), fixed, and autoradiographs prepared. Silver grains over the nucleolus, chromatin, and cytoplasm are counted. After appropriate corrections for background labeling the num-

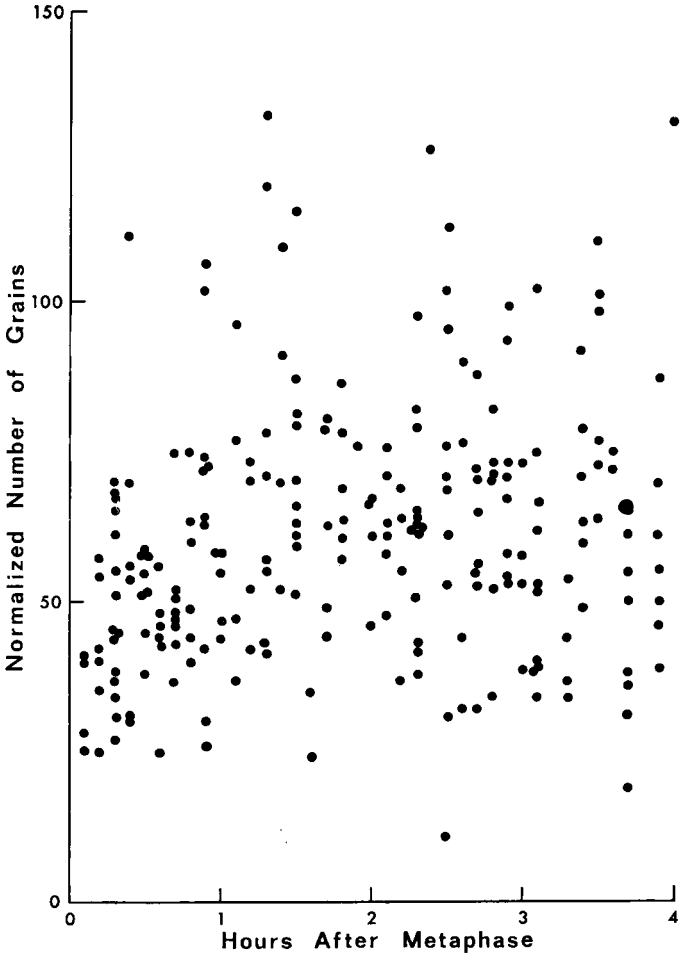


FIG. 3. Distribution of normalized grain counts over the nucleus of individual monkey kidney cells during the early G_1 phase of the cell cycle.

Cell ages were recorded by time-lapse cinephotomicrography prior to a 10-minute label with uridine-5-H^3 and cytidine-5-H^3. The data are compiled from 14 separate experiments.

ber of grains per compartment is plotted against the age of the cell. Since the number of cells in a field is limited, the experiment must be repeated several times to obtain sufficient data. It is important to maintain uniform conditions of labeling, exposure, and processing. The data from the separate experiments are normalized for compilation.

The abrupt rise in nuclear RNA synthesis observed immediately after mitosis in experiments with naturally synchronized cells is illustrated in Figure 3. Both the rate of synthesis of rapidly labeled RNA (Seed, 1963; Showacre *et al.*, 1967) and total cellular RNA (Killander, 1965; Killander and Zetterberg, 1965; Seed, 1966) have been followed through interphase by time-lapse methods. These studies were carried out on cells with a variable G_1 period and serve to establish trends but may overlook brief discontinuities.

Studies of protein synthesis using cinephotomicrography are based on methods similar to those described for the RNA experiments. Labeled amino acids are incorporated into protein that appears throughout the cell and nuclear label is generally not clearly separated from cytoplasmic label. Zetterberg and Killander (1965) followed the incorporation of labeled amino acids into mouse fibroblasts in relation to cell age. Total protein, measured as dry weight with a microinterferometer, has also been studied (Killander and Zetterberg, 1965; Zetterberg and Killander, 1965; Seed, 1966).

Recent reports have dealt with the effects of physical and chemical factors on the cell cycle. Temperature (Sisken *et al.*, 1965; Das *et al.*, 1966), radiation (Froese, 1966; Marin and Bender, 1966), and pH (Sisken and Kinosita, 1961) were examined. The actions on mitosis of inhibitors such as Colcemid (Stubblefield, 1962; Kleinfeld and Sisken, 1966), puromycin, and actinomycin D (Donnelly and Sisken, 1967) have been followed. Fluorophenylalanine was used to time the synthesis of mitosis-associated protein (Sisken and Wilkes, 1967). In addition to studies with naturally synchronized cells, time-lapse photography serves to monitor the effectiveness of physically or chemically induced synchrony in subsequent generations (Kasten and Strasser, 1966; Stubblefield and Klevecz, 1965).

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Chapter 8

Method for Reversible Inhibition of Cell Division in Tetrahymena pyriformis Using Vinblastine Sulfate[1]

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I. Introduction

Studies on control mechanisms operating during the cell growth and division cycle depend to a large extent on available techniques for obtaining large populations of cells all at the same stage of the cell cycle. Synchronization or phasing of cell division has been accomplished in *Tetrahymena* by subjection to repeated heat shocks (Scherbaum and Zeuthen, 1954), repeated cycles of cold shocks (Padilla *et al.*, 1966), and by manually isolating dividing cells (Prescott, 1959).

The techniques which permit reversible blockage of cell division not

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only allow for more precise studies of the temporal sequence of events during the cell cycle but often provide information on the timing of specific events through analysis of the action of the blocking agent.

Studies with vinblastine (VLB) indicate that one effect of this drug is to interfere with the mitotic spindle in vertebrate cells both *in vivo* and *in vitro* (Cutts, 1960; Palmer *et al.*, 1960) and that this inhibition can be partially overcome by administration of excess glutamic acid and other amino acids *in vivo* (Johnson *et al.*, 1960). Because of possible reversible inhibition with VLB, *Tetrahymena* cells were treated with this drug as a tool for obtaining populations at similar stages of the cell cycle.

II. Growth of Cells

A. Cell Strains

Two strains of *Tetrahymena pyriformis*, WH6 mating type I (micronucleate) and GL (amicronucleate), are compared in their response to vinblastine. *Tetrahymena* is usually binucleate containing a small diploid micronucleus which divides mitotically and a large polyploid macronucleus which does not appear to have a mitotic apparatus during division and merely pinches into two approximately equal halves. Some strains, i.e., GL, have lost their micronucleus but are capable of continued vegetative growth in culture. This difference in the two strains offers the advantage that any specific effect on the mitotically dividing micronucleus by VLB may be detected.

B. Culture Conditions

Both strains are grown under sterile conditions in 25 or 50 ml of a tryptone-glucose medium [Holz *et al.* (1962) modified by Frankel (1965)] in 250- or 500-ml flasks in constant temperature water baths without shaking. The components of the medium are given in Table I. This medium is, in all aspects, identical to the one used by Frankel and is repeated here because of the apparent importance of the medium and method of sterilization in the effectiveness of VLB to act as an inhibitor of cell division. When it became apparent (Section III,B) that autoclaving the medium made a difference in the ability of VLB to act as an inhibitor, the medium was subsequently autoclaved after Millipore filtering. In the techniques reported here the glucose was present during autoclaving of the complete mixture of medium components. Autoclaving the glucose and Bacto-tryptone mixture separately, and then mixing has

TABLE I
TRYPTONE-GLUCOSE MEDIUM[a]

Component	Amount (mg/liter)[b]
Bacto-tryptone	3000.0
Glucose	5000.0
KH_2PO_4	1000.0
$K_2HPO_4 \cdot 3H_2O$	1000.0
$MgSO_4 \cdot 7H_2O$	300.0
Nicotinic acid	1.8
Calcium pantothenate	1.5
Thiamine·HCl	1.0
Riboflavin monophosphate	0.9
Pyridoxamine·2HCl	0.2
DL-6-Thioctic Acid	0.04
Folic Acid	0.04
Biotin	0.004
Fe	2.700 [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ = 14.2]
Mn	1.000 ($MnSO_4 \cdot 4H_2O$ = 1.6)
Zn	0.400 ($ZnSO_4 \cdot 7H_2O$ = 4.5)
Cu	0.080 ($CuSO_4 \cdot 5H_2O$ = 0.3)
Co	0.100 [$Co(NO_3)_2 \cdot 6H_2O$ = 0.5]
Mo	0.012 [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ = 0.1]

[a] Frankel (1965).
[b] Vitamins and salts are made up 1000 × concentration.

not been tested as yet. Strain GL and WH6 are maintained at 27°C and 33°C, respectively. These temperatures are near the optimum for each strain.

Experiments are begun 18–24 hours after inoculation of logarithmically growing cells into new medium. Only cultures with a cell concentration between 15,000 and 30,000 cells per milliliter are used. Concentration of cells is determined with a Coulter electronic particle counter using 1 ml of culture for each determination. Up to 15 ml of culture has been removed without producing erratic results in control cultures in logarithmic growth.

III. Inhibition of Cell Division

A. Concentration of VLB Required to Block Cell Division

Vinblastine sulfate (Velban, Eli Lilly Co.) is obtained as a dry powder and diluted with Bacto-tryptone medium. Detectable loss of activity has been noted between 2 to 4 weeks after dilution and storage in the

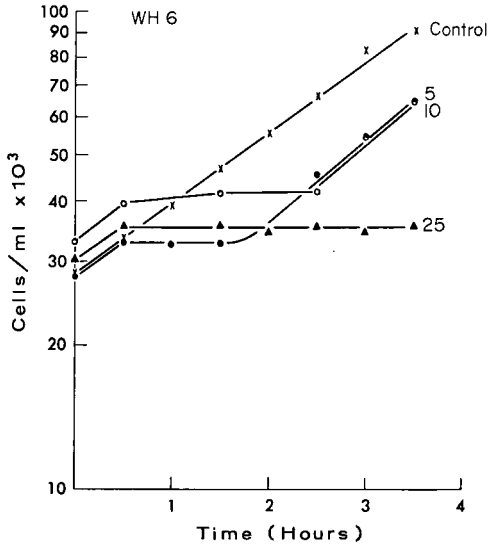


FIG. 1. Inhibition of cell division in strain WH6 growing in Bacto-tryptone medium autoclaved 40 minutes using different concentrations of VLB. Control generation time is 2 hours.

refrigerator. At the beginning of an experiment VLB is added to the culture with a pipette to give the desired concentration. An identical amount of diluent is added to the control culture. Figure 1 illustrates the concentration required to block cell division for strain WH6 in medium autoclaved for 40 minutes at 15 pounds pressure at 121°C. The lowest level of VLB concentration illustrated which produces blockage in strain WH6 is 25 μg/ml. Cell division has been blocked for as long as 11 hours with no obvious loss of viability using this concentration. Inhibition can also occur with 15 μg/ml, but results are variable from one experiment to another when different batches of medium are used.

As seen in Fig. 2, VLB does produce blockage of cell division in strain GL (amicronucleate), the difference being that the lowest concentration tried that produces blockage in strain GL is 50 μg/ml, twice as much as is required for strain WH6. Recently, 35 μg/ml has been examined and found to be ineffective as an inhibitor for strain GL.

Concentrations of VLB which inhibit cell division do not become completely effective until about 30 minutes after the addition of the inhibitor. This probably indicates that those cells in division are able to continue and complete this process, but no additional cells are able to enter division. Since it takes approximately 20–25 minutes for cytokinesis, at least in other strains of *Tetrahymena* (Stone *et al.*, 1964), this is not an unreasonable assumption.

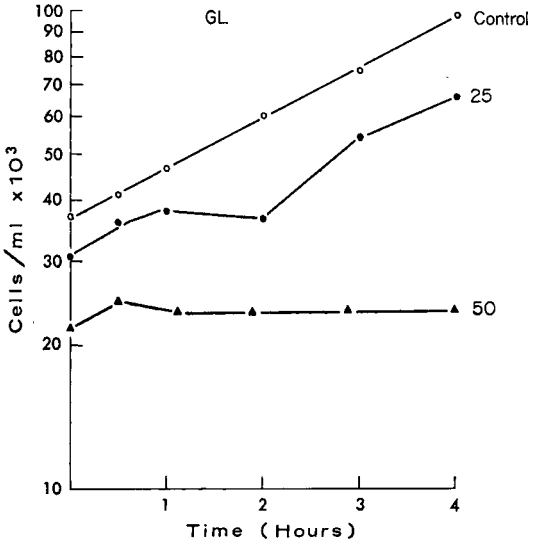


FIG. 2. Inhibition of cell division in strain GL growing in Bacto-tryptone medium autoclaved 40 minutes. Only two different concentrations of VLB used, 25 μg/ml and 50 μg/ml. Control generation time is between 2 hours 45 minutes and 3 hours under these conditions.

A comparison of the two strains demonstrates that a micronucleus is not necessary for VLB to act as an inhibitor. The implications are that VLB is acting on some undefined division mechanism in the macronucleus or at some site unrelated to nuclear division. One explanation for the difference in concentrations of VLB required to produce inhibition in the two strains is that the micronucleus is more sensitive to VLB than other sites, i.e., macronucleus. Consequently strain GL, without a micronucleus, requires more VLB to block the less sensitive events. However, explanations based on differences in culture temperature or other undefined genetic differences cannot be ruled out as yet.

One other point should be mentioned here and that is the concentrations required to produce blockage in *Tetrahymena* are massive when compared to 0.01 μg required for blockage of mammalian cells in culture (Palmer *et al.*, 1960); the type of medium in which the cells are grown may be responsible, as judged by results on the method of sterilization.

B. Method of Medium Sterilization

Only strain WH6 is discussed in this section because of the low concentration requirements of VLB. Growth curves for control cultures grown in medium, autoclaved 20 and 40 minutes, and Millipore filtered with no autoclaving are shown in Fig. 3. The results of these curves indicate little

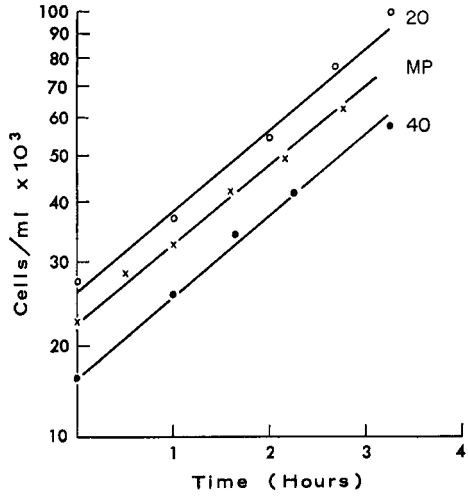


FIG. 3. Growth curves of cells (WH6) grown in Bacto-tryptone medium sterilized by Millipore filtering (MP) only, autoclaved for 20 and 40 minutes. Generation time is approximately 2 hours in all media.

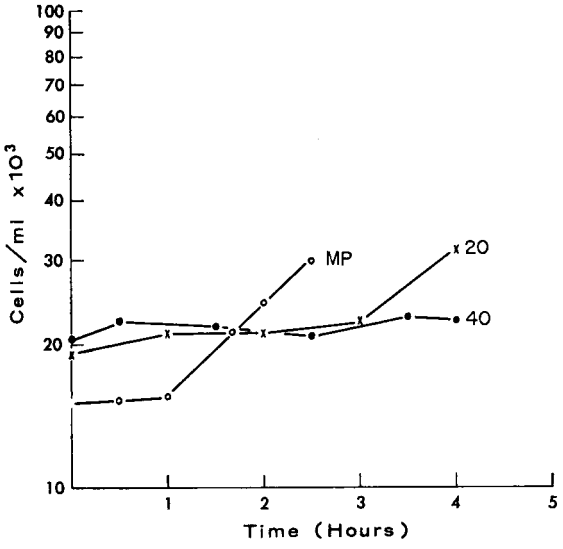


FIG. 4. Effectiveness of VLB at 25 μg/ml as a division inhibitor for cells grown in medium sterilized by Millipore filtering (MP), autoclaved for 20 and 40 minutes.

or no effect of autoclaving on the generation time. Figure 4 illustrates the effectiveness of VLB concentration at 25 μg/ml when cells are grown in medium sterilized by Millipore filtration and autoclaved for 20 and 40 minutes at 121°C and 15 pounds pressure. VLB has little or no effect when the medium is sterilized by Millipore filtration. Autoclaving of the medium for 10 (not shown in figure) and 20 minutes inhibits cell division, but the culture then begins growing after a brief lag period. Thirty minutes of autoclaving blocks division for as long as 5 hours, but the culture will eventually escape from the inhibition. Autoclaving for 40 minutes results in complete inhibition of cell growth for as long as 11 hours.

These results indicate that the medium contains a component(s) which overcomes VLB inhibition but is also destroyed by autoclaving. Until this component is identified and its effects minimized care should be taken that comparative experiments on the effects of VLB should always be performed in the same batch of medium.

IV. Recovery from Inhibition

Cultures of strains WH6 and GL recover from the inhibition of VLB when washed free of this agent. Cultures blocked with VLB and control cultures are removed from constant temperature water baths and rapidly poured into 50-ml conical centrifuge tubes. The cultures are centrifuged for 5 minutes at 800 rpm (150–200 g) at room temperature. Excess medium is siphoned off leaving cells in 0.5 ml of the medium. Prewarmed medium is then added to restore the volume and the cells are immediately resuspended with a Pasteur pipette and replaced in the constant temperature baths. This procedure takes 8–10 minutes and recovery time is measured from the time the new medium is added. Control cultures are treated in an identical manner to determine the effect of the washing procedure. The addition of fresh medium represents between 75- to 100-fold dilution of VLB which is sufficient to overcome inhibition. Determination of cell concentrations is made immediately before and after the washing procedure to determine how many cells are lost during centrifugation. Loss of cells is reflected in the lower cell concentrations during recovery experiments.

Figures 5 and 6 indicate the length of time required before release of the inhibition takes place after being blocked for 4 hours. These figures also indicate the degree to which the cells are synchronized or phased

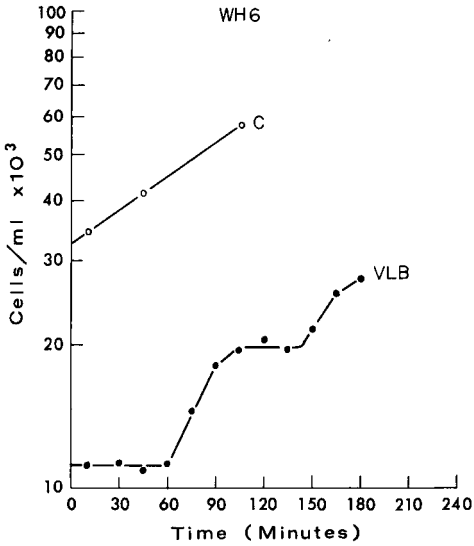


FIG. 5. Recovery of strain WH6 from 4 hours of division inhibition with 25 μg/ml VLB. Zero time represents the point at which fresh medium is added to the centrifuged pellet of cells for both control and experimental. The generation time of control culture (C) after washing is approximately 2 hours indicating little or no effect of the washing procedure.

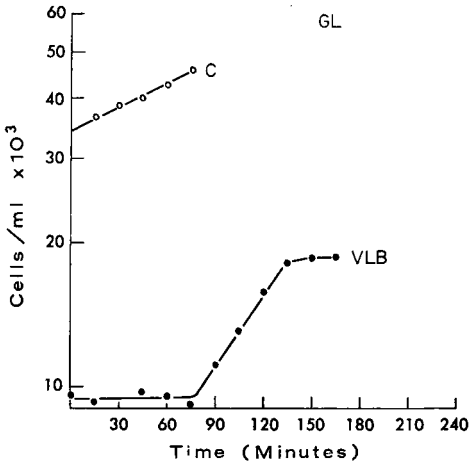


FIG. 6. Recovery of strain GL from 4 hours of division inhibition with 50 μg/ml of VLB. Control culture (C) indicates a generation time of 2 hours 45 minutes.

with respect to division. An increase in the cell population in strain WH6 is usually detected between 60 and 90 minutes after addition of fresh medium and continues for 30 to 45 minutes when population growth reaches a plateau.

Details for conditions resulting in the highest degree of cell division synchrony following removal of VLB are under study. For example, the effect of the length of inhibition prior to washing on recovery time and the degree of synchrony has not as yet been determined. The 4-hour inhibition is used to demonstrate the technique because in preliminary experiments this length of time gives the best results in terms of shortest time interval for the division burst. The increase in population during the first division burst varies between 70 and 100%.

V. Concluding Remarks

The action of VLB remains unknown, but it apparently acts at a specific point in the cell cycle of *Tetrahymena* judging from the phasing of division after the release of inhibition.

The observation that unautoclaved medium overcomes the inhibition by VLB is an indication that it may be possible to learn more about the mode of action of VLB. The approach that is being taken is to grow *Tetrahymena* on synthetic medium and to vary the concentration of the components to determine the effectiveness of VLB as an inhibitor. Also, studies on the effects of VLB on protein and nucleic acid synthesis are underway.

Only qualitative observations are available on the possible time when inhibition occurs during the cell cycle. Cytologically the micronuclei after Feulgen-staining reaction in strain WH6 appear to be in division after 4 hours of inhibition. However, no readily apparent differences at the light microscope level exist between macronuclei of VLB-inhibited cells and control cells.

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Chapter 9

Physiological Studies of Cells of Root Meristems

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I. Introduction

Growing roots provide an excellent and convenient system for the study of cell development. The three phases of plant cell morphogenesis, viz. (1) mitosis and mitotic cycles, (2) cell enlargement, and (3) the development of cells with specialized functions and structures, occur in more or less distinct zones of the root. This spatial separation of different developmental phases follows from the growth pattern of primary plant organs. Primary growth is linear, occurring along one axis. Thus, new cells are generated, by mitosis, at the apex and with continuous mitotic activity the daughter cells are gradually displaced, along the axis of growth, away from the region of active cell proliferation. The morphogenesis of the cell, from a proliferative stage to a nondividing and specialized stage occurs within the first 1–3 mm of the root meristem.

The linear pattern of growth results from an organizational polarity that affects both dividing and expanding cells. Superimposed on this system is the presence of rigid cell walls. The walls prevent cell migration and preserve the cellular architecture of the meristem; they allow analysis of the patterns that existed before an experimental treatment and make possible the study of individual cell lineages in the succeeding period of recovery.

Root meristems, therefore, are a source of readily accessible proliferating cells; the mitotically active cells are grouped together at the root apex and, basal to them, their daughter cells are arranged in vertical columns. In growing roots, under standard conditions, the size of the population of proliferating cells remains fairly constant, i.e., the original group of meristematic cells generates, in the mean doubling time, an equal number of cells that are displaced from the meristem and enter the region of cell elongation. This system can be prolonged, in its steady state condition, by organ culture of the roots.

It should also be noted that many root systems develop secondary or lateral roots. These roots arise *de novo* and eventually reproduce the linear growth pattern of the parent root, i.e., they show the development of organizational polarity. The groups of meristematic cells, the primordia, that give rise to lateral roots undergo, while still mitotically

active, a series of morphogenetic changes. As primordia develop they show distinct changes in mitotic activity and the duration of mitotic cycles, in sensitivity to various drugs and antimetabolites, in sensitivity to X-rays, and in their ability to incorporate labeled precursors of DNA.

Root systems, particularly in species with a small number of large chromosomes, have been used intensively in studies at three levels: (1) *chromosomal*—structure of chromosomes, the replication of chromosomal DNA and synthesis of histones, the partition of DNA between daughter chromosomes, and the stability of DNA molecules; (2) *cell populations*—meristems as steady state populations, stem cell and proliferating components of meristems, the effects of experimental treatment on the steady state system and the behavior of heterogeneous populations, such as mixtures of diploid and tetraploid cells; (3) *root growth*—the effects of changes in the supply of growth factors and metabolites on organized growth. The object here is to describe the materials and techniques by which such studies are being made and also to indicate some of the problems of cell growth and morphogenesis in growing roots.

II. Growing Roots

Some of the techniques of germinating seeds and growing roots have been described (Wolff, 1964).

A. *Vicia faba*

This species is excellent for cytological studies. The diploid complement consists of 12 chromosomes. *Vicia faba* var. *minor* is the dwarf bean. The seeds are about 2.0×1.5 cm, somewhat smaller than var. *major*, whose seeds are $2.5–3.0 \times 1.5 \times 2.0$ cm. The larger seeds are easier to handle.

1. Soak seeds for 24 hours in distilled water at 20°–22°C. It is best to soak the beans, 1 or 2 layers deep, in flat dishes and keep them just covered with water.

2. Remove seed coat taking care not to break the radicle.

3. Stand the beans, radicle down, on the surface of 3 to 4 inches of moist sand. When all the beans have been planted, cover with moist sand and leave for 2 to 3 days at 20°–22°C. Sand should be washed and sterilized before use. Vermiculite may be used instead of sand.

4. Remove the beans and gently wash in distilled water. Mount the beans on fairly rigid wires (2.5 mm stainless steel) and suspend over

water. Large numbers of beans can be grown in Perspex tanks ($12 \times 10 \times 14$ inches).

5. The wire should be inserted near the top of the cotyledons, then the whole base of the bean can be kept in water, insuring that the base of the root never dries out.

6. Beans will grow well for 4 to 6 days in distilled water (pH should be kept at about 6 to 6.4) but growth is better if nutrients are added. Half-strength Hoagland's solution is satisfactory (Hoagland and Broyer, 1936).

7. The water should be aerated and changed every 24 hours. Small aquarium pumps provide adequate aeration; one will serve 2 tanks.

During germination and the early growth of seeds of *V. faba* in water culture many seeds succumb to bacterial or fungal infection. In general we find it necessary to plant 100 seeds for every 50 growing roots that are needed for an experiment.

The wires used to suspend the roots in water provide a convenient method of handling 10–12 beans. Beans can also be transferred from wire to wire in order to group beans at the same stage of development. Lateral roots appear about 4 days after the beans are placed in water culture. Other species of *Vicia* have smaller seeds and cannot be mounted on wire. They must be handled like peas (*Pisum sativum*).

B. *Pisum sativum*

The chromosomes of the pea ($2n = 14$) are smaller than those of *V. faba*. Though *Pisum* has been used less extensively for cytological studies it has been a valuable tool in studies of cell populations and cell cycles. It has the added advantage that it can readily be used for root organ culture.

Peas can be germinated and handled in the same way as beans. It is not necessary to remove the seed coat before planting in moist sand or vermiculite. Peas can be mounted on wire but it is more difficult to make sure that the bases of the roots do not dry out overnight. An alternative method of culture is to suspend them in water on metal or plastic mesh. It may be necessary to coat the metal mesh with a spray plastic to prevent metallic poisoning (Van't Hof, personal communication).

The disadvantage of using mesh is that the peas cannot be selectively isolated once they have developed lateral roots. If lateral roots are to be used in addition to primaries, the peas should be mounted on wires and an apparatus set up to drip water into the culture tanks to replace water loss due to evaporation.

C. *Allium cepa*

This is also good cytological material ($2n = 16$). The dried scales at the base of the bulb should be removed and the bulbs then suspended over water at 20°–24°C. The water should be changed every 24 hours. It may also be necessary to remove the dry outer leaves.

In buying onions it is best to go to a seed merchant. The bulbs available in supermarkets have generally been treated to prevent sprouting and such bulbs are a poor source of growing roots.

Allium cepa does not usually produce lateral roots, though it may do so from older roots that growing slowly or have stopped growing. Lateral root formation can also be induced (Section VI,A).

D. *Hyacinthus orientalis*

This is excellent material for chromosomal studies ($2n = 16$). The hyacinths available commercially constitute a remarkable aneuploid series ranging from diploids to hypertetraploids (Darlington *et al.*, 1951). In view of recent reports of the deleterious effects of aneuploidy on development and the changed patterns of thymidine-H^3 incorporation in unbalanced complements, it would seem worthwhile to reinvestigate the various chromosome races of the hyacinth using modern techniques.

The plants should be handled in the same way as onions.

E. *Zea mays*

Maize roots have been used in studies of meristem organization. The chromosomes ($2n = 20$) are small and less satisfactory than those of *V. faba* or *A. cepa* for cytological studies. The boundaries of the histogens, however, are more clearly defined in roots of *Zea* and other grasses than in roots of *Vicia* or *Pisum;* thus, they are excellent for studies of the organization of the quiescent center and root histogens (Clowes, 1961 a,b; Whaley *et al.*, 1960).

Maize can be germinated in moist sphagnum or vermiculite at room temperature. Growing roots are ready for use after 4–6 days.

F. Grasses

Grasses, in general, do not have a large caryopsis (*seed*) and for ease of handling they are germinated in petri dishes on moist filter paper. They should be kept at 22°–25°C, in the dark, and after 3–4 days the

primary root will be 5–10 mm long. *Hordeum vulgare* ($2n = 14$) is satisfactory for cytological studies but many grasses are not; they have large numbers of small chromosomes. The thin, threadlike roots of grasses are excellent material for the study of root elongation since the roots will grow between 2 glass slides and their growth can be followed photographically (Brumfield, 1955).

Grass roots also provide material for the study of asymmetrical cell divisions. In the epidermis of the roots, hairs are formed. The cell that produces the hair, the trichoblast, is the smaller of two cells produced by an unequal cell division (Sinnott and Bloch, 1939). As initiation of the hair begins, marked changes occur in the enzyme complement and RNA content of the trichoblast (Avers and Grimm, 1959) and the nucleoli increase in size (Rothwell, 1964; Lowary and Avers, 1965); these changes do not occur in the adjacent hairless sister cell. Thus, within one mitotic cycle there is an asymmetrically placed mitotic spindle followed by divergent development of the two, unequal sister cells. The cells undergoing these changes are epidermal and readily accessible.

III. Culture of Roots and Root Cells

Excised roots can be grown in culture. The growing conditions can be varied in many ways but the variations of physiological interest are those that (1) affect the ability of the cells to grow and divide; (2) change the pattern of organization of groups of cells; (3) lead to the production of calluses and perhaps suspensions of single cells or small clumps of cells; (4) control the pattern of differentiation or senescence. The main experimental approaches using root cultures attempt to maintain the growth of an excised meristem, to convert a meristem which is organized into a disorganized callus, to induce, in culture, mitotic activity in cells that were nonmitotic *in vivo*, and to induce organ formation in groups of cells stimulated to become meristematic.

A. Culture Media for Excised Roots

White (1942, 1943) has defined a basal medium (Table I) for root growth, and there are now a number of modifications and improvements designed to satisfy the requirements of particular species. Addition of ferric ethylenediaminetetraacetic acid (FeEDTA) helps to maintain adequate available amounts of ferric ions. Street and McGregor (1952) also recommend the addition of 0.005 ppm Cu^{2+} (as sulfate) and 0.001 ppm Mo^{3+} (as acid).

TABLE I
BASAL MEDIUM FOR ROOT GROWTH[a]

Component	Amount	Component	Amount
$MgSO_4$	360	$MnSO_4$	4.5
$Ca(NO_3)_2$	200	$Fe_2(SO_4)_3$	2.5
Na_2SO_4	200	$ZnSO_4$	1.5
KNO_3	80	H_3BO_3	1.5
KCl	65	KI	0.75
NaH_2PO_4	16.5		
Glycine	3.0	Pyridoxine	0.1
Nicotinic acid	0.5	Thiamine	0.1
Sucrose	20 gm		
Agar	5 gm		

[a] Composition of basal medium given in milligrams/liter of double-distilled or demineralized water except where indicated (White, 1943).

Torrey's basal medium for pea roots (1954) differs slightly from White's medium. He uses the following inorganic constituents in addition to those used by White (Table I):

$Ca(NO_3)_2 \cdot 4\ H_2O$	242 mg
$MgSO_4 \cdot 7\ H_2O$	42 mg
$FeCl_3$	1.5 mg
$CuSO_4 \cdot 5\ H_2O$	0.04 mg
$Na_2MoO_4 \cdot 2\ H_2O$	0.25 mg

and added only thiamine·HCl (0.1 mg), nicotinic acid (0.5 mg), and sucrose (40 gm) to each liter of medium. Roots of *Convolvulus arvensis* grow on the same inorganic medium as pea roots and require the addition of only thiamine and sucrose (Torrey, 1967).

B. Culturing Roots

Growing roots from young seedlings should be excised and surface sterilized. They may be grown on an agar medium or in liquid culture. Van't Hof (1966b) transferred pieces of pea roots 1 cm long to 125 ml Erlenmeyer flasks containing 50 ml of White's medium. In each flask 10 or 11 root apices were cultured. They were kept at 23°–25°C and were shaken at 60 cpm. The composition of the medium was varied by changing the amount of sucrose present. In the absence of sucrose, mitotic activity in the meristem decreased; it was low 24 and 48 hours after transfer and was absent by 72 hours. The roots were transferred to flasks of fresh media that contained sucrose and tritiated thymidine and the onset of DNA synthesis was followed.

The gradual fall in the number of cells entering the S-phase can be shown to be due to the depletion of sucrose and it provides a system for studying requirements for energy sources. This type of system in which the composition of a meristematic population can be controlled, i.e., changing it from a mixed population of G_1, S, G_2, and mitotic cells into one that consists mainly of G_1 cells and lacks mitotic cells completely, presents a chance to define the differences between the types of synthetic events that occur in G_1 compared with G_2 cells. It could be particularly valuable in studies of the synthesis of spindle proteins and the time of action of growth substances.

Segments of roots that do not include the meristem have also been cultured. Pieces of roots of *Convolvulus arvensis* were excised and transferred to Torrey's pea medium containing agar (Section III,A). The pieces were 1.5 cm long and were taken 1.5–16.5 cm behind the apex. These cultured segments develop new meristems from pericycle cells. Some of the newly developed meristems give rise to lateral roots but some produce shoot buds (Bonnett and Torrey, 1966).

C. Root Calluses

Segments of roots can readily be stimulated to produce calluses in culture. One crucial addition to the basal medium appears to be the presence of an auxin [for example, indoleacetic acid (IAA) or 2,4-dichlorophenoxyacetic acid (2,4-D)] at a concentration of about 10^{-6} *M*. In many of the early studies coconut milk was also added. Caplin and Steward (1948) used White's medium with 2% sucrose and 0.8% agar at a final pH of 5.6 for the culture of cells from the secondary phloem of the carrot tap root. Added IAA stimulated growth: in 21 days explants weighing 4 mg increased to 8.2 mg in the presence of 0.01 mg per liter IAA. With 15–20% coconut milk the 4-mg explant increased to 184.0 mg in 21 days. Coconut milk, like yeast extract, has the great drawback of being a mixture of unknown composition, and it has gradually been replaced by artificial media of known composition.

Root calluses have been initiated from explants of mature regions of growing roots and from secondary tissue of storage roots, such as the carrot.

1. Carrot Root Callus

Young roots, 3–5 cm in diameter, should be washed and surface sterilized. Wash thoroughly and cut the root transversely into disks. A cork borer (diameter 2-4 mm) should be sterilized and can then be used to cut out sections of secondary phloem. Disks containing cambium

or parts of a lateral root should be discarded. The disks are then placed in culture.

Foskett and Roberts (1965) used four media: (1) 2% sucrose and 1% agar (SA); (2) Heller's mineral medium + SA (NA); (3) NA medium + 10^{-5} gm per liter 2,4-D (H4); (4) H4 medium + 15% coconut milk (HW). With SA or NA media there was little mitotic activity and no callus formation. With H4 and HW, cell division continued for 6 days. Subsequently (9–15 days) the H4 explants showed poor development while the HW explants produced macroscopically visible calluses. The enzymatic activity of these cultured explants has been studied.

2. Cultures from Growing Roots

For callus cultures small segments of growing roots should be excised somewhere behind the apical meristem. Segments of 5 to 10 mm are suitable. On normal basal medium (Table I), with the addition of 10^{-6} 2,4-D, calluses will form. Their formation and growth are generally improved by the addition of myoinositol and adenine sulfate. *Convolvulus* roots produce callus growth readily on Torrey's basal medium (Section III,A) with the addition of the following (in milligrams per liter):

L-Glutamine	146
Myoinositol	90
Adenine sulfate	40.3
Pyridoxine HCl	0.5
Nicotinic acid	0.5
2,4-D	0.22

Callus production by pea roots occurs on a similar medium. The calluses can be transferred and maintained in culture and they will continue to grow as unorganized masses of cells. Slight changes in the culture conditions, however, can lead to the formation of free, single cells or small clumps of cells. One such change is to substitute a liquid medium for the agar substrate and to shake the cultures (Steward and Shantz, 1956). The addition of kinetin (10^{-6} *M*) can also produce this effect. The cells no longer adhere together so readily and many become free floating. Eventually the free cells turn the medium cloudy.

D. Studies of Single Cell Cultures

The cell suspensions obtained by liquid culture of calluses induced on tissue explants have played an important part in recent studies of the physiology of cell growth. Each change in cell behavior, it should be noted, calls for a different medium. From the suspension obtained

from the calluses, the cells are induced to re-form multicellular masses, produce organized tissues and organs, and give rise to differentiated cells.

Isolation procedures for single cells involve either filtration through a coarse cloth, such as fiberglass filter cloth, or through stainless steel sieves (Halperin, 1966). The single cells and small clusters can be plated on agar medium or grown in liquid culture. The studies of these cultures have revealed that in its morphogenesis the single cell may pass through phases closely similar to those that the zygote undergoes in embryogenesis (Steward *et al.*, 1958; Halperin, 1966) though in some cases this is not so (Vasil and Hildebrandt, 1965).

Gamborg (1967) has described a medium on which soybean root cells have been cultured without undergoing any detectable phenotypic change for 3 years. The mineral composition should be noted (Table II, cf. Table I).

TABLE II
COMPOSITION OF THE B5 MEDIUM FOR SUSPENSION CULTURES OF SOYBEAN CELLS[a]

Component	Amount	Component	Amount
$NaH_2PO_4 \cdot H_2O$	150	$MnSO_4 \cdot H_2O$	10
KNO_3	2500	H_3BO_3	3
$(NH_4)_2SO_4$	134	$ZnSO_4 \cdot 7H_2O$	2
$MgSO_4 \cdot 7H_2O$	250	$Na_2MoO_4 \cdot 2H_2O$	250 μg
$CaCl_2 \cdot 2H_2O$	150	$CuSO_4$	25 μg
Iron[b]	28	$CoCl_2 \cdot 6H_2O$	25 μg
Nicotinic acid	1	KI	750 μg
Thiamine·HCl	10	Sucrose	20 gm
Pyridoxine·HCl	1	2,4-D	2
m-Inositol	100		

[a] Gamborg, 1967.

[b] Sequestrene 330 Iron obtained from Geigy Agricultural Chemicals, Saw Mill River Road, Ardsley, New York. Compounds in milligrams/liter except where indicated; pH 5.5.

Cell division has been observed in free-cell cultures maintained on a complex medium (Torrey and Reinert, 1961) but has not been studied to any great extent. Mitotic indexes of suspension cultures of *Linum usitatissimum* were low (Henshaw *et al.*, 1966), though that in itself should not prevent studies of the cytological changes that occur in some cultures, for example, the formation of polyploid cells. Furthermore, Pollard *et al.* (1961) and Muir (1965) have shown that different clones of cells established from single roots may respond differently to growth stimulators. Analysis of this response and of the effects of mutagenic

agents on single plant cells in culture could reveal a great deal about cell morphogenesis.

IV. Use of Isotopically Labeled Precursors

A. Incorporation of Tritiated Thymidine

Tritiated thymidine (TdR-H^3) is incorporated specifically into the DNA of cells undergoing replication. From the initial experiments (Taylor *et al.*, 1957) it has become clear that labeling with TdR-H^3 provides a tool for analyzing the replication of chromosome segments, whole chromosomes, and complements and populations of cells.

TdR-H^3 penetrates root cells rapidly and incorporation can be detected, in cells undergoing S, within a matter of minutes. Long treatments are chosen for specific purposes, not to insure satisfactory incorporation, which is achieved with samples of TdR-H^3 of moderate radioactivity given for as little as 30 minutes (Table III). The speed with which incorporation occurs indicates that the exogenous thymidine is rapidly phosphorylated. The cells must possess the necessary kinases to produce thymidine triphosphate at the time of treatment; without them there would be no incorporation or incorporation only after a lag period during which the kinases were induced (see this Section, A,5).

1. Chromosome Replication

Chromosome replication and the stability of the newly synthesized polynucleotides in subsequent interphases have been studied in cells exposed to TdR-H^3 for several hours (Taylor *et al.*, 1957; Taylor, 1958b; LaCour and Pelc, 1959; Peacock, 1963). Long exposures, 4 hours or more, have also been used in determining the onset of S and mitosis in roots of germinating seeds (Davidson, 1966) or excised roots deprived of an energy source (Van't Hof, 1966b).

The questions concerning (1) the stability of a newly synthesized DNA polynucleotide of a chromosome, i.e., whether or not sister exchanges occur, (2) the number of polynucleotides that make up a G_1 chromosome or a G_2 chromatid, and (3) whether the DNA polynucleotides extend from one end of a chromosome to the other have been under consideration for the past 10 years. To a large extent the questions have been formulated on the basis of results from experiments using TdR-H^3. The experimental systems used, however, have certain aspects that suggest some caution when considering the results.

TABLE III

TREATMENT OF ROOTS WITH TRITIATED THYMIDINE

Species	μc/ml	Specific activity (c/mmole)	Treatment duration	Exposure	References
Vicia faba	30	About 3	8 hours	—	Taylor *et al.*, 1957
	0.2	0.208	1 hour	11–45 days	Howard and Dewey, 1960
	2.0	3.0	5–60 minutes	3 weeks	Evans, 1964
	2.0	1.9	4, 12, 24 hours, and continuous	12 days	Davidson, 1966
Tradescantia paludosa	0.7	0.118	8 and 10 hours	—	Sisken, 1959
	0.5	3.6	30 minutes	3 months	Wimber, 1966
Pisum sativum	1.0	6.7	Up to 24 hours	2 days	Van't Hof, 1966c
Scilla campanulata	5.0	5	30 minutes	10 days	Evans and Rees, 1966

(*a*) Thymidine itself and colchicine, which has been used in a number of experiments for periods of treatment that are considerably in excess of the period needed to induce tetraploid cells, have effects on the mitotic cycle. They both affect mitosis and colchicine affects the rate at which cells progress to mitosis.

(*b*) Pools of thymidine or pools that contain some TdR-H^3 may exist and they would distort study of labeling pattern at X_2 or X_3 metaphases. Even in situations in which part of the change in labeling intensity between two successive fixations is due to the fact that some of the cells were about to complete S, the results suggest that incorporation continued for some time after roots were removed from the TdR-H^3 solution. Wimber [(1961), cf. Figs. 1 and 2)] shows two cells, one fixed an hour after the other; the intensity of labeling is greater in the cell from the root left for an additional hour after the 15-minute treatment, even though the root was treated during that hour with unlabeled thymidine at a concentration of ca. 3×10^{-5} *M*. This is approximately 100 times the strength of the labeled thymidine solution.

The incorporation of exogenous TdR-H^3 also depends upon the ability of a cell to phosphorylate the thymidine. Cells vary in their ability to carry out phosphorylation of thymidine (this Section, A,5).

(*c*) Almost without exception the plants (and animals) used in studies of chromosome replication have large chromosomes and contain comparatively large amounts of DNA. A number of species that are used in cytological and physiological studies were examined by McLeish and Sutherland (1961). The DNA values are shown in the following tabulation.

Species	DNA per cell (gm $\times 10^{-11}$)	Ratio
Tradescantia ohiensis	10.77	1.78
Scilla campanulata	9.46	1.56
Allium cepa	7.87	1.3
Vicia faba	6.05	1.0
Pisum sativum	2.14	0.35
Zea mays	1.55	0.25
Lupinus albus	0.55	0.091

The first 4 species are used in cytological studies; *P. sativum* is used in cell cycle studies, less frequently for chromosomal studies, and *Z. mays* is used in studies of meristem organization. *Vicia faba* has been shown to have 7 times more DNA than the related species *V. sativa* (Rees *et al.*, 1966) though both have 12 chromosomes. It appears that

the advantages of large size may be offset by a complexity of structural organization. The effect of such complexity on the behavior of labeled strands could be examined either by comparing the response of *V. faba* and *V. sativa,* or by comparing the behavior of the different chromosomes in a species hybrid.

Asynchrony of replication has been described in several plants (Taylor, 1958a; Pelc and LaCour, 1959; Wimber, 1961; Evans and Rees, 1966). Asynchrony is an inevitable result if S lasts several hours and if there is sequential replication along a linear template. Where asynchrony affects a specific chromosome or segment, such as a heterochromatic region, it suggests genetic control of the behavior of that segment. The other type of asynchrony appears to be of variation in the rate of synthesis of DNA (Howard and Dewey, 1961). That will also affect the pattern of labeling intensity found at different times.

2. Mitotic Cycles

The rapidity of penetration, incorporation, and exhaustion of exogenous TdR-H^3 has made it possible to pulse-label meristematic populations and, by following changes in the numbers of labeled mitotic figures with time, to estimate the durations of the G_1, S, G_2, and mitotic phases of the mitotic cycle. These phases were defined in a study using P^{32} (Howard and Pelc, 1953), and a method using a pulse label was developed by Quastler and Sherman (1959). The way in which the curves of labeled mitotic figures or metaphases are analyzed has been described and its limitations discussed by Sisken [(1964), pp. 393–397]. Mitotic cycles have also been analyzed using a technique of double labeling with C^{14}- and H^3-labeled thymidine (Wimber and Quastler, 1963).

In most roots growing at temperatures between 19° and 24°C, the mean duration of a mitotic cycle varies from 14 to 20 hours. For a study of the duration of mitotic cycles, growing roots or excised roots in liquid culture are handled as follows: (1) Transfer roots to a medium containing TdR-H^3. Solutions of TdR-H^3 (2.0–5.0 μc/ml) with a specific activity of about 3 c/mmole (Table III) provide satisfactory results. (2) Wash thoroughly and return to culture medium or, if a chase with nontritiated thymidine is to be used, the roots should be washed and transferred to the thymidine solution before being returned to the culture medium. (3) Roots should be fixed in freshly mixed and chilled acetic acid-alcohol (1:3) at approximately hourly intervals. Longer intervals between fixation may result in a curve of labeled mitoses that misses the first peak of labeled divisions. (4) Root squashes can be made in the usual way and the coverglass removed by the dry-ice method

(Section VIII,A,2). The cells adhere to the slide. Wash the slide in water and dry. (5) Dip warmed, dry slides in liquid emulsion then dry, store, and develop after a suitable period of exposure [the method is given in Prescott (1964), pp. 366–370. Exposure times are discussed in this Section A,5].

This method has been used to estimate intermitotic times in normal roots and to study the effects of drugs and growth factors (Sections V and VI). It has also revealed that the duration of the mitotic cycle changes during root development. It changes, for example, within a meristem (Clowes, 1961b); cells in different parts of the meristem having different mitotic cycle times.

The intermitotic time can be determined, in the same root system, in cells of fully formed lateral and primary roots and in cells of developing primordia. Whole root systems are treated with TdR-H^3 for 30 minutes to 1 hour and serial fixations are made. After hydrolysis and staining (Section VIII) squashes are made of all meristems; those of fully formed roots are simply cut off, those of primordia are dissected out of the primary root (Section VIII,E).

In *V. faba* roots, cells of small primordia (about 1200–1500 cells) have a mitotic cycle time of 11.4 hours; in primary roots the cycle time is 17.8 hours (Davidson and MacLeod, 1967). This initial phase in the morphogenesis of primordia is followed by one, in primordia of greater than 1500 cells, in which no incorporation of H^3-TdR can be detected. We have already seen that in *Convolvulus* some primordia develop into buds, not into roots (Section III), and there is evidence that the response of cells of primordia to various experimental treatments differs from that of cells of fully formed roots (Section VI). At present little is known of the systems controlling the changes that occur in developing primordia.

3. Responses of Primordia to TdR-H^3

Large primordia of roots of *Pisum* (Hummon, 1962) or *Vicia* (Davidson and MacLeod, 1967) fail to show evidence of incorporation of TdR-H^3. Small primordia (i.e., less than 1500 cells) incorporate TdR-H^3, though the rate of incorporation is slow initially. The exogenous thymidine may not penetrate their cell membranes, though it does enter cells of small primordia. This change occurs, in *Vicia* roots, about the stage when a primordium consists of 1200–1500 cells, i.e., within one mitotic cycle the cells suddenly fail to incorporate exogenous thymidine. It may be that the cells of the large primordia are temporarily incapable of phosphorylating thymidine. This would be difficult to explain. The cells must either suddenly destroy or inactivate the thymidine kinases that

may have been present in the previous interphase, or, unlike cells of small primordia, they must fail to activate or synthesize the kinases.

The cells of the small primordia complete two mitotic cycles after labeling with TdR-H^3 since, when treated with colchicine immediately after TdR-H^3, they eventually form octoploid cells. The time of appearance of these cells gives no evidence of any mitotic delay. With long treatments with TdR-H^3, 12–24 hours (Hummon, 1962; Stein and Quastler, 1962) instead of 1 hour (Davidson and MacLeod, 1967), there is an inhibition of cell division of pericycle cells and of the growth of the primordia. Thus, it must be borne in mind that treatments with TdR-H^3 may have adverse effects.

4. Effects of Thymidine on Cells

Most treatments with TdR-H^3 use a concentration of thymidine that ranges between 10^{-5} and 10^{-6} *M*. Given for short periods, treatments with such concentrations would not be expected to produce cell aberrations. Some cells resist higher concentrations for longer periods. Barr (1963) found no effect of 10^{-2} *M* thymidine on barley roots treated for 26 hours. This is probably a unique case since other cells are affected. HeLa cells show some metaphase delay (Barr, 1963) and in rat liver cells the response to exogenous thymidine is to begin thymidine kinase production (Hiatt and Bojarski, 1960).

Metaphase delay has also been reported in roots of *Haplopappus gracilis*. The roots were treated with a 2.9×10^{-6} *M* solution, either of tritiated or unlabeled thymidine, for 30 minutes, returned to water, and fixed 6.3 hours after the end of treatment (Ames and Mitra, 1967). Both treatments resulted in an increase in the number of metaphases and of the mitotic index. Though fixations were made at only one time it seems unlikely that the effect will be confined to the cells in mitosis 6 hours after treatment. The effects of thymidine must obviously be studied in other organisms and after other periods of recovery in view of the widespread use of TdR-H^3 in studies of cell cycles.

5. Uptake of TdR-H^3

In *V. faba* roots, treatments with TdR-H^3 (2 μc/ml; specific activity, 3.0 c/mmole) for 5, 10, or 20 minutes and exposure of emulsion for 3 weeks produce nuclei with low grain counts. After these treatments the mean number of grains per nucleus varied from 5 to 8. Values around 20 grains per nucleus were found only after a 30-minute treatment and 3 weeks exposure (Evans, 1964). The duration of S in *V. faba* roots is between 6 and 7 hours: thus, it appears that reasonable numbers of grains

are obtained only if at least 10–15% of any S period occurs in the presence of labeled precursor. A similar situation occurs in *Pisum* (Van't Hof, 1966c).

Detection of incorporated TdR-H^3 also depends upon an adequate exposure time. In some systems, exposure for 1 or 2 days may be sufficient, but in roots a period of at least 10 days appears to be needed.

It has been pointed out in the previous section that large primordia of *V. faba* (Davidson and MacLeod, 1967) and *P. sativum* (Hummon, 1962) fail to show evidence of incorporation of TdR-H^3, even after exposures of 1 hour or more. A similar observation, i.e., failure of cells to incorporate TdR-H^3, has also been made on growing roots though, at best, sporadically. We have found that some lateral roots of *V. faba* fail to incorporate TdR-H^3. A similar observation has been made in roots of *P. sativum* (J. Van't Hof, personal communication). This type of observation and those on the effects of thymidine on cell cycles described in the previous section indicate that not enough attention has been paid to the biochemical versatility of the root cells or to the presence of stable pools of labeled precursors. Such phenomena are highly relevant to the discussion of any results that describe the labeling of chromosomes or chromatids 2 or more metaphases after the S period in which TdR-H^3 was supplied to the cells (cf. Section IV,A,1).

B. Tritiated Amino Acids

There has been relatively limited investigation so far of the synthesis and distribution at mitosis of nuclear and chromosomal proteins, as far as they can be studied using tritiated amino acids. There are limitations to a study of chromosomal protein in mitotically active cells that result from the solubility of histones in fixatives containing acids. But given suitable fixatives and staining (see Section VIII,D,2) the histones can be preserved. In addition to the use of neutral formalin and of TCA instead of HCl in the Feulgen procedure, Bloch *et al.* (1967) have also used enzyme treatments instead of acetic acid to soften cells.

The levels of radioactivity that provide satisfactory numbers of grains in autoradiographs (Table IV) are similar to those used with TdR-H^3 (Table III).

Roots of *V. faba* and *A. cepa* have been used in studies of the turnover of chromosomal proteins and the S period for histones. In *Vicia,* mitotic chromosomes had a mean grain count of 15.6 ± 4.8 at the first division after labeling but by the second division the count had fallen to 2.6 ± 2.7 (Prensky and Smith, 1964). These results should be extended using treatments that preserve histones.

TABLE IV
TREATMENT OF ROOTS WITH TRITIATED AMINO ACIDS[a]

Amino acid	Fixative	Hydrolysis	Radioactivity (μc/ml)	Specific activity	Treatment	Exposure	Observed	References
Arginine-H^3	10% neutral formalin	TCA	5	0.1 c/mmole	5–15 minutes	18 days	Nuclei	Mattingly, 1963
Lysine-H^3	10% neutral formalin	TCA or HCl	10 and 100	0.25 c/mmole	5–30 minutes	8 days, 5–20 days	Nuclei and chromosomes	Mattingly, 1963
Tryptophan-H^3	10% neutral formalin	TCA	10	0.5 c/mmole	5–15 minutes	50 days	Nuclei	Mattingly, 1963
Arginine-H^3	10% neutral formalin	HCl	2	c.100 c/mole	9.5 hours	116 days	Chromosomes	Prensky and Smith, 1964
Valine-H^3	Acetic-alcohol	HCl	20–40	40 c/mole	2 hours	10–20 days	Chromosomes	Alvarez, 1965
Argine-H^3	Neutral buffered formalin	TCA	1	1.05 c/mmole	30 minutes	2 weeks–2 years	Nuclei and chromosomes	Bloch *et al.*, 1967
Lysine-H^3	Neutral buffered formalin	TCA	1	0.2 c/mmole	30 minutes	2 weeks–2 years	Nuclei and chromosomes	Bloch *et al.*, 1967

[a] Activity of solutions, specific activity of the amino acid, and hydrolyzing acid are given.

C. Isotopic Studies of Meristem Organization

Meristems are not simply a group of cells undergoing perpetual mitotic cycles. In studies of cell cycles it may be convenient to treat meristems as a system of uniform components, but they are, in reality, highly complex. If angiosperm roots are exposed to TdR-H^3, incorporation, as we have seen (this Section IV,A), is rapid. The labeling index (LI) can be determined after a short treatment, for example, 10 minutes, with a labeled DNA precursor (however, see this Section, A,5). This value gives the percentage of cells in S; for a steady state system the LI should be a constant value. As the duration of treatment is extended, LI will increase, as more and more cells enter S in the presence of the labeled precursor.

Treatments of roots of *V. faba, A. cepa,* and *Z. mays* (Clowes, 1956, 1961a) for 1 to 3 days with adenine-C^{14} resulted in heavy labeling of all regions of the meristem except the dome-shaped group of cells that occupies the apex of the meristem. These cells constitute the quiescent center. They incorporate labeled precursors of DNA slowly, or not at all; they divide rarely (Jensen and Kavaljian, 1958) and exhibit low rates of protein synthesis (Jensen, 1957). However, following irradiation, these quiescent center cells enter S and can be labeled and become mitotically active (Clowes, 1961a).

The meristem consists of columns of cells, each of which is derived from its most apical cell. A gradient of mitotic activity exists along each column: (1) low mitotic activity and long mitotic cycles in the quiescent center; (2) high mitotic activity and short mitotic cycles in the region of the apical initial cells; and (3) a gradual decrease in mitotic activity and an increase in mitotic cycle time along the root. This system and that leading to the establishment of a group of nondividing cells within a meristem, as happens when a primordium develops into a lateral, suggest an elaborate mechanism of control of cell division operating within distances occupied by a row of 5 or 6 cells.

V. Treatments with Drugs and Antimetabolites

Analyses of cell population have been carried out using TdR-H^3 (Section IV), mitotic inhibitors, of which the best known is colchicine, and inhibitors of either DNA or protein synthesis, such as 5-aminouracil and actinomycin D. Radiomimetic chemicals have also been used, particularly

in studies of the relative sensitivity of chromosomes to such agents at different stages of the cell cycle.

A. Colchicine

1. Metaphase Accumulation and Chromosome Contraction

Perhaps the best known property of colchicine is its ability to inhibit anaphase. The consequences of suppression are an increase in the number of metaphases and an increase in the degree of chromosome contraction. The whole complex of effects on mitotic cells produces a complement of shortened chromosomes that separate easily on squashing and whose constrictions are clearer than in normal cells. Thus, in karyotype analysis or in chromosome number determinations a pretreatment with colchicine immediately before fixation has become standard practice.

2. Colchicine Solutions

Colchicine is readily soluble in water and can be kept at 2°–5°C as a stock solution of 0.1% or 0.05%. Pure colchicine consists of pale yellow crystals but not all samples purchased are pure. It may be necessary to dissolve in water, filter, to remove a white precipitate that forms, and recrystallize. Since some crude extracts of colchicine contain ethyl acetate the solution should be aerated before it is allowed to crystallize. Roots to be treated should be transferred to 0.01–0.5% solutions for 2 to 4 hours. The solutions should be aerated and should be brought to the desired temperature before the roots are placed in the solution. Fix roots immediately after treatment (see Section VIII).

3. Induction of Polyploid Cells

If roots treated with colchicine are washed and allowed to continue to grow, the cells at metaphase undergo restitution, i.e., all the chromatids are included in a single nucleus. In a diploid cell, the restitution nucleus that forms has 4C DNA and is tetraploid. After one mitotic cycle the tetraploid cells enter mitosis.

This technique marks one population of cells and their cycle times, duration of S and response to other agents can be determined.

a. Pisum sativum. The technique described by Van't Hof (1966a) for the induction of a population of tetraploid cells is as follows:

1. Seedlings are grown in Hoagland's nutrient solution at 23°C. When the primary roots are about 3.5 cms long, transfer to a solution of colchicine (3.76×10^{-4} *M*).

2. Treat with colchicine for 30 minutes. Wash and return to culture tanks.

3. Make serial fixations (every 1 or 2 hours) in chilled acetic acid-alcohol.

4. Prepare roots as Feulgen squashes (Section VIII).

The frequency of dividing cells that are tetraploid is determined. A typical plot of percent tetraploid mitoses shows that their peak occurs 14 hours after treatment. This value is taken as the mean intermitotic time of the tetraploid cells.

The curve for the tetraploid cells shows the variation in cycle time inherent in any cell population; of the cells at one metaphase only 60%, in *Pisum*, are still in division together at the subsequent metaphase.

b. Vicia faba. Using a 3-hour treatment with colchicine we have found that the concentration of colchicine used affects the number of tetraploid cells subsequently seen in division (Table V). It appears from

TABLE V
RESULTS ON METAPHASE AFTER TREATMENT WITH COLCHICINE[a]

Hours from beginning of treatment	Percent of Colchicine					
	0.000125	0.00125	0.0125	0.025	0.5	1.0
9	—	—	—	—	—	—
11	—	—	3.6	—	—	—
13	—	6.2	5.8	1.6	—	—
15	1.4	17.2	11.0	21.2	20.2	0.4
17	—	11.4	9.2	25.4	3.2	1.6

[a] Results are expressed as percent metaphases that are tetraploid in roots of *V. faba* following a 3-hour treatment with colchicine. Each value is based on 500 metaphases.

these results and those using *Pisum* (Van't Hof, 1966c) that colchicine not only induces tetraploidy but it alters the duration of the mitotic cycles of the tetraploid cells. This effect occurs both in cells induced to become tetraploid and in diploid cells that are in interphase at the time of treatment (MacLeod and Davidson, 1968).

4. ANALYSIS OF MIXED CELL POPULATIONS

The result of a colchicine treatment is the formation of a population that includes diploid, tetraploid, and perhaps octoploid cells. These mixed populations have been used to study the effects of inhibitors such as dinitrophenol (DNP) (Van't Hof *et al.*, 1960). They have also yielded

information on the effects of auxins, such as IAA, on mitotic cycles (see Section VI), confirming results obtained on normal diploid populations.

Though mitotic activity continues for about 24 hours at normal or above normal levels in roots treated with colchicine, it eventually falls to low levels (Davidson *et al.*, 1966; MacLeod, 1966; MacLeod and Davidson, 1966). Thus the colchicine-induced stimulation is temporary. The mixed condition of the meristem is also temporary, and the roots gradually revert to the diploid condition (Davidson, 1961, 1965). However, the apical tumor induced by colchicine gives rise to laterals that have mixed meristems (Davidson, 1965), and they appear to remain as stable chimeras for a period of up to 10 days. They also provide an excellent system for comparative studies of the behavior of diploid and tetraploid cells. Thus by varying the colchicine concentration it is possible to produce laterals that have mainly diploid cells and some tetraploids, or mainly tetraploid roots with some diploid cells.

B. 5-Aminouracil

5-Aminouracil (5-AU) is an analog of thymine. Treatments of root meristems with 5-AU leads to a much extended S period. The results are (1) cells in G_2 at the time complete this phase, pass through mitosis, and enter G_1; (2) cells in S are held there for periods 25–40% longer than the normal S (Jacob and Trosko, 1965); (3) G_2 is also extended, both in cells in G_2 at the time of treatment and in cells that enter G_2 after a delay in S; (4) the consequence of the effects listed in (1)–(3) is a shift in the relative proportions of nuclei in G_1, S, and G_2 from the normal distribution to an excess of cells in G_1 and S and also a marked decrease in mitotic index (MI), which may fall from 7 or 8% in normal roots to 0.5% or less after treatment.

The weakest concentrations that are effective contain 125 ppm 5-AU, but in general solutions of 250 or 500 ppm are used. Growing roots should be transferred to aerated solutions and fixations made periodically. Primary or lateral roots of *A. cepa* of *V. faba* provide convenient expermental material.

In *A. cepa,* Duncan and Woods (1953) found that after 24 hours of continuous treatment, 31 or 63 ppm had reduced the MI to 5%; 125, 250, and 500 ppm reduced it to 1 or 2%. The control MI was 8%. In *V. faba* the effect on the MI is more pronounced, and after 24 hours less than 1% of cells may be undergoing mitosis. *Allium cepa* and *V. faba* recover from the effects of 5-AU treatment, and sometime after they are returned to water a rise in the MI occurs. For example, 14 hours after 24 hours in 500 ppm 5-AU, the MI in *V. faba* reaches 42% (Smith

et al., 1963), in *A. cepa*, 14 hours after a 24-hour treatment with 250 ppm 5-AU, the MI is 48%, (Prensky and Smith, 1965).

Studies of the response of meristems to 5-AU have been concerned with: (1) the effect on the duration of S and the basis for the inhibition induced by 5-AU, (2) effects on other phases, for example, G_2, (3) effects on chromosome structure and organization.

1. Effect on S

5-Aminouracil does not inhibit S, it prolongs it. Thus, cells will eventually complete S and later appear in division. This is shown by the spontaneous recovery of cells and by the fact that in roots treated with colchicine and then 5-AU, tetraploid cells appear in mitosis (Mattingly, 1966a). A direct demonstration is made by studying incorporation of TdR-H^3 or CdR-H^3 (Jacob and Trosko, 1965).

TABLE VI
Results of Treatment with 5-Aminouracil on *V. faba* Nuclei[a]

5-Aminouracil					
Concentration (ppm)	Treatment (hours)	Labeled precursor	Period in label (hours)	Labeling index	Grains/ nucleus
500	6	CdR-H^3	2	67.5	—
—	—	CdR-H^3	2	42.7	—
500	6	TdR-H^3	2	62.2	—
—	—	TdR-H^3	2	46.1	
750	4	TdR-H^3	1/2	51.7	5.4
—	—	TdR-H^3	1/2	42.7	20.9

[a] Labeling of nuclei in roots of *V. faba* treated with 5-aminouracil and TdR-H^3 (4μc/ml) or CdR-H^3 (5 μc/ml). Data from Jacob and Trosko (1965).

Roots of *V. faba* were treated with 5-AU and then with 5-AU containing TdR-H^3 or CdR-H^3. The labeling index and grain counts for nuclei were determined (Table VI). It is clear that incorporation of labeled precursors occurs in the presence of 5-AU, that after 4 to 6 hours of treatment with the inhibitor the number of nuclei in S increases (by approximately 50%), and that the rate of incorporation of TdR-H^3 is decreased to about 25% of the normal rate. The effect on DNA synthesis also appears to extend to CdR-H^3 incorporation, which is affected in the same way as TdR-H^3 incorporation. This would be expected if the supply of thymidine is rate limiting in DNA synthesis. Jacob and Trosko's results (1965) reported in part in Table V do not agree with those of Prensky and Smith (1965), and the matter requires examination.

Since the response of large primordia to exogenous TdR-H^3 differs from that of primary and lateral roots, we have examined the response of primordia to 5-AU (D. Davidson, S. H. Socher, and M. J. Preece, unpublished). They show a similar inhibition of the MI initially, i.e., at 6 and 12 hours when treated with 500 ppm 5-AU, but by 24 hours their MI has risen to 20, while in laterals it is less than 0.5 This spontaneous recovery, even in the presence of 5-AU, is similar to the recovery seen at later times in roots (Mattingly, 1966a).

In view of the spontaneous recovery in the continued presence of 5-AU, it appears to be anomalous that the number of nuclei that incorporate TdR-H^3 immediately after a 24-hour treatment with 5-AU is so low. Smith *et al.* (1963) report that 13.8% of nuclei in *V. faba* lateral roots became labeled after a 1-hour exposure to TdR-H^3 (1.5 μc/ml), whereas one might expect a high labeling index on the basis of results reported by Jacob and Trosko (1965), which show that S is apparently extended by 5-AU, thus increasing the number of cells in S. The labeling index did not reach 51% (cf. Table VI) until 4½ hours after removal from 5-AU, 3 hours in water, and 1½ hours in TdR-H^3.

5-AU is potentially useful in controlling cell cycles chemically but at present there are inconsistencies between reports of its effects.

2. Effects of 5-AU on G_2

Roots have been labeled with TdR-H^3 and then treated with 5-AU. Labeled cells are delayed in their passage through G_2, indicating that 5-AU also affects cells not in S (Prensky and Smith, 1965; Mattingly, 1966a).

3. Chromosome Structure

Anaphase chromatids show unstained, i.e., Feulgen negative, gaps when treated with 5-AU, and cells that have completed telophase may contain micronuclei (Duncan and Woods, 1953). Some micronuclei may be trailing segments that result from an unstained gap but some result from chromosome breakage. 5-AU induces chromosome breakage (Chu, 1965) though the breakage frequency per cell is low (0.1). Thus, in addition to its effects during S and DNA synthesis, 5-AU also affects the structural organization of chromosomes.

4. Differential Response to 5-AU

Recovery from 5-AU and the more or less synchronous division of large numbers of cells some time after the beginning (though not necessarily the end) of treatment does not occur simultaneously in all cells of a meristem (Clowes, 1956; Mattingly, 1966b) nor at the same time in roots and primordia (this Section B,1). The extent of the effect of 5-AU

on S and G_2 may be related to the duration of the mitotic cycle; it differs in different cells, and that may be involved in their differential response.

C. 5-Fluorodeoxyuridine

Another analog of thymine is 5-fluorouracil. Its deoxyriboside, FUdR, is an effective inhibitor of DNA synthesis. On entering the cell, FUdR is phosphorylated and the monophosphate inhibits the action of thymidylate synthetase (Cohen *et al.*, 1958). FUdR induces chromosome breakage, and it has been suggested that it does so by interfering with the endogenous availability of thymidine and its phosphorylated derivatives (Taylor, 1963). It has been shown, however, that the radiomimetic action of FUdR is not confined to S but also occurs in G_2 (Bell and Wolff, 1964).

The endogenous supply of thymidine is clearly involved in the effects induced by FUdR since treatment with thymidine can lead to a reduction in the yield of aberrations, provided there is an excess of thymidine. While a 4-hour treatment with FUdR ($3 \times 10^{-6} M$) and thymidine ($10^{-5} M$) results in a slight, though probably not significant, increase in the percentage of damaged cells as compared with treatment with FUdR ($3 \times 10^{-6} M$) alone (Ahnström and Natarajan, 1965), when the concentration of thymidine exceeds that of FUdR by a factor of 100, the frequency of damaged cells is reduced. Thus, after 4 hours in $10^{-5} M$ FUdR and thymidine, there were 73% damaged cells with $10^{-4} M$ thymidine and only 13% damaged cells with $10^{-3} M$ thymidine (Bell and Wolff, 1964). The mechanism by which a change in levels of endogenous thymidine could lead to chromatid breakage and reunion in G_2, is unknown; it would be of interest to relate it to the sister-strand exchanges that can be detected in chromosomes labeled with TdR-H^3.

As with 5-AU, FUdR affects more than one system in meristematic cells. It may interfere with RNA synthesis or the base composition of the RNA produced in treated cells. Some treatments have, therefore, included uridine (Bell and Wolff, 1964). The effects of many base analogs and base ribosides that contain sugars other than deoxyribose or ribose (see Kihlman, 1966) would repay close study.

VI. Growth Factors—Auxins and Cytokinins

There is an extensive literature on the effects of auxins and cytokinins, particularly on the growth and differentiation of cultures of roots, cal-

luses, cell suspensions, and tumors. Studies of the effects induced by these growth factors on intact roots are much less extensive and they relate mainly to effects on cell division.

A. Effects of Auxins on Cell Division

Treatments with IAA (3-indoleacetic acid) for short periods do not affect the percentages of cells in the various stages of mitosis (Chouinard, 1955; Davidson and MacLeod, 1966). Within about 4 to 6 hours, however, a fall in mitotic index is apparent. With 6.26×10^{-4} *M* IAA, the MI in *V. faba* falls to 3.4 (control value = 6.9) 5 hours after the end of a 3-hour treatment. A similar, though less marked effect, is induced by 10^{-5} *M* IAA for 3 hours (Davidson and Webster, 1967; Webster and Davidson, 1967). By labeling with TdR-H^3, it was found that the IAA had no effect on cells in G_2 at the time of treatment but was delaying a population of cells in S. The delay lasted about 6 hours. The result of the IAA treatment is to reduce the number of labeled prophases entering division in the 6–9 hours following treatment.

IAA treatments have also been used in studies of mitotic cycle times of tetraploid cells. Treatments with 10^{-4}, 10^{-5}, and 10^{-6} *M* IAA delay the appearance of tetraploid cells in mitosis; but though the progress of these cells through interphase is temporarily slowed down, they eventually exceed the numbers of tetraploid cells seen in division in control roots.

IAA and α-naphthaleneacetic acid (NAA) also stimulate the entry into division of pericycle cells and cortical cells; the latter type of cell does not normally divide.

NAA solutions (0.0002%) were used to treat roots of *A. cepa* for 4 hours. Roots were examined after 12 hours recovery, and it was found that primordia were forming from pericycle cells (Witkus and Berger, 1950). This appears to be a genuine stimulation since pericycle cells do not normally give rise to lateral root primordia in roots of *A. cepa*. This result was repeated with 0.0005% NAA, also after a 4-hour treatment (Berger and Witkus, 1948). Pericycle cells were dividing and were found to be diploid, as expected. Roots from the same onions were examined after 48 hours. Cell divisions were also taking place in cortical cells, and these cells were tetraploid.

The responses to IAA or to NAA are pertinent to the question of the mechanism controlling the pattern of distribution of dividing cells in root meristems (Introduction and Section IV,A,2); are the regions of low and high mitotic activity controlled by gradients of auxins?

B. Induction of Lateral Roots

A consequence of IAA or NAA stimulation of division of pericycle cells is the formation of lateral roots. It should be noted that IAA stimulates their initiation but it will prevent the growth of the primordia once they have been formed (Goldacre, 1959).

C. Kinetin

Kinetin (6-furfurylaminopurine) is required by cells growing in culture and appears to be a factor involved in cell division. It has also been found to stimulate cell division in intact roots and in root segments in culture.

a. Effects on Mitosis in Growing Roots. Seeds of lettuce (*Lactuca sativa*) were sowed on one piece of filter paper in petri dishes at 33°C in the dark. To each dish 4 ml of one of the following solutions were added: water, 5×10^{-5} *M* kinetin, 5×10^{-5} *M* 6-benzylaminopurine, or other 6-substituted aminopurines. Germination and the onset of cell division were followed for up to 72 hours.

Germination of seeds treated under these conditions was zero if they had been sowed on water. Addition of kinetin or 6-benzylaminopurine induced almost 50% of the seeds to germinate. Mitotic activity was increasing after 18 hours of treatment, and by 23 hours the number of cells in mitosis was higher in the treated roots than in controls (Haber and Luippold, 1960). The number of divisions observed in the lettuce roots were, however, low. Greater increases were seen in pea root segments developing as calluses in culture. A 7-day treatment with 3.2×10^{-6} *M* kinetin stimulated mitotic activity; in particular, it affected tetraploid cells, which constituted about 50% of the cells seen in mitosis (Torrey, 1961). This stimulation of mitotic activity did not occur in apical meristems.

VII. Radiation Effects

The effects of irradiation on growing roots are complex since so many aspects of cell and root growth are affected by radiations. The early studies of the effects of radiations, particularly of X-rays, on *V. faba* roots have been described by Read (1959), who gives a thorough consideration of the classic work of Gray and Scholes on the changes that occur in meristems. The implications of the physiological and organiza-

tional changes in irradiated roots have also been described (Davidson, 1960, 1961; Clowes, 1961a).

Growing roots can be used for studies of cell survival (Hall *et al.*, 1962) and for studies of the differential sensitivity of different meristems. The smallest primordia are more sensitive to a given dose of X-rays than larger primordia; thus there is a fall, with time, in the number of lateral roots emerging from an irradiated primary root (Howard, 1966). This effect is a futher example of the changes that occur, in the response of cells of primordia to experimental treatments, as the primordia develop. The relationship of this growth inhibition by X-rays to the level of endogenous growth factors is important in considering the potential development that can be shown by groups of mitotically active cells.

An excellent system for the study of cell growth and differentiation in the absence of DNA synthesis and cell division occurs in irradiated wheat plantlets (Foard and Haber, 1961). Wheat is irradiated heavily (800 kr) and then sowed. The wheat germinates, and the primary and first 2 lateral roots emerge and elongate. Their final length is 9–15 times that of the primordia from which they grow. These "gamma plantlets" undergo several changes that occur in the differentiation of normal plants, for example, hair formation, xylem differentiation, and lignin formation. Though the life of these irradiated plantlets is limited, they show many changes within 12 days of sowing, and they are a source of cells that develop without the complications of DNA synthesis or mitosis.

VIII. Fixation and Staining

Squash preparations of root meristems continue to be useful in the following light microscope studies: (1) chromosome structure and karyotype analysis; (2) autoradiographic analysis of the synthesis and stability of chromosome constituents (DNA and histones) and of mitotic cycles. They are also used in making quantitative determinations of the amounts in cells of DNA, RNA, and proteins, particularly histones.

A. DNA of Chromosomes and Nuclei

In a large number of studies, chromosomes continue to be stained with the DNA-specific Feulgen's reagent.

1. Fixation

Excellent fixation of chromosomes is achieved with La Cour's 2BD, an osmic acid fixative (Darlington and La Cour, 1953). Roots must be

bleached before hydrolysis and staining, and Ford's technique for bleaching has been described (Wolff, 1964). For most cytological studies, however, roots may be fixed in acetic acid-ethyl alcohol (1:3), the fixative in general use, or in Carnoy's fluid—ethyl alcohol-chloroform-acetic acid (6:3:1). The fixative should be mixed immediately before use and chilled.

Fixed roots may be stored but it is generally better to make preparations as soon as possible.

2. Hydrolysis and Staining

Roots should be thoroughly washed to remove all fixative.

For *Vicia, Allium,* and *Hyacinthus* roots fixed in acetic acid-alcohol or Carnoy, 8 minutes hydrolysis in 1 *N* HCl at 60°C is adequate. Whole root systems of *Vicia* may require 10 minutes, particularly if it is intended to dissect out primordia. For *Pisum,* 12–15 minutes hydrolysis is required. After hydrolysis roots are transferred to Feulgen's reagent and left for 2 to 3 hours. The meristems are prepared as squashes.

This procedure for fixation, hydrolysis, and staining is used in cell cycle studies and in autoradiography of labeled DNA in interphase nuclei and in chromosomes.

The preparation of a meristem squash is simple; the following points should be borne in mind: (1) Use only the meristem, i.e., discard the root cap and the lightly stained region basal to the meristem; (2) Mount in 1–2 drops of 45% acetic acid but do not flood the slide; (3) Separate the cells as much as possible, by gentle tapping, *before* putting on the coverglass; (4) Do not move the coverglass once it is on the squash, i.e., it must be held in place when tapping the coverslip (to separate the cells still further) and when squashing. Permanent preparations of squashes can be made using the dry-ice method (Conger and Fairchild, 1953) or by smearing coverglasses with albumin before they are placed on the squash (Darlington and La Cour, 1953). The dry-ice method is generally used in autoradiographic studies.

In some cases, particularly after pretreatment with some inhibitors of DNA synthesis, Feulgen staining of nuclei may be light. In those cases the final preparation can be improved by staining in Harris' hematoxylin for 5 to 10 minutes. The squashes can be made in a drop of stain or the squash can be stained before dehydration is begun.

3. Quantitative Determinations of DNA

If squashes are to be used for a microphotometric determination of amounts of DNA the general procedure described above is modified. (1) Fix in acetic acid—alcohol, Carnoy's fluid or 4–10% formaldehyde. Formaldehyde is often used if it is also intended to determine amounts of

histone in the nuclei. (2) Feulgen's reagent is prepared from basic fuchsin and this is generally a mixture of dyes. It may be desirable, therefore, to standardize the Feulgen's reagent; appropriate methods have been described by Stowell (1945). (3) Conditions and times of hydrolysis must be kept uniform for repeat experiments since the intensity of Feulgen staining is dependent on hydrolysis conditions (Deitch, 1966, see pp. 332–334). (4) After staining for 1 to 2 hours, the preparations should be taken through 3 baths of freshly prepared sulfurous acid (referred to as SO_2 water). They are then washed in water, dehydrated and mounted. Absorption by the Feulgen stained nuclei or chromosomes can be determined. Patau and Srinivasachar (1959) measured absorption, by the two-wavelength method, of nuclei of onion roots. McLeish (1959) determined absorption at a wavelength of 553 mμ with an integrating microdensitometer. His determinations were made on isolated nuclei. From these Feulgen absorption studies the amount of DNA is expressed in arbitrary units. McLeish (1959) determined values for DNA content of G_1 and G_2 nuclei in diploid, triploid, and tetraploid strains of different species, and the ratios calculated for DNA content in nuclei at different stages of the cell cycle fitted the ratios expected on the basis that a normal haploid complement has a constant and fixed DNA content (C value).

The reproducibility of measurements obtained by these techniques, for example the two-wavelength method, is excellent. Thus a difference of 10% between any 2 measurements can be taken to be significant (Hale, 1966).

Other stains that reveal the presence of DNA include methyl green, azure B, and methylene blue. Azure B and methylene blue are also bound by RNA. The ability of DNA and RNA to bind these dyes can be changed significantly by the fixation used (particularly if it includes formaldehyde), the duration of fixation period, the state of the nucleic acid (DNA, for example, loses its ability to bind methyl green if the double helix is destroyed) and the extent to which the nucleic acid is complexed with a protein (the effect of one component of a nucleoprotein complex on the ability of the other component to take up a dye is also discussed in this Section, D. Also see Deitch, 1966; aond Swift, 1966).

B. RNA

Several qualitative staining methods of demonstrating the location of RNA in cells have been available for some time. They include (1) the Unna-Pappenheim-Brachet method using methyl green-pyronin, (2)

azure B, and (3) methylene blue. These can be used, particularly in combination with selective extraction of DNA with DNase, to demonstrate the presence of RNA in nuclei, nucleoli, and cytoplasm. The criteria that must be satisfied in using them for quantitative studies of RNA have been discussed by Swift (1966).

In autoradiographic studies of RNA synthesis, roots are exposed to solutions of uridine-H^3 and cytidine-H^3 and then treated as follows.

1. Fixation

Treated roots can be fixed in chilled acetic acid-alcohol or 10% formalin. Short fixation periods, 1–3 hours, should be adopted where possible since long periods of fixation may interfere with the ability of the fixed cells to undergo extraction or digestion. Extended periods of exposure to 10% formalin, for example, may reduce or eliminate sensitivity to DNase (Swift, 1966).

2. Softening

In studies of RNA metabolism roots cannot be hydrolyzed in HCl since the acid removes the RNA. Squashes can be prepared by digesting the middle lamella between adjacent cells. Wash roots thoroughly. Place in a 1% solution of pectinase at room temperature for 24 hours (Davidson, 1964). The pectinase solution should be buffered with a potassium hydrogen phosphate-citric acid mixture to about pH 3.4–4.0. With pectinase pretreatment the cells separate easily and adequate squash preparations can be made. With *V. faba*, the pectinase-treated roots are sufficiently soft to allow dissection of primordia out of primary roots.

3. Staining

Squashes prepared after pectinase treatment can be stained with toluidine blue after developing and fixing the autoradiographs. Permanent preparations can be made in the usual way.

C. Nucleoli

Nucleoli are generally constant in number in diploid cells of an organism but their size may vary; with increasing metabolic activity of a cell there is often an increase in nucleolar volume. Nucleoli are the site of synthesis of ribosomes and they are clearly key structures of cells. Nucleoli are made up of RNA and protein; and part, at least, of the protein may be histone (Birnsteil and Flamm, 1964; Gifford and Dengler, 1966).

Nucleoli take up RNA stains (this Section, D). Two double-staining

techniques that involve staining the protein component of the nucleoli and give satisfactory results were described by Semmens and Bhaduri (1941) and Kurnick and Ris (1948). The nucleoli, however, are never very heavily stained in comparison with the staining of the chromatin. Denser staining of nucleoli can be obtained using Rothwell's modification (1964) of Rattenbery and Serra's technique.

1. Fix in absolute alcohol-formalin-acetic acid (6:3:1) for 12 to 24 hours.
2. Wash roots thoroughly.
3. Hydrolyze in 1 *N* HCl at 60°C for 15 to 30 minutes.
4. Stain whole roots in iron-acetocarmine for 12 hours.
5. Strips of epidermis or other cells or squashes of meristems are made and destained in 45% acetic acid until the nucleoli can be clearly distinguished from the rest of the nucleus.
6. Permanent preparations can be made using the dry-ice method.

Rothwell used this technique to study changes in nucleolar size, particularly in the nucleus of the developing hair cell of grass roots. It has also been used to follow the nucleoli that persist through mitosis in some species, for example, members of the Panicoideae (Brown and Emery, 1957). If formalin is omitted from the fixative no persistent nucleoli can be observed in cells undergoing mitosis, and this suggests that some acid-soluble fraction of the nuceloli is removed, either by the fixative (acetic acid-alcohol) or during hydrolysis. Nucleolar histones and nucleoli that persist during mitosis are, as yet, only partially understood and are clearly problems that invite reinvestigation. They may be related problems. The grasses are an excellent experimental subject since some (Panicoideae) have persistent nucleoli while others (Festicoideae) do not.

D. Proteins

The fixatives generally used to precipitate cellular proteins include formalin, heavy metal salts, and alcohol, in various combinations.

1. Fixation and Staining of Cell Proteins

A general demonstration of cellular proteins can be carried out using the well-known Millon reaction. Material may be fixed in acetic acid-alcohol (1:3) or 10% neutral formalin. Tryptophan and tyrosine residues can be treated to form nitrosomercurial derivatives; that of tyrosine is colored and absorbs visible light (500 mμ). Millon's reaction provides a reliable demonstration of proteins but it does not provide a dependable

basis for quantitative estimates of proteins (Rasch and Swift, 1960). Cell proteins may also be stained by fast green at an acid pH (2.0).

2. Histones

These basic proteins are important constituents of nucleoprotein complexes of the cell. Our knowledge is biased at present toward the nuclear histones though their cytoplasmic counterparts clearly deserve more investigation. Further information is needed on their site of synthesis, movement between nucleus and cytoplasm, what role they play in translation events in the cytoplasm, and the relationship of histones to nucleoli that persist throughout mitosis (see this Section, C).

The two best-known techniques for staining histones are the alkaline fast green method and the Sakaguchi reaction.

a. Alkaline Fast Green. The standard technique was developed by Alfert and Geschwind (1953).

1. Fix in neutral formalin (5–10% solution).
2. Wash thoroughly to remove all fixative. Overnight washing in running water is recommended.
3. Embed and prepare sections (6–10 μ thick sections are convenient).
4. Remove embedding medium and hydrate sections. Warm to 90°C.
5. Extract DNA in a 5% solution of trichloroacetic acid (TCA) at 90°C for 15 minutes.
6. Wash sections in water. A final wash can be made in water buffered to pH 8.1.
7. Stain in 0.1% fast green at pH 8.1. Staining for 30 minutes is usually adequate.
8. Rinse in distilled water and then dehydrate in 95% alcohol and xylene. Each rinse should be not longer than 5 minutes. Make preparations permanent or mount in index of refraction oil.

The pH of the dye solution may be adjusted with NaOH, 0.005 *M* phosphate buffer (Deitch, 1966), or with tris-HCl buffer (Bloch, 1966).

The staining of histones by fast green does not provide a quantitative measure of the total number of basic amino acid residues in the protein, but reflects its net positive charge. The ability of histones to bind fast green, or eosin, depends upon the removal of polyanions such as DNA. The removal of DNA, generally with hot TCA, appears to expose certain groups that are necessary for stain binding. These are α-amino and α-carboxyl groups. If TCA-extracted nuclei are deaminated or acetylated, staining with alkaline fast green is abolished (Gifford and Dengler, 1966; Gorovsky and Woodard, 1967).

At present we know that removal of DNA enables us to locate

nuclear histones and to determine how they change, quantitatively, during a cell cycle or as a cell becomes specialized (Rasch and Woodard, 1959; McLeish, 1959, 1960; Bloch *et al.*, 1967). This does not mean, however, that DNA is the only polyanion capable of complexing with histones. Other proteins, i.e., nonhistone, do so *in vitro* (Bloch and Goodman, 1955) and may do so *in vivo;* it also appears that RNA will do so. When it is removed, cytoplasmic staining with alkaline fast green is observed.

b. Fast Green Staining of Cytoplasm. When cells of onion roots are treated with TCA or DNase, fast green is taken up by the nuclei and may or may not be taken up by the cytoplasm. But if the cells are treated with RNase, which would not remove DNA, staining is weak in the nucleus and positive in the cytoplasm (Gifford and Dengler, 1966). These authors conclude: "this strongly suggests an association between fast green staining basic proteins and RNA . . . in the cytoplasm." If the proteins are histones it appears that they differ in some way from nuclear histones since they are not revealed by TCA extraction, which removes RNA as well as DNA. The difference may be in molecular weight or in a molecule with which they are complexed. What is now needed, using the techniques available, is to determine (1) whether the basic proteins of the cytoplasm are all histones, (2) if they react with other histone stains, such as the Sakaguchi stain (see this Section, 2,C) and (3) how they react to combined treatments (Bloch, 1966) with fast green and eosin, which reveal differences between nuclei of one tissue and indicate functional differences. Growing meristems provide excellent material for such studies since it would be possible to follow changes in nuclei and cytoplasm along columns of cells and thus to relate any changes to the stage of development of the cell.

c. Sakaguchi Reagent. One of the basic amino acids present in basic proteins, histones, and protamines, in high concentrations is arginine. Sakaguchi showed that the arginine residues of basic proteins can be stained with α-naphthol. The colored complex formed fades rapidly, however. More intense staining is obtained with 2,4-dichloro-α-naphthol but it also tends to fade (McLeish *et al.*, 1957). In part, this appears to be the result of digestion by the high concentrations of NaOH in the reaction mixture. The alkali not only removes arginine but also destroys tissue sections. Barium hydroxide has been substituted for sodium hydroxide (Deitch, 1966) and has less deleterious effects. McLeish's method (1959) is as follows:

1. Fix roots in 4% formalin (pH 6.0–6.8).

2. Wash roots thoroughly. Homogenize in 45% acetic acid, add more acetic acid, and collect isolated nuclei by slow centrifugation.

3. Place nuclei on a slide, add a coverslide, and squash. Warm gently. Most nuclei adhere to the coverslide.

4. Place the slide, inverted, in a dish of 45% acetic acid. The coverslide will fall off. Remove from the acetic acid and wash with distilled water.

5. Coat the coverslides with celloidin and allow to dry in air. They are then taken through an alcohol series to water.

6. Stain for 6 minutes at 20°C. The staining solution has 47 ml of 1% NaOH, 1 ml of 1% 2,4-dichloro-α-naphthol in 70% ethanol (these should be shaken before adding the last ingredient), and 2 ml NaOCl. When the NaOCl is added the mixture should be shaken again.

7. Drain coverslides, rinse in an excess of 1% NaOH for 10 minutes, and mount in glycerol (pH 11.3).

This procedure produces intensely stained isolated nuclei. Quantitative estimates of histones in these nuclei have been made using an integrating microdensitometer at a wavelength of 517 mμ.

The Sakaguchi reaction can also be applied to sections. Deitch (1966) has substituted BaOH for NaOH. She adds tri-*N*-butylamine to the dehydrating and mounting media. The result, using 2,4-dichloro-α-naphthol, was intensely stained cells that did not fade over a period of 14 days. As with alkaline fast green the Sakaguchi method also stains cytoplasm.

The important conclusions that have been reached from the studies using alkaline fast green and 2,4-dichloro-α-naphthol are that nuclear histones are synthesized during the S period of DNA, that there is a remarkable constancy in the amount of histone in a nucleus, and that chromosomal histones are distributed to daughter cells in a manner similar to that of chromosomal DNA (Rasch and Woodard, 1959; McLeish, 1959, 1960).

d. Fixation of Histones. The standard fixative for nuclear histones is neutral formalin, and general cytological fixatives have been thought to be unsatisfactory in preserving basic proteins. Thus any fixative that removes chromosomal histones would change the fixation image of the chromosomes of dividing cells.

Acetic acid-alcohol (1:3) produces less satisfactory fixation of chromosomes than 2-BD (in Darlington and La Cour, 1953) or chromic acid-formalin. The chromosomes swell slightly and have small, lightly stained regions.

The same effect is produced by stain fixatives such as acetocarmine or aceto-orcein, though these and acetic acid-alcohol fix chromosomes adequately for most purposes, such as mitotic studies and autoradiography. Some of the effects attributed to acetic acid fixation are really due to the subsequent hydrolysis in hot HCl. This undoubtedly removes histones. Thus, even isolated nuclei of *Vicia* may lose little or no basic

protein when treated with 45% acetic acid, whereas they lose considerable amounts of such proteins when treated with dilute inorganic acids (Dick, 1966 cited by Gorovsky and Woodard, 1967). Gifford and Dengler (1966) have demonstrated the effect of hydrochloric acid on histones of *Allium* roots. They showed that hydrolysis with 0.01 *N* HCl before TCA extraction resulted in the loss of the ability to take up alkaline fast green.

It is clear that acetic acid does not remove all basic proteins and may not even result in any appreciable loss of stain binding. Root cells fixed in 30% acetic acid can be stained subsequently with fast green (pH 8.1). Gifford and Dengler (1966) found that interphase nuclei of some roots stain somewhat less intensely than those fixed in 10% neutral formalin or 70–100% ethanol, but they still classified the stain uptake as "good"; chromosome staining was described as "excellent," i.e., as good as that observed after formalin fixation.

Of the fixatives tested by Gifford and Dengler the following resulted in satisfactory binding of fast green: (1) 70% or 100% ethanol; (2) 100% methanol; (3) 10% neutral formalin at room temperature or 10°C, (4) methanol-chloroform-acetic acid. It should be emphasized that the evaluation of staining intensity was by eye and that all that could be concluded is that several fixatives preserve sufficient cellular basic protein to enable them to produce a satisfactory staining reaction.

McLeish (1959) using roots of *Scilla campanulata* has also studied the

TABLE VII

EFFECT OF DIFFERENT FIXATIVES ON THE AMOUNT OF COLOR COMPLEX PRODUCED BY THE SAKAGUCHI REACTION[a]

Fixative	Period of fixation		
	30 minutes	6 hours	24 hours
Lewitsky[b]	243 ± 3.1	254 ± 3.6	220 ± 4.8
Baker[c]	248 ± 3.5	252 ± 3.6	250 ± 3.1
Bouin[d]	229 ± 4.2	242 ± 5.4	201 ± 3.9
4% formalin	240 ± 3.0	249 ± 2.9	251 ± 3.3
Acetic acid-alcohol	110 ± 5.4	129 ± 5.5	117 ± 6.8
5% acetic acid	—	97 ± 6.5	—
1% chromic acid	198 ± 6.1	226 ± 6.3	220 ± 6.7

[a] Mean absorption value (measured at 517 mμ) based on twenty 2C nuclei of roots of *Scilla campanulata*.

[b] Lewitsky fixative, equal volumes of 4% formaldehyde and 1% chromic acid.

[c] Bouin fixative, mixture of 25 ml 40% formaldehyde, 75 ml saturated aqueous picric acid, and 5 ml acetic acid.

[d] Baker fixative, formaldehyde-calcium.

effects of various fixatives on the subsequent ability of nuclei to take up 2,4-dichloro-α-naphthol. He measured absorption at 517 mμ by 20 diploid nuclei; all were in G_1 (i.e., had 2C amount of DNA). His quantitative estimates of stain binding by nuclei in the same stage of interphase showed (Table VII) that maximum staining occurs only if formaldehyde is present in the fixative. With acetic acid-alcohol there is a 50% loss of arginine-containing histone (see next section).

3. Fixation of Nuclear and Chromosomal Proteins for Autoradiography

Synthesis of nuclear and chromosomal proteins has been studied using isotopically labeled amino acids (Section IV). Roots were fixed in neutral formalin or acetic acid-alcohol; hydrolysis was in hot 1 *N* HCl or TCA (Table III).

Hydrolysis with HCl of acetic acid-alcohol fixed roots does not remove all chromosomal label (Alvarez, 1965), indicating that some protein has remained. However, this may not be histone and in view of McLeish's observations (Section VIII,D,2,d), it seems unlikely that the protein remaining after the treatment used by Alvarez includes a normal concentration of histones. Mattingly (1963) reports that grain counts found after incorporation of lysine-H^3 are not reduced by HCl hydrolysis, but it must be recalled that (1) the determination is based on nuclei, not chromosomes, taken from the first 5 mm of the root; this region includes differentiated cells whose nuclei may contain proteins that could change the effect of acid hydrolysis; (2) the fixative was 10% neutral formalin, not acetic acid-alcohol. Thus, it does not seem reasonable to conclude that labeling seen after incorporation of basic amino acids indicates the presence of histones.

TCA can be substituted for HCl both in preparing the Feulgen stain and in hydrolysis (Bloch and Goodman, 1955). This technique is particularly useful in autoradiographic studies of chromosomal histones since it makes it possible to study incorporation of amino acids in Feulgen-stained squashes of complete chromosome complements (Bloch *et al.*, 1967).

E. Dissection of Primordia

Growing primary root systems yield not only the meristems at the root apices but also the primordia that will develop into lateral roots. These can be dissected out very easily either after hydrolysis and staining with Feulgen, or after softening roots in pectinase. The epidermis is split

and peeled off, and cortical cells are removed. Primordia are obvious, particularly after Feulgen staining, as groups of densely stained cells. They can be removed and prepared as squashes in the usual way.

IX. Conclusions

Growing roots provide a convenient system for the study of cell growth and the physiology and biochemistry that growth represents. The organizational complexity shown by meristems, from control of mitotic rates to patterns of polymer arrangement in cell walls, is capable of more precise description. The polarized orientation shown by spindles and the axes of elongation shown by cells indicates the existence of a dynamic pattern that may extend to chromatid segregation. Polarized segregation occurs in meiosis and it may occur in mitosis (Lark, 1967; cf. Heddle *et al.*, 1967). Thus polarity indicates a specific control of the interrelationships between cells, which, because they cannot migrate, must be capable of adapting to neighbors.

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Chapter 10

Cell Cycle Analysis[1]

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I. Introduction

In cell biology, one of the most exciting and intriguing questions is how one cell grows and reproduces to yield two cells of similar nature. A better understanding of the mechanisms involved in cell reproduction depends upon having markers spaced around the cell cycle, which allow the determination of how far the cell has progressed towards the next cell division.

Mitosis with its four familiar phases and cytokinesis are the most obvious markers in the cell cycle. Mitosis itself is well known as the event which brings about the equal partition of full sets of genetic information to the two new daughter cells. Those who have witnessed it cannot help but be awed by this dramatic event. However, what must be emphasized is that this marvelous and observable process of cell division is the culmination of many more subtle but no less important parallel chains of structural and biochemical preparations.

Another marker in the cell cycle was demonstrated in 1953 by Howard and Pelc, who showed with a radioactively labeled precursor of DNA that the replication of the genetic material of the cell (i.e., chromosomal DNA) takes place during a discrete period of interphase. Thus, the interphase portion of the cell cycle could be subdivided into the DNA-synthesis phase, S period, and the pre- and post-DNA synthesis phases, the G_1 and G_2 periods, respectively. We soon may be approaching the position of being able to give the G_2 and other portions of the cell cycle chemical definitions also (Sisken and Wilkes, 1967).

Other cell cycle markers hopefully will be found. The time in the cell cycle at which an inhibitory agent can no longer affect the coming cell division may serve as an operational physiological marker even if the sensitive process is unknown (Nachtwey and Giese, 1968). There are even indications that some processes necessary for a division may be completed during a previous cell cycle, thus suggesting the possibility of operational cell cycle markers outside the cell cycle under study. The

processes involved in the differentiation of cells may have their own markers.

If the number of markers increases and if the cell cycle can be subdivided into smaller and smaller phases, adequate quantitative description of the phase durations and positions becomes imperative. Even if no more markers other than mitosis and the DNA synthesis period are found, exploitation of the known markers requires refined analyses to obtain the most information from the data. Although there are numerous elegant experimental methods for dissecting the cell cycle into its component parts, the analyses of the results frequently are crude, in many cases, because better analytical methods are not available, and in other cases, because of a lack of understanding of the underlying factors producing the observed results. One of the greatest problems in cell cycle analyses is that most of the analytical procedures are based upon the idealized, invariant cell. Real cells, however, spend different times in the cell cycle and in each particular phase of the cell cycle, exhibiting variability around some central time value. This chapter is primarily concerned with this variability between the cells of a population. It introduces some methods for measuring the degree of variability and for dealing with the variability in making more accurate temporal dissections of the cell cycle. The investigator who approaches an analysis of the cell cycle armed with more refined methods of data analysis is likely to get more information from his experiment. Also, familiarity with these methods and the theoretical considerations underlying them before the experiment is run can sometimes simplify procedures of data collection.

One of the analytical methods presented is primarily directed at experiments using radioactively labeled DNA precursors. Briefly, the method involves a matching of the behavior of real cell populations to the behavior of a computer-generated model population. The computer program for generating the curves is based upon a relatively nonmathematical "graphic" approach that does not require mathematical sophistication on the part of the reader. The results of the computer program are expressed as correction factors that may be used for the refinement of the analyses of empirical data. Even if the factors are not used to correct the results, they will at least allow assessment of the possible errors involved in particular determinations and provide an understanding of the limits of application of the various methods.

If our model is eventually looked on as yielding poor approximations, we are still left with the reassuring fact that our approximations are closer than those based upon the concept of an invariant cell. If this approach introduces more complexity into our thinking, it is because the

TABLE I

METHODS OF ESTIMATING THE GENERATION TIME AND THE PHASES OF THE CELL CYCLE IN POPULATIONS OF CELLS[a]

A. Generation Time

1. Time for cell number to double obtained from counts of periodic samples with an electronic particle counter (e.g., Coulter Counter) or hemacytometer,[b] or from direct repeated counts of entire populations of cells in microchambers[c]
2. Time for average of one division obtained from determination of number of divisions per unit time in clones from cells originally isolated in hanging drops,[d] drops under oil,[e] or attached to glass or plastic
3. Time between two 50% points of two ascending portions of labeled mitoses curves after thymidine-H^3 pulse labeling[e,f,h]
4. Metaphase accumulation rate in cells blocked with colchicine or Colcemid[i,j,k]
5. Time required to halve radioautographic grain count after pulse labeling with thymidine-H^3 [l]
6. Time between two successive divisions of individual cells determined by time-lapse cinematography[m]

B. Mitotic Phase (M)

1. Time spent in mitosis by individual cells determined by time-lapse cinematography or direct observation[m]
2. Time for mitotic index to decrease to zero after X-irradiation.[n]
3. * Percentage of cells in mitosis[b,j]

C. G_1 Phase

1. Time between cell division and appearance of labeled cells by a combined method of time-lapse cinematography and radioautography,[m,o] or selection of dividing cells with a micropipette and pulsing of the cells at intervals[p]
2. Time required for the percentage of labeled cells to reach a plateau after treatment with Colcemid or colchicine in a continuously labeled cell population[i]
3. Time required for the percentage of labeled cells to reach the first plateau after X-irradiation in a continuously labeled cell population[q]
4. * The fraction of nonlabeled cells with low DNA content, by the combined use of radioautography and microspectrophotometry[r]
5. * The increase in the percentage of the labeled cells in a Colcemid-treated and continuously labeled cell population[i]
6. * The increment in the percentage of labeled cells after X-irradiation of a continuously labeled cell population[q]

D. S Phase

1. Time between the appearance and the disappearance of labeled mitotic cells after pulse labeling[g,h]
2. Rate of accumulation of the labeled cells after Colcemid or colchicine treatment in a continuously labeled cell population[i]
3. Difference in percentage-labeled cells between a single and a double labeling[s,t]
4. Difference in percentage of cells labeled with thymidine-C^{14} and percentage of cells labeled only with thymidine-H^3 after double pulsing[u,v]
5. Time required for labeled metaphase cells to reach a plateau of grain counts after a pulse[w]
6. * Percentage of labeled cells shortly after pulse labeling[h,w]

TABLE I (*Continued*)

E. G_2 Phase

1. Time between the start of labeling and the time of appearance of labeled mitotic cells[x]
2. Time between accumulation of mitotic cells and accumulation of labeled mitotic cells after Colcemid treatment in a continuously labeled cell population[i]
3. * Difference between the total mitotic cells and the labeled mitotic cells after Colcemid treatment[i]
4. * Difference between the percentage of the labeled cells at the plateau of nonirradiated cells and the percentage at the plateau of the X-irradiated cells in a continuously labeled population[q]
5. * The fraction of nonlabeled, nonmitotic cells with high DNA content[r]

[a] After Watanabe and Okada (1967).
[b] Merchant *et al.*, 1964.
[c] Prescott, 1959.
[d] Christensen and Giese, 1956.
[e] Nachtwey, this chapter.
[f] Thrasher, 1966.
[g] Dewey and Humphrey, 1962.
[h] Quastler and Sherman, 1959.
[i] Puck and Steffen, 1963.
[j] Leblond, 1959.
[k] Hooper, 1961.
[l] Kimber cited in Wimber, 1963.
[m] Sisken, 1963.
[n] Knowlton *et al.*, 1948.
[o] Sisken, 1964.
[p] Stone and Cameron, 1964.
[q] Kim and Evans, 1963.
[r] Mak, 1965.
[s] Pilgrim and Maurer, 1962, 1965.
[t] Wimber and Quastler, 1963.
[u] Baserga and Lisco, 1963.
[v] Thrasher, 1966.
[w] Stanners and Till, 1960.
[x] Defendi and Manson, 1963.

reproductive behavior of real cells is complex. Hopefully, methods which take into account this complexity will lead us closer to a true understanding of cell reproduction.

II. Experimental Methods for Obtaining Cell Cycle Data

A. Methods

The duration of the cell cycle and its phases can be determined from data obtained by a variety of methods. Table I, based upon one compiled by Watanabe and Okada (1967), outlines most of these procedures. [Methods in Table I marked with an asterisk are used for determining the fraction of the population in a particular phase, which can then be converted to the duration of the phase by further analysis (see Section III,A and B).] Many of these methods are familiar and have been discussed at length in Vols. I and II of "Methods in Cell Physiology" and elsewhere in the literature, so Table I will serve mainly as a reference source.

Before continuing to a discussion of the approaches that can be used to analyze the data collected by these methods, it is important to comment briefly on the possible inherent errors, the assumptions, and the major criticisms of these methods.

B. Precautions and Criticisms

Almost all of the methods for estimating the phases of the cell cycle use tritiated thymidine (thymidine-H^3) as a specific labeled precursor of newly synthesized DNA. The labeled cells are radioautographed and then analyzed under the microscope.

One of the most widely used methods for dissecting the parts of the cell cycle is to pulse label a population of cells, and then sample frequently for radioautography. The percentage of labeled mitotic or metaphase figures is then scored from the radioautographed slides.

A pulse labeling is accomplished in one of two ways. One way is to introduce thymidine-H^3 into a cell culture and then wash the cells free of the isotope with unlabeled wash fluid. The use of unlabeled thymidine in the wash fluid as a means of "chasing" the labeled thymidine and thereby facilitating the pulse effect is, in fact, a dangerous procedure because thymidine at a concentration of 2 m*M* or less can arrest the further progress toward division of mammalian cells that are either in the S phase at the time of exposure or that reach the G_1–S transition during a continued exposure. Continuous exposure of cells to concentrations of 0.2 m*M* thymidine causes a significant depression of clonal growth (see Painter *et al.*, 1964). The cell cycle is therefore known to be disturbed by these concentrations of thymidine. Another criticism of the washing procedure is that the wash solution itself may cause perturbations in the normal cell cycle. This is a valid criticism which is *too often* ignored! A partial compensation for these perturbations may be accomplished by use of "conditioned" media in the washing and subsequent culture procedures.

The second pulsing approach is used for labeling mammalian cells *in vivo*. Thymidine-H^3 is injected into the animal, and it is assumed that it is distributed throughout the body and that every cell in the process of nuclear DNA synthesis—and only those cells—incorporates the label in detectable amounts. In practice, a group of animals is injected and then the individual animals are killed at various time intervals. After histological and radioautographic procedures, the tissue cell populations are scored for the percent of labeled mitoses. The thymidine-H^3 which is not used by the cells in DNA synthesis presumably undergoes rapid catabolism (less than 20 minutes) by the liver. Thus thymidine-H^3 is

cleared from the circulation and there is assumed to be no pool of thymidine in the body or in any of the cells. Thrasher has reviewed these assumptions in Vol. II and in general the assumptions are well founded (also see Steiner *et al.*, 1966). However, a recent study of the cell cycle in regenerating mammalian liver suggests that a small but significant pool of thymidine-H^3 may persist in the body for some time after injection (Stöcker and Pfeifer, 1967). The indication is that cells not in DNA synthesis at the time of thymidine-H^3 administration do eventually appear as weak but distinctly labeled cells upon entering the S period at some later time. The weakly labeled cells in question were seen only in those radioautographs with longer exposure times. Perhaps this phenomenon is caused by the reduced metabolic capacity of the remaining portion of the liver, which, if complete, would have rapidly catabolized all of the unincorporated thymidine-H^3. However, large cellular pools of labeled thymidine derivatives can be produced by rather large tracer doses of thymidine-H^3 (e.g., 2.5×10^{-6} *M*), (Cleaver and Holford, 1965), so it is possible that small cellular pools of thymidine (or its derivatives) may be more common than we now recognize. For example, Stone *et al.* (1965) reported that in the ciliate *Tetrahymena pyriformis* a pool of thymidine-H^3 derivatives is carried from one S period to label nuclear DNA in the next S period of the resulting daughter cells.

Another problem in pulse labeling of cell cultures as well as in the intact mammal can arise when some of the thymidine-H^3-labeled cells die; the breakdown products from the labeled DNA in the dead cells are then reutilized and incorporated into the DNA of other cells which were not previously labeled at the time of the pulse (Rieke, 1962; Bryant, 1963, 1965; Hill, 1961; Stöcker and Pfeifer, 1967).

Radiation damage to chromosomes resulting from incorporation of high doses of thymidine-H^3 has been demonstrated by several investigators (McQuade *et al.*, 1956; Wimber, 1959; Hsu and Zenzes, 1965). Marin and Prescott (1964) have demonstrated that thymidine-H^3 concentrations above 1 μc/ml cause division delay in Chinese hamster fibroblasts. Unpublished experiments cited by Puck and Steffen (1963) suggest that there may be delays in the G_2 period at concentrations as low as 0.1 μc/ml.

For cell cycle studies it is recommended that no larger quantity of thymidine or of thymidine-H^3 be used than is absolutely necessary. Judicious choice of radioautographic exposure duration may help in eliminating very weakly labeled mitoses, while still recording all those cells which were labeled at the time of the pulse. A considerable amount of care and caution must always be used in methods involving grain counting of radioautographs (Lang *et al.*, 1968). It also seems reasonable

to assume that any radiation damage or pharmacodynamic effects of thymidine-H^3 will be increased whenever the labeled compound is in continuous contact with the cell population under study.

Thrasher states that the double-labeling techniques for determining S period duration are based on a number of assumptions that are quite often not warranted in many cell populations (for details see Thrasher, 1966).

Use of the drugs Colcemid or colchicine and other spindle poisons to block the mammalian cell cycle is subject to some criticism. In all cases a lag period between drug administration and its cell-block effect is to be expected and in some *in vivo* experiments it has been shown that mitotic figures which have been blocked for 4 or more hours undergo necrosis and disintegration (Leblond, 1959; Hooper, 1961). These drugs may very well affect cells in nonspecific ways thereby reducing their usefulness, especially in those studies which involve experimental manipulation of the normal cell cycle. Stevens-Hooper (cited by Leblond, 1959) has stated requirements that must be met to obtain dependable results from experiments using spindle poisons.

The use of X-irradiation as a cell cycle-blocking agent is perhaps subject to more criticism than is the thymidine-H^3 labeling technique or the Colcemid blocking technique. The use of X-irradiation is based on the finding that the G_2 period of mammalian cells is most easily blocked by this method (Puck and Steffen, 1963). In fact, depending upon the dosage, one may get anything from a slight lag of cells passing through the G_2 period to complete death of the cell population. However, the lack of specificity in blocking cells in one particular stage of the cell cycle and the unknown effects of radiation on stages that are presumably radiation-resistant limits the value of X-irradiation in cell cycle studies.

One can see from this brief review that practically every technique so far devised, with the possible exception of direct and nonperturbing observation of the cytologically recognizable parts of the cell cycle, is subject to criticism in one way or another. It would seem on this basis, that, when possible, thymidine-H^3 be used as a terminal label and that this terminal pulse be used on cells with established histories as determined by means of time-lapse cinematography or direct observation of individual cells. When such a procedure is not possible, it is necessary to exercise caution and provide controls for detecting perturbations and false labeling. This brief review is not meant to frighten people away from using these methods of cell cycle analysis, but rather to make them aware of the types of criticisms these methods can encounter. A more extensive discussion of criticisms and precautions may be found in the recent book by Feinendegen (1967).

III. Analysis of Empirical Data—Conversion of Fractions of Cells in a Phase to the Duration of the Phase

A. Steady State Systems

If a population of cells is in an ideal steady state, the fraction of the population in a phase is directly convertible to the fraction of the generation time spent in that phase. In the ideal steady state, the number of cells remains constant because, on the average, one daughter cell of a pair is lost by death or migration immediately after every division—in some cases two daughter cells remain, in others both are lost.

Unfortunately, the ideal steady state system is rarely, if ever, found in nature. Usually cells that are destined to be lost will remain in the population for some time. Other cells may be present but will not enter the cell cycle until a later time (i.e., in G_0). Quastler (1963) has reviewed some of the problems in the analysis of multicompartment, cell-proliferation systems and has concluded that to describe completely a system with 6 compartments (G_0, G_1, S, G_2, M, and transitional and maturing forms) requires the estimation of 53 parameters. Therefore, in order to be able to convert directly from fraction of the population in a phase to fraction of the generation time spent in that phase, and from that to duration of the phase, one must know that the number of proliferating cells remains constant and must be able to distinguish proliferating cells from the G_0 or transitional and maturing forms.

B. Exponential Cell Proliferation Systems

1. The Age Gradient and Equations

With data obtained on exponentially growing cultures, conversion of the fraction of a population in a phase to the duration of the phase requires an understanding of the so-called age gradient. Figure 1A illustrates the relative number of cells of particular ages in a sample from an ideal, exponentially growing culture. Because one cell at the end of its cell cycle divides into two cells, there are twice as many just-divided cells as there are cells just ready to divide. Between these two extremes, the frequency of cells of any particular age decreases exponentially (Scherbaum and Rasch, 1957; James, 1960; Edwards *et al.*, 1960; Cook and James, 1964).

In Fig. 1A the relations of the shaded areas under the curve represent the ratios of cells in the various phases relative to the total number of

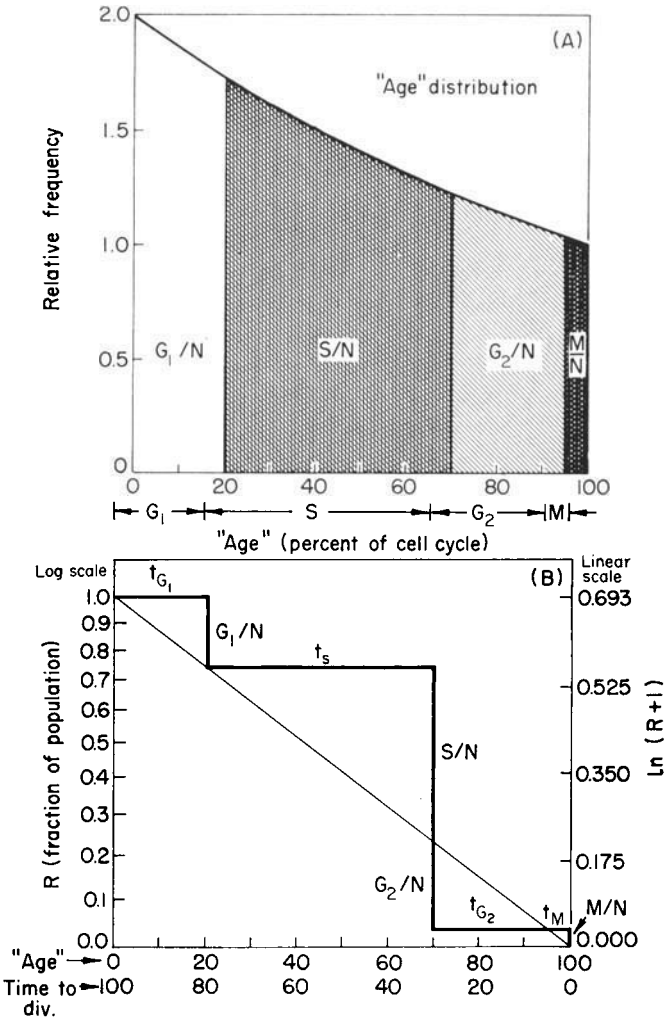


FIG. 1. (A). Example of the ideal age distribution in an exponentially proliferating cell system (see text for details). (B). Example of a simplified graphic method for determining the durations of the various phases from the fractions of cells in the phases (see text for details).

cells. For example, the shaded area labeled M/N represents the mitotic index or ratio of mitotic cells to total number of cells. The shaded area S/N represents the DNA-synthesis index or the fraction of cells in the population that are labeled when given a short pulse of thymidine-H^3. Because of the age gradient, the proportion of cells in any particular

phase is not necessarily equal to the proportion of the generation time spent in that phase. To determine the proportion of time spent in a phase from the empirically determined proportions of cells in that phase, a correction of the data is necessary. The basic formula [Eq. (1)] (after Scherbaum and Rasch, 1957) and the formulas to apply to the proportion data (after Watanabe and Okada, 1967) are as follows:

$$\frac{t_{\text{term}}}{T} = \frac{\ln[(N_{\text{term}}/N) + 1]}{\ln 2} \tag{1}$$

$$t_{\text{M}} = T/\ln 2 \ln\left(\frac{\text{M}}{N} + 1\right) = k \ln\left(\frac{\text{M}}{N} + 1\right) \tag{2}$$

$$t_{\text{G}_2} = k \ln\left(\frac{\text{G}_2}{\text{M} + N} + 1\right) \tag{3}$$

$$t_{\text{S}} = k \ln\left(\frac{\text{S}}{\text{G}_2 + \text{M} + N} + 1\right) \tag{4}$$

$$t_{\text{G}_1} = k \ln\left[\frac{1}{1 - (\text{G}_1/2N)}\right] \tag{5}$$

where t_{term} is the time spent in a terminal phase, T = generation time, N_{term} = number of cells in some terminal phase of the cell cycle, N = total number of cells observed, ln = natural logarithm ($\log_e$), M, G_2, S, or G_1 = number of cells in mitosis, G_2, S, or G_1, and the constant, $k = T/(\ln 2)$ or $T/0.693$.

To apply these formulas for analysis of the entire cell cycle, at least the numbers of cells in M, G_2, and S and the total number, N, must be known; the G_1 duration can be obtained by subtraction of the sum of the other phases from the generation time. However, except when using the Mak (1965) technique, the number of cells in G_2 is unknown. Usually in cell cycle analyses, N and the number of cells in M and S are counted and t_{G^2} is determined from the time to 50%-labeled mitoses. Therefore, to use Eq. (4), t_{C_2} must be converted to the number in G_2. Equation (6) satisfies this requirement.

$$\text{G}_2 = (\text{M} + N)(e^{t_{\text{G}_2}/k} - 1) \tag{6}$$

The term e is the base of the natural logarithm, and $e^{t_{G_2}/k}$ results from making both sides of one of the intermediate equations (not shown) a power of e in order to obtain the antilog$_e$ of the right side. The other symbols refer to the same quantities listed for Eqs. (1)–(5). It should be emphasized that, in Eq. (6), N is the number of cells counted in the experiment where M and S are determined, not in the experiment where t_{G_2} is measured.

In Eq. (7), all of the needed elements for the determination of t_S are combined to yield one equation.

$$t_S = k \ln\left(\frac{S}{(M + N)\exp(t_{G_2}/k)} + 1\right) \tag{7}$$

The term, $\exp(t_{G_2}/k)$, is e raised to the t_{G_2}/k power, which may be found in most compilations of mathematical tables.

The mitotic time can be further subdivided into prophase (P), metaphase (Meta), anaphase (A), and telophase (T). The durations of these phases can be calculated using Eq. (2) and substituting successively T, T + A, and T + A + Meta for M. After determining the durations of T, T + A, T + A + Meta, and T + A + Meta + P($=t_M$), the durations of the individual phases can be calculated by subtraction. Alternatively, when the total mitotic time is short relative to the generation time (e.g., less than 10%), the error introduced by the age gradient is negligible so the durations of the various mitotic phases can be closely approximated simply by multiplying t_M by the fractions of *mitotic* cells in each of the phases. For example, if 0.3 of the mitotic cells are in prophase, then prophase occupies close to 0.3 of the mitotic time.

2. Simple Graphical Approach to Application of the Age Gradient Formulas

In Fig. 1B a simple graphical approach is presented which allows one to make the appropriate corrections required by the age gradient. This graphic method, devised by Okada (1967), is based on Eq. (1) which states that the time to the end of the cell cycle is related logarithmically to the proportion of cells in this terminal state of the cell cycle. The right hand ordinate (linear scale) in Fig. 1B is simply the relationship of Eq. (1) expressed graphically, where $R = N_{term}/N_{total}$ and the abscissa value for "Time to Division" is $t_{term}/T \times 100$. The left hand ordinate of Fig. 1B shows an easier method employing this same relationship. On the logarithmic scale of semilog paper, subtract 1 from each of the ordinates labeled 1 and 2, thus yielding 0 and 1. In between on the logarithmic scale, mark off the tenths of the population. Draw a line connecting the 1 point on the ordinate to the generation time on the abscissa, which must be measured by some independent method (see Section V). In the example shown, the generation time is given as 100 arbitrary units, but any generation time could be marked off on that scale. Mark off the time to division in reverse order.

After plotting the line, the durations of the various phases can be determined graphically. For example, the mitotic index, M/N, is plotted

vertically above the generation time point. Measuring from this point to the left to the diagonal line gives a measure of the duration of the mitotic phase. A measure of the G_2 time can be obtained by determining the time to 50%-labeled mitoses after application of tritiated thymidine. The G_2 duration then is marked off horizontally from the intersection of the t_M line. The G_2 index, G_2/N, then becomes the distance between the t_{G_2} line and the diagonal line. The height of the diagonal line above the abscissa at this point is the $(G_2 + M)/N$ index. The S index, S/N, is continued on from the G_2/M line and from that point horizontally to the diagonal line. The time spent in the S phase can then be calculated from the abscissa values by subtracting the beginning from ending times. Similarly, the G_1 time can be calculated, either by using the G_1 index, i.e., the difference between $(S + G_2 + M)/N$ and 1, or, as is more frequently done, the time of $S + G_2 + M$ is simply subtracted from the generation time.

The analytical methods discussed so far fail to take into account the fact that generation times and phase durations vary from cell to cell and even the relative durations of the phases in the cycles of individual cells may vary from cell to cell (Sisken and Morasca, 1965). In addition, the independently measured generation time is frequently not the mean generation time (see Section V). Therefore, the durations calculated from the proportions of cells in a phase are approximations that, for the most part, cannot be given statistical terms such as "mean mitotic duration" or "median S-phase duration." The exact relationships of the calculated durations to the mean, or median durations have not as yet been resolved, so one can only safely say that they constitute an approximation to some measure of central tendency (see Section IV).

The remainder of this chapter deals with the variability of populations of cells as they pass through the cell cycle and its phases, and some of the effects this may have on cell cycle analyses.

IV. Review of Generation-Time and Phase-Duration Distributions

A. General Considerations of Distributions

Before discussing the effects of the variation of individual generation times or phase durations on cell cycle analyses, some definitions and a review of some of the characteristics of distributions are in order. The "generation time" is defined for our purposes as the interval between

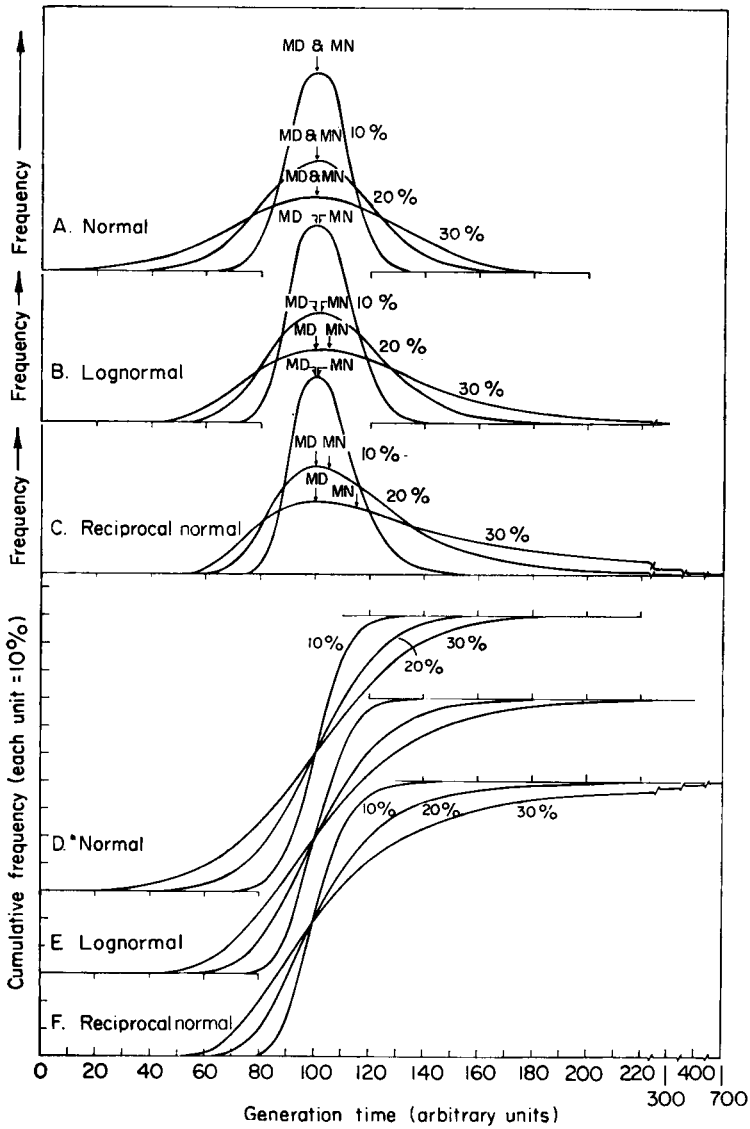


FIG. 2. Examples of three types of frequency distributions (A–C) and corresponding cumulative frequency distributions (D–F) of generation times. MD, median; MN, mean. The percentage values shown are coefficients of variation. The means for the reciprocal normal curves were determined empirically by graphic integration. The other means were calculated (see text for further details).

the times of two successive "cell separations" or division into two separate physiological entities. (Generation time could as easily be defined as the time between two successive anaphases, or two successive metaphases, or any other identifiable identical points in the cell cycle.) "Phase duration" is defined as the interval between entry into and exit from a particular phase, e.g., S phase, metaphase. The term "distribution" has a precise mathematical meaning when used in probability theory (Feller, 1957, 1966), but for our purposes it denotes the way that the generation times or phase durations vary between cells, i.e., the way that they are distributed over a range of values. The meaning of the term will become clearer as we discuss particular types of distributions.

In that which is to follow, we will be speaking about distributions in general, but we will use generation-time distributions to exemplify the concepts involved. These concepts are equally applicable, in most cases, to phase-duration distributions.

Although the generation times of individual cells in a large population will vary over a fairly large range, there is a tendency for most of the cells to have generation times near the middle of the range. This central tendency may be stated in terms of probability; the probability of an individual cell having a particular generation time is greatest at some middle point and decreases on either side of that point. In practice, the probability of occurrence of generation times is inferred from the frequency with which each particular generation time is found in a large population. The results are usually presented in the form of a frequency distribution or a cumulative frequency distribution. Figure 2 illustrates examples of possible generation-time distributions. Three examples each of three different types of continuous frequency distributions, (A–C), are presented. Also shown are the same distributions plotted as cumulative frequency distributions (D–E). A point on a frequency distribution curve shows the number of cells with the particular generation time listed on the abscissa. A point on a cumulative-frequency distribution curve shows the percentage of the population with a generation time equal to or less than the time listed on the abscissa. That is, the cumulative frequency distribution curve is the integral of the curve for the frequency distribution (expressed as percentages) as a function of generation time.

The basic features of the familiar, bell-shaped, normal frequency distribution curve (Fig. 2A) are obtainable from any elementary statistics text. Briefly, the equation describing the curve is

$$f(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-(1/2)[(x-\mu)/\sigma]^2} \tag{8}$$

The position of the peak and the amount of spread of the curve are determined by the terms μ and σ, respectively. The term, μ (mu), represents the mean generation time, i.e., the average of all of the generation times of the cells in the population. The term, σ (sigma), corresponds to the standard deviation of the population. The limits, plus and minus 1 standard deviation from the mean, enclose 68.2% of the population. The coefficient of variation (c.v.) of the distribution is σ expressed as a decimal fraction of μ, c.v. $= \sigma/\mu$, or as a percentage, $\sigma/\mu \times 100$.

A logarithmic-normal (lognormal) distribution of generation times is a normal frequency distribution of the logarithms of the generation times. A reciprocal normal distribution of generation times is simply a normal distribution of the reciprocals of the generation times, that is, a normal distribution of the generation rates (generation rate = 1/generation time).

A fourth type of distribution (not shown) has been proposed for the distribution of generation times, namely the Pearson type III (Powell, 1956). This distribution is based upon mathematics that we are not prepared to evaluate at this time.

The three types of generation-time distributions shown are all based on the normal distribution function, Eq. (8), and therefore, are all characterized by μ and σ. As mentioned above, with the normal distribution, μ is the mean generation time; however, with the lognormal or reciprocal normal distributions, μ is the mean logarithm or mean reciprocal of the generation time. These latter two values when transformed back to generation times do not equal the mean generation times.

Another measure of central tendency is the median. The median generation time is the time by which 50% of the population has divided. Its value is independent of the shape of the distribution; since it is always just the 50% point. Therefore, as a measure of central tendency, it is frequently preferred over the mean when distributions are asymmetrical and the mean could be overloaded by a relatively few very high values. Statistically speaking, the median is not as efficient as the mean but, as will become apparent, it is sometimes the best measure we have; and, where the distribution is known to be lognormal, the mean generation time can be calculated from it.

The curves shown in Fig. 2 illustrate these points about different types of generation-time distributions. In Fig. 2A, it should be noted that the normal distribution curve is symmetrical, that the median and the mean generation times are identical for all three coefficients of variation. In Fig. 2B, it may be seen that the lognormal frequency distribution, when plotted on linear coordinates, is skewed to the right, and the greater the coefficient of variation, the greater the skewness. This skewness results in

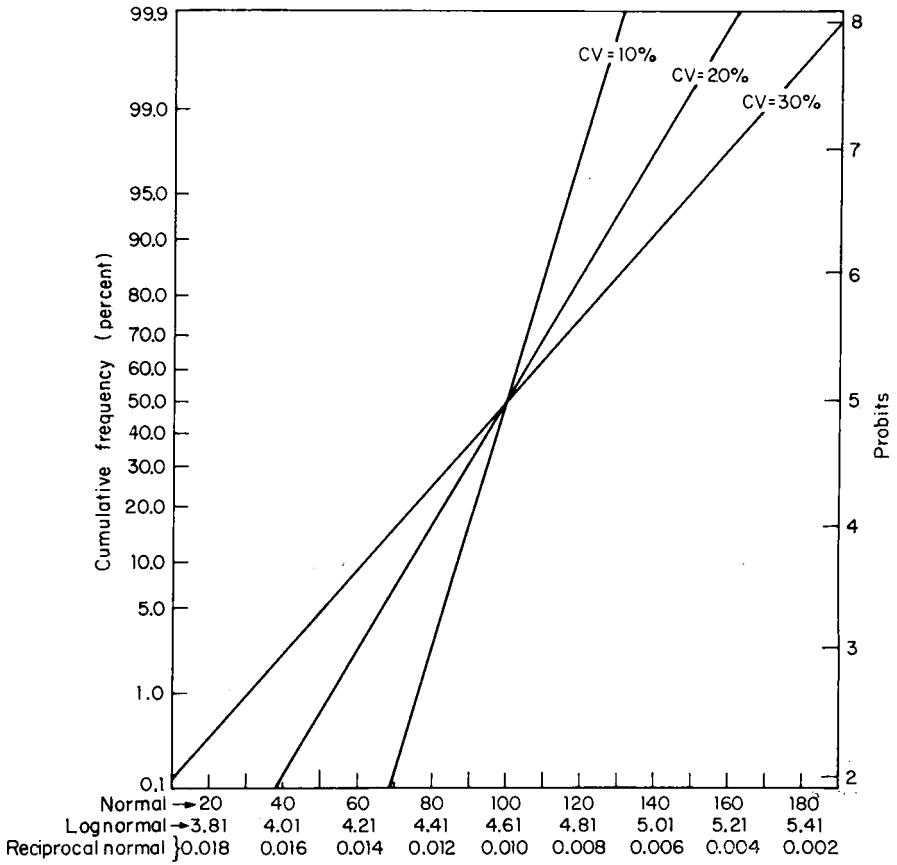


FIG. 3. Plot of the three types of cumulative frequency distributions from Figs. 2D–F on probability paper. CV, Coefficient of variation. Note three abscissa scales (see text for details).

a difference between the median and the mean generation times. In the reciprocal normal curve, Fig. 2C, the mean is even more displaced from the median at any particular coefficient of variation because the curve is even more skewed to the right. In the cumulative frequency distributions plotted in Figs. 2D–F, this skewness is also apparent. This skewness is completely eliminated by transforming the generation time to either the logarithm of the generation time for the curves of Fig. 2E or the reciprocal of the generation time for the curves in Fig. 2F. Then the lognormal distribution and the reciprocal normal distribution would be superimposable upon the normal distribution curve. Reference to Fig. 3 may help make this clear, but first it should be explained.

Figure 3 shows cumulative distribution curves with three different coefficients of variation plotted on probability paper. The ordinate scale of probability paper is based upon a transformation of the cumulative percentage curve to a linear value expressing the number of standard deviations from the mean. The commonly used linear value is the probit. A probit of 5 represents the 50% point. Probit of 6 is up 1 standard deviation from the mean at 84.1%. A probit of 7 is up 2 standard deviations from the mean at 97.7%. Going the other way, minus 1 standard deviation from the mean is at 15.9% or probit 4; minus 2 standard deviations from the mean is 2.3% or probit 3. As will become apparent later, probits or probability paper are very useful in the analysis of generation-time and phase-duration distributions, so a table of percentages with their corresponding probit values are given (Table II). From such a table, one can convert percentages to probits and use linear coordinates for plotting cumulative distribution curves. Probability-scaled paper may be obtained from Keuffel & Esser Company with a variety of abscissa scales, e.g., linear, and 2- or 3-cycle logarithmic.

Returning to Fig. 3, it may be seen that there are 3 lines representing three different coefficients of variation. There are three sets of abscissa

TABLE II

SHORT TABLE OF PROBIT TRANSFORMATION OF PERCENTAGE RESPONSE[a]

% Response	Probit value									
	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
% Response	Probit value									
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
97	6.88	6.90	6.91	6.93	6.94	6.96	6.98	7.00	7.01	7.03
98	7.05	7.07	7.10	7.12	7.14	7.17	7.20	7.23	7.26	7.29
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

[a] After Fisher and Yates, 1953.

values corresponding to normal, lognormal, and reciprocal normal distributions. Note that the reciprocal normal scale is reversed; the symmetry of the transformed distribution allows this. Thus, any distribution which can be transformed to a normal distribution can be plotted on probability paper and yield a straight line by a proper choice of the abscissa values.

Qualitatively, the steeper the slope of the line, the lower is the coefficient of variation or standard deviation. The standard deviations for all three distributions are the differences between the abscissa values at which the 15.9% and 50% values fall, and the coefficients of variation for the normal and reciprocal normal distribution, which are based on these standard deviations, have the usual form of σ/μ.

The coefficient of variation for the lognormal distribution is calculated differently

$$\text{c.v.} = (e^{\sigma^2} - 1)^{1/2} \tag{9}$$

(Aitchison and Brown, 1957). (The term, e^{σ^2}, can be calculated using a table of natural exponential functions.) This relationship results in the coefficient of variation for a lognormal distribution being independent of the mean and approximately equal to the standard deviation for values up to about 0.40, which, in turn, results in distributions with different means, but the same coefficients of variation, being parallel on log-probability coordinates. (In the case of the normal distribution, parallel lines are the result of equal standard deviations, but different coefficients of variation.)

The coefficient of variation is important because it expresses the variability of a population in terms of its mean, and therefore allows one to determine if two populations which have very different mean generation times have the same degree of variability. For example, one can visualize that under one set of conditions leading to a short generation time the spread of generation times around the mean may be small in terms of minutes, yet under another set of conditions leading to a long generation time, the spread may be large even though the inherent variability of the population is the same. It is as though the situation leading to the long generation time allows time for the cells to spread out. So also, one can visualize that if a population of cells started out synchronously and progressed through a series of steps toward the next division the spread of times to the first step would be small and that to the last step large, yet the inherent variability of the population might not have changed. Thus the coefficient of variation may be a more meaningful measure than the standard deviation in some cases. With the lognormal distribution, this problem is automatically resolved.

B. Consideration of Distributions as Applied to Cells

Of the three types of distributions shown in Fig. 2, the normal distribution fits the published data on generation-time distributions least well, so we will not consider it further. Almost all the published generation-time distribution data show a noticeable skewness to the right (Cook and Cook, 1962; Killander, 1965; Killander and Zetterberg, 1965; Dawson *et al.*, 1965; Marin and Bender, 1966; in addition to those mentioned below). These data appear to fit either a reciprocal normal or lognormal distribution. Kubitschek (1966) has presented a case for the generality of the reciprocal normal distribution of generation times; i.e., the normal distribution of generation rates. On the other hand, Schmid (1967) has reanalyzed statistically some of the published data for goodness of fit to see which of the two distributions might be better. He tested the data of Prescott (1959) and McDonald (1958) for *Tetrahymena pyriformis*, the data of Sisken and Morasca (1965) for human amnion cells, the data of Kubitschek (1962) for *Escherichia coli*, and the data of Powell (1956) for *Bacillus megaterium*. All of the data he analyzed were found to be consistent with a lognormal distribution of generation times (the linearity χ^2 values resulting from a maximum-likelihood regression of the weighted probit of percentage frequency against logarithm of generation time are below the expected χ^2), but only some of the data were found to be consistent with a reciprocal normal distribution. Therefore, he concludes that the lognormal distribution is more widely applicable to generation-time distributions. Barrett (1966) also finds that the assumption of a lognormal distribution for his Monte Carlo approach to generating labeled mitotic waves allows a better fit of his data than does assumption of a normal distribution of generation rate or of generation time.

In statistical treatments of data, the only justification needed for using a particular transformation function is that it adequately fits the data (Finney, 1947). However, it is desirable to have some theoretical basis for expecting the data to follow a particular function. We have such a theoretical basis for expecting either a normal distribution of generation rates or a lognormal distribution of generation times. Koch (1966) has reviewed many of the factors that can produce normal and lognormal distributions. The following discussion is based upon his review.

As is well known, in biology many variables are normally distributed, e.g., the heights of human male adults. Normal distributions are expected on the basis of the central limit theorem: " . . . the sum of k stochastically independent variables of any sort converges stochastically with increasing k towards a normal distribution" (Anonymous, 1962). A normal distribu-

tion of a measured variable results from the addition of a large number of small independent variables, or from the addition of a smaller number of normally distributed variables; for example, head size, trunk length, leg length, foot size. So also it could be with the overall generation rate of a cell; a summation of a sequence of independent rates of progression around the cell cycle could lead to a normal distribution of overall generation rates. On the other hand, a lognormal distribution results from multiplicative or proportionate effects in a series of independent variables. For example, the sizes of two precursor pools might be normally distributed in a population of cells but the size of one of the pools might alter the rate of a bimolecular reaction involving the other pool. The multiplicative interaction of the two pools could generate a lognormal distribution of the times required to produce a given amount of, say, a division-essential substance. In fact, Koch (1966) has pointed out that the logarithm will always enter from the kinetics of a first order process —a process that abounds in the biochemistry of cells.

At present, we have no overriding reason for choosing one or the other of the two distributions or some other distribution, as yet unknown, as being more descriptive of cells in general or certain cells in particular. We can only say that the frequency distribution is likely to be skewed to the right. However, in our attempt to assess the effect of generation-time and phase-duration distributions on cell cycle analysis, we have chosen the lognormal distribution for study at this time because it is intermediate between the normal distribution, which is unskewed, and the reciprocal normal distribution, which is greatly skewed. In addition, some of the mathematical properties of the lognormal distribution discussed in Section IV,A make it easier to handle in computer simulation. Therefore, judged from Schmid's analyses, the assumption that generation times are lognormally distributed is a sufficiently close approximation to enough data to warrant using it for our purposes. Even if we are wrong, some of the concepts to be presented apply qualitatively to skewed distributions in general.

V. Effect of the Distribution of Generation Times on the Empirical Determination of Generation Times

The generation time plays a primary role in assigning time durations to the phases of the cell cycle from the determination of the fractions of the population in particular phases. Furthermore, any error in its determination may magnify the errors in phase-duration determinations.

Therefore, a fairly extensive discussion of methods of determining generation times may be warranted.

A. Inequality of Mean Generation Time and Culture-Doubling Time

Many students of cell biology determine generation times from the time taken for the cell number in a culture to double, the implication being that the mean generation time is being determined. Lately, this value is being called more properly the culture-doubling time because of the increasing awareness that not all cells in the culture continue to proliferate, i.e., they may die but not lyse, or just not divide. This phenomenon is especially true of mammalian cell cultures. To correct for the death of cells in a population, the percentage dead cells at each sampling time (determined by direct observation or from the loss of the ability to exclude dyes, e.g., eosin, trypan blue, nigrosin) may be subtracted from the total cell number to obtain a measure of live cell number which can then be plotted on semilog axes to obtain the rate of exponential increase in cell number or a doubling time that is more realistic. Correction for nondividing but living cells requires determination of plating efficiency or growth fraction.

Even with these corrections, however, the culture-doubling time is not a measure of the mean generation time, especially if the generation-time distribution is skewed. Reference again to Figs. 2B and C may make this clear. Figures 2B and C show various lognormal or reciprocal normal frequency distributions plotted on linear coordinates. All of these distributions have the same median generation time. However, note that the skewness to the right introduces some cells with very long generation times which are not counterbalanced by correspondingly very short generation times at the left side of the distribution. Therefore, the mean generation time in such skewed distributions is always greater than the median generation time. Furthermore, intuitively, one can visualize that if 50% of the cells have a short generation time and the range of these short generation times is narrow, these cells will contribute more to the geometric increase in cell number than will the cells with longer and more spread-out generation times. The discrepancy between culture-doubling time and mean generation has been pointed out by Powell (1956), Cook and Cook (1962), Barrett (1966), and by Painter and Marr (1967). Barrett provides an approximation equation for use when the distribution of generation times is normal and $\sigma \ll T$, and Painter and Marr provide an approximation equation and a table of correction factors for use when the distribution is a Pearson type III. The mathe-

matically inclined reader is referred to these latter two papers for further details. The influence of a lognormal distribution of generation times on the culture-doubling time has been tested by computer simulation and it has been found that the culture-doubling time under these conditions is less than the median generation time when the standard deviation of the distribution is greater than about 0.15 (Nachtwey, in preparation).

B. Accurate Methods

Methods are available for the determination of median and mean generation times that are more accurate than the culture-doubling time method. These methods either circumvent, or measure and use the distribution of generation times.

1. Exact Method

The true mean generation time of cells can be determined most accurately by taking an average of the generation times of a number of individual cells determined from time-lapse cinematographic records or from very frequent observations. (See Sisken's chapter in Vol. I for a review of the cinematographic method.) From such data, the median and mean generation times and the standard deviation can be obtained by the usual statistical procedures. This method is usually very tedious, but some shortcuts are possible. Some tediously obtained data on the generation times of *Tetrahymena pyriformis* (Nachtwey and Dickinson, unpublished) will be used to illustrate methods for handling such data and in Section V,B,2 to illustrate a less tedious method for obtaining it.

With a mouth-tube-controlled micropipette, 60 furrowing *Tetrahymena* cells were selected from a mass culture in groups of 10 cells per selection and pipetted individually into 1 μl drops of conditioned medium under paraffin oil.[4] The selection and pipetting into drops required less than 1 minute for the 10 dividers. The cells were examined every minute initially for the time of separation of the just-selected cells and every 2–6 minutes thereafter until all 120 of the daughter cells had divided. The time of initial cell separation was subtracted from the time at which a cell was observed to have separated again to obtain the generation time. The uncertainty in the measured generation times was at most 5 minutes,

[4] The drops were prepared by tapping a Falcon Plastics Co. 35 × 10 mm tissue culture petri dish (catalog No. 3001) with a fire polished 1-mm diameter capillary tube filled with about 1 cm of medium. The drops were covered immediately with thick paraffin oil in order to prevent evaporation (Nachtwey and Dickinson, 1967). Other less wettable plastic surfaces do not allow drop formation as well as the tissue culture-type plastic. Silicone oil takes up water or allows evaporation.

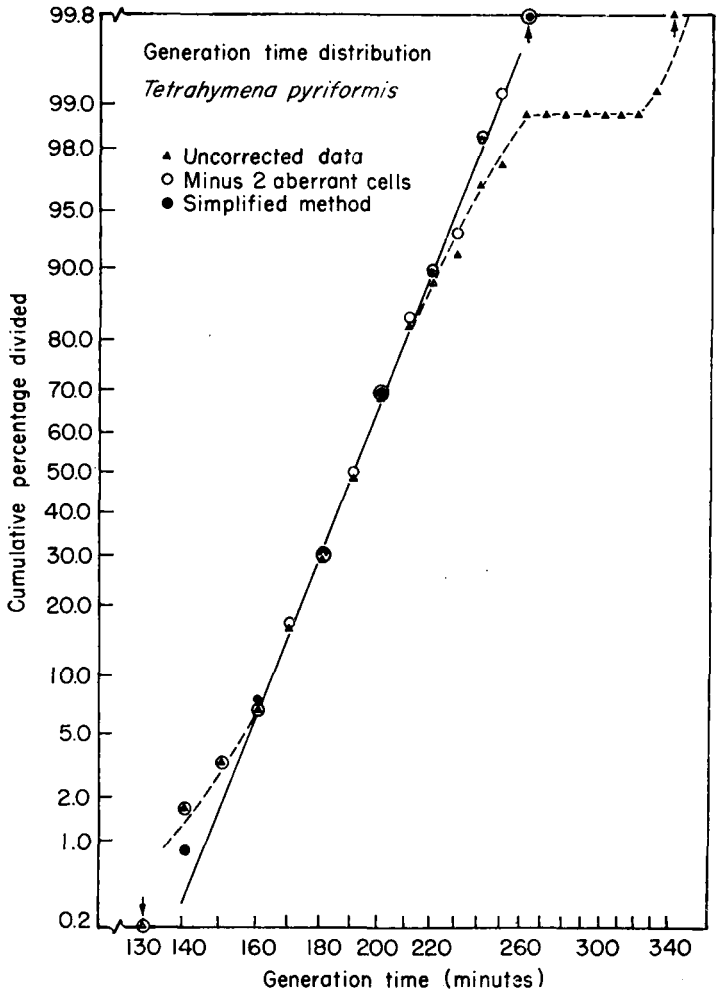


FIG. 4. *Illustration of methods of handling generation-time distribution data for Tetrahymena pyriformis.* The arrows indicate points that are off-scale at 0 or 100% (see text for details).

but the majority of generation times were accurate to within 2–3 minutes. The mean generation time, calculated by simple averaging, was 193.6 minutes; the median generation time was 191.5. The cumulative distribution of the measured generation times is shown on logarithmic-probability coordinates in Fig. 4 (triangles). For clarity, only the points at the end of each 10-minute interval are plotted. It may be seen that, at the upper end, the points deviate from the straight line representing a lognormal

distribution. This deviation results from two sister cells which divided much later than the other 118. From their very long generation times only, these outlying cells appear to be from another population, that is, not in any way typical of the population under study; therefore, they may be eliminated from consideration (Bliss, 1967). (That these two cells were abnormally small and abnormal morphologically is further justification for eliminating them.) After elimination, the mean and median generation times were 192.5 and 190 minutes, respectively.

The cumulative distribution curve obtained after eliminating the aberrant cells is indicated by open circles. Except at the low end, the points follow the straight line fairly well, indicating that the generation times are lognormally distributed. The points at the lower end do not deviate sufficiently to warrant eliminating any cells from them. With relatively small samples, one can expect deviations at the extremes as a result of chance alone, even if the population sampled is known to be distributed lognormally. In drawing the line through the points, the middle points, e.g., in the range 16–84%, should be given more weight than the extremes and if these middle points fall on the line, this is adequate for most studies. More exact mathematical methods can be used to obtain the best fitting lines. Finney (1947) has written a monograph on probit analysis in which the subject is exhaustively treated, Aitchison and Brown (1957) present the elements needed for a computerized approach to probit analysis, and Litchfield and Wilcoxon (1949) have devised a simplified method of fitting lines to apparently normal or lognormal cumulative distributions. Bliss (1967) treats several methods of mathematical analysis of cumulative distributions in a clear manner. Further discussion of these methods, however, is beyond the scope of this chapter.

Having constructed a curve of the cumulative distribution of generation times, one can use it to obtain information about the distribution. The median generation time is obviously interpolated from the intersection of the plotted line with the 50% line. If the distribution is lognormal, the standard deviation of the distribution can be obtained from the following equation (from Aitchison and Brown, 1957):

$$\sigma = \ln\left[\frac{1}{2}\left(\frac{X_{50}}{X_{16}} + \frac{X_{84}}{X_{50}}\right)\right] \tag{10}$$

where X_{50}, X_{16}, and X_{84} represent the linear time values (i.e., in minutes) read from the abscissa corresponding to the intercepts of the plotted line with the 50, 16, and 84% ordinate values. In the example presented, $\sigma = 0.112$. Knowing σ, one can refer to Table III and interpolate the mean to median ratio and then calculate the mean generation time, which, for

TABLE III

RELATIONSHIPS OF THE CUMULATIVE LOGNORMAL DISTRIBUTION[a]

Sigma	Coefficient of variation cv = $(e^{\sigma^2} - 1)^{1/2}$	Ratio of mean to median $e^{\sigma^2/2}$	Ratio of mean to mode $e^{3\sigma^2/2}$
0	0	1.0000	1.0000
0.05	0.0500	1.0013	1.0038
0.10	0.1003	1.0050	1.0151
0.15	0.1508	1.0113	1.0343
0.20	0.2020	1.0202	1.0618
0.25	0.2540	1.0317	1.0983
0.30	0.3069	1.0460	1.1445
0.35	0.3610	1.0632	1.2017
0.40	0.4165	1.0833	1.2712
0.45	0.4738	1.1066	1.3549
0.50	0.5329	1.1331	1.4550

[a] The values were extracted from a table compiled by Aitchison and Brown (1957).

the example, is 192.3. This agrees well with the 192.5 minutes obtained by mathematical averaging.

2. SHORTCUT METHOD

The less tedious approach to the determination of generation-time distributions and the median and mean generation times therefrom, is also based upon the construction of a cumulative distribution curve and the use of the appropriate mean to median ratios. The same experimental observations used for the treatment described above were treated as though they were observations obtained with a different experimental design to see if fewer observations and fewer calculations could provide about the same information. We simulated an experiment by listing the actual times of first- and second-cell separations. We then simulated an observation by setting an arbitrary time and examining the division times to see if the observation time was greater. If it was, we counted that as a separated cell. For the initial cell-separation time, we examined the data at 5 and 10 minutes and found that roughly half had divided by 5 minutes and the remainder by 10 minutes; those not dividing in the 10 minute interval were crossed off. (If, in an actual experiment, some cells had not divided within the 10-minute interval, they could have been eliminated from further consideration by merely withdrawing them from their drop.) Without calculation, we assumed that the "average" cell-separation time for every group was 5 minutes after selection and used this value as the initial cell-separation time for all of the cells in the

group. We then examined the data for each group at 20-minute intervals after the starting point for the group and determined the number of cells that had divided at any time previous to the observation time. (In an experimental situation, this is easily done by determining the number of drops with 3 or 4 cells and counting one division where there are 3 cells and two divisions where there are 4.) We did not need to record whether a particular cell had divided during one interval or another; we recorded just the number in the group that had divided by a particular time. Summing the numbers for all groups having divided by the various times after the starting point (multiples of the 20-minute interval) and expressing the sum as a percentage of the total number of cells in all groups yields the cumulative percentage dividers as a function of time.

The results are plotted as filled circles in Fig. 4. (As before, the two aberrant cells were eliminated from consideration.) The points, in most cases, are superimposed on those obtained by the more tedious method and the position and slope of the line needs no alteration, so the sigma value and the median and mean generation times are not altered.

The results from this simulated experiment indicate that it is possible to obtain generation-time distributions and median and mean generation times with many fewer observations, records, and calculations than are required in the method in which generation times of individual cells are measured. The method is not restricted to cases in which dividing cells are able to be selected; all that is required is that the cells can be kept track of and that the initial division time be known to fall in a short time interval relative to the period during which the population divides. The time interval of examination for subsequent division should be chosen so that at least 5 or 6 observations are made during the period when the cells are dividing. If the generation-time distribution is skewed, the time intervals may be made progressively longer toward the upper extreme of the distribution without significantly affecting the results.

The study of generation-time distributions per se can have greater significance than merely as an adjunct to the determination of the mean generation time. For example, Cook and Cook (1962) showed that the coefficient of variation of the distributions of generation times of *Euglena gracilis* was greater when grown in a simple medium than when grown in complex medium. Prescott (1959) made a similar finding using *Tetrahymena.* These findings suggest that a difference in the coefficients of variation might result from requiring the cell to provide more of its precursor substances and thus increase the chance for accumulating errors in a step-by-step progress to division. [This idea was first expressed by Kendall (1948, 1952) who based it upon the initial suggestion of Rahn (1932)· that variation in generation times might result from

temporal errors in only the gene-duplication process (see also Kubitschek, 1966).] In experimental studies of the effects of an agent on the generation-time distribution, such as those of Cook and Cook (1962) and Prescott (1959) the shortcut method presented may prove useful.

3. Accurate Method for Use over Several Generations

Another accurate and unbiased measure of the rate of cell proliferation is the mean number of divisions per unit time (Christensen and Giese, 1956). This measure may be obtained in situations where a single starting cell and its progeny may be reidentified and followed with time, for example, cells in drops under oil or in hanging drops, mapped or photographed cells plated-out on agar, or attached to glass or plastic. The number of cells in each of, say, 25–50 clones arising from a single cell is determined by occasional microscopic observation. The number of cells/clone (N) at any particular time is related to the number of divisions (D) that have occurred. For binary fission, the equation is

$$N = 2^D \tag{11}$$

The number of divisions can be determined from the number of cells per clone by

$$D = \frac{\log_{10} N}{\log_{10} 2} \tag{12}$$

or, what is the same thing,

$$D = \log_2 N \tag{13}$$

Table IV gives the most frequently used $\log_2 N$ values. Averaging the number of divisions/clone at the various observation times and plotting them as a function of time yields a straight line for cultures showing

TABLE IV

Number of Divisions ($\log_2$) versus Number of Organisms/Clone[a]

	0	1	2	3	4	5	6	7	8	9
0	—	0.00	1.00	1.58	2.00	2.32	2.58	2.81	3.00	3.16
10	3.32	3.46	3.58	3.70	3.80	3.91	4.00	4.10	4.18	4.25
20	4.33	4.40	4.47	4.53	4.59	4.65	4.70	4.76	4.80	4.86
30	4.91	4.96	5.00	5.04	5.09	5.13	5.17	5.21	5.25	5.29
40	5.32	5.37	5.40	5.43	5.46	5.50	5.53	5.56	5.59	5.62
50	5.64	5.67	5.70	5.73	5.76	5.78	5.81	5.83	5.86	5.88
60	5.91	5.93	5.96	5.97	6.00	6.03	6.05	6.07	6.09	6.11

[a] Number of organisms/clone appears at top and side of table with the corresponding number of divisions appearing in the body of the table.

exponential growth. The reciprocal of the slope of the line is the generation time, or more precisely, the time for an average of one division.

This analytical method reduces the biasing effect of cells with short generation times "swamping out" those with longer ones. Furthermore, in experiments where an inhibitor is applied, deviations from exponential growth by a sensitive portion of the population is not masked by the least affected cells.

VI. Effect of G_2-Duration Distributions and Mitotic Durations on Percentage-Labeled Mitoses Curves

The duration of the G_2 phase is usually measured by adding a radioactively labeled precursor of DNA and determining the time required for labeled mitotic cells (or dividing cells) to appear. In the ideal case in which all cells have identical G_2 durations, the time for the first labeled mitotic cell to appear is the G_2 duration and the time at which the last unlabeled mitotic cell has divided is the $G_2 + M$ duration. The time at which half of the mitoses are labeled is the $G_2 + \frac{1}{2}M$ duration. This results from the cells at the very end of S being labeled, the cells that are an instant older (at the very beginning of G_2) being unlabeled, and from the cells remaining in mitosis for a period of time. The percentage of labeled mitotic cells is the relationship between labeled cells entering mitosis and unlabeled cells leaving it.

In practice, cells do not have identical G_2 durations; as with generation times, the G_2 durations are distributed around some modal value. In an attempt to determine the effect of a distribution of G_2 durations on the percentage-labeled mitoses curves, a computer program was written to simulate such curves. This approach is predicated on the concept that curves generated with known input variables may allow us to interpret empirically determined curves where the underlying variables are not known. For example, with the computer approach it is possible to establish the relationship of the generated 50%-labeled-mitoses time to the input median G_2 duration, and the relationship of the slope of the generated percentage-labeled mitoses curve to the slope of the input G_2 duration.

A. Description of Graphical Approach to Simulation of Percentage-Labeled Mitoses Curves

The computer program uses a "graphical" approach. In Fig. 5, the approach is schematically represented by an example. In Fig. 5A, we

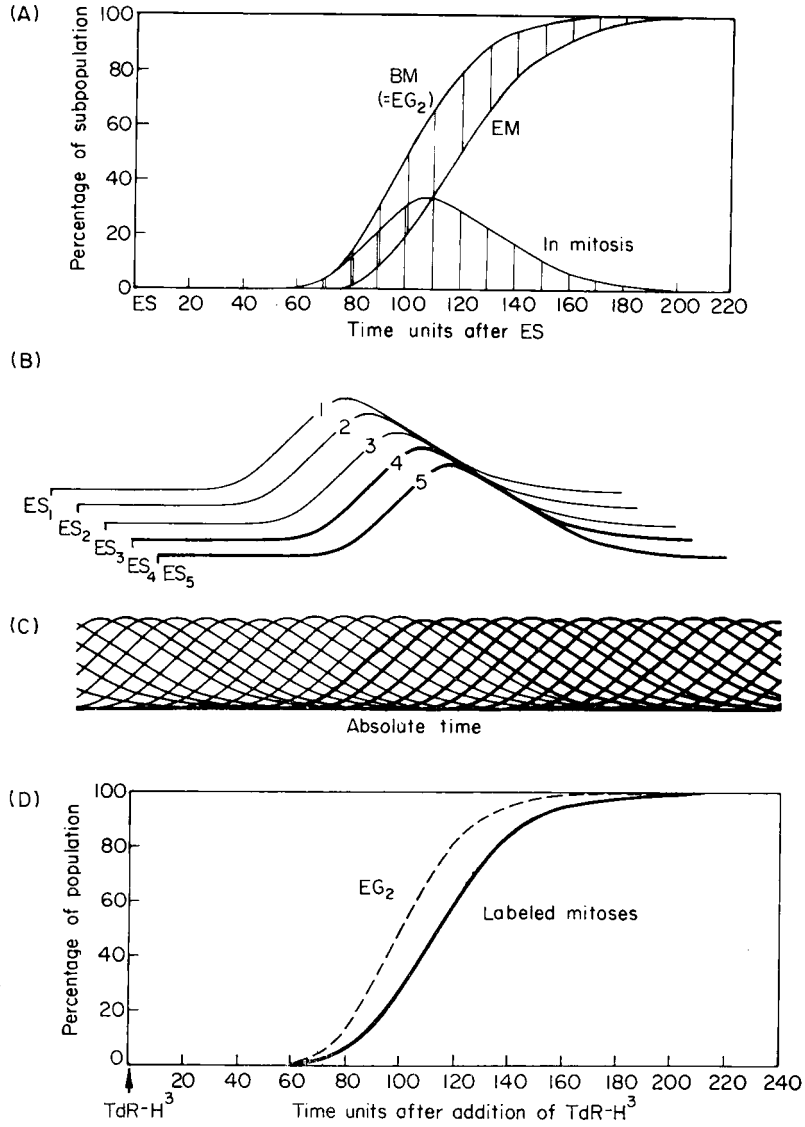


FIG. 5. Schematic illustration of the graphical approach upon which is based the computer program for generation of percentage-labeled mitoses curves. BM, begin mitosis; EG_2, end G_2; ES, end DNA synthesis; ES_x, end of S phase for subpopulation X where $X = 1, 2, 3, \ldots$; TdR-H^3, tritiated thymidine (see text for further details).

have assumed that we have a subpopulation which is perfectly synchronized at the end of the S phase (ES), and that the cells in this subpopulation will begin mitosis (BM) and simultaneously end G_2 (EG_2) at times that are lognormally distributed around a median time 100 units later and will end mitosis (EM) at times distributed around a median of 120 units after ES (i.e., the median time in mitosis is 20 time units). We will assume also that σ for the lognormal distribution of times to beginning of mitosis is equal to σ for the times to the end of mitosis. (It may be recalled that with lognormal distributions, when σ's are equal, the coefficients of variation are equal.) The percentage of this particular subpopulation that is in mitosis at any given time after ES is the difference between the percentage that has begun mitosis and the percentage that has passed into interphase. Thus if 80% of the subpopulation has entered mitosis and 10% has left mitosis, 70% of the subpopulation is still in mitosis. The percentage of cells from this single subpopulation in mitosis at various times after ES is also shown in Fig. 5A. This curve only shows the percentage of cells in mitosis; it doesn't say anything about whether they are in early, middle, or late stages of mitosis.

Now let us consider subpopulations with other ES times. The curves for these different subpopulations will be identical to those shown in Fig. 5A, but they will be offset on an absolute time axis. This is illustrated in Fig. 5B, which shows cells-in-mitosis curves for 5 subpopulations with different ES times; the axes for these curves are staggered to show the ES times. In Fig. 5C, curves for many such subpopulations are plotted on the same axis. The ordinates of Figs. 5B and C are numbers of cells from each subpopulation in mitosis, directly converted from the percentage values, shown in Fig. 5A, by assuming that each subpopulation consists of 100 cells. (This conversion is the theoretically correct one for simulating a steady-state cell-proliferation system. For an exponentially growing system, a correction for the age gradient could be introduced here, but the period during which the labeled mitoses appear is usually short compared to the generation time, so the effect of the age gradient is practically negligible.) From Fig. 5C it should be apparent that, at any particular time, an actively proliferating cell system will have mitoses from many subpopulations that synchronously ended the S phase some time earlier.

Now let us consider that a labeled precursor of DNA is added (e.g., thymidine-H^3, TdR-H^3) to the proliferating cell system. A subpopulation that was just at the beginning of G_2 at the time the labeled precursor was added will be unlabeled, as will those that ended S at earlier times. A subpopulation that is just at the end of S at the time of addition will be

labeled, as will those ending S at later times. Labeled subpopulations are represented in Figs. 5B and C by heavier lines. The number of labeled mitoses relative to total mitoses at any particular time can be determined graphically by taking a vertical slice through the set of curves in Fig. 5C at that time, and summing the numbers of labeled mitoses contributed by all of the labeled subpopulations and the numbers of mitoses contributed by all subpopulations in the cell system. By taking observations at various times, the percentage-labeled mitoses curve as a function of time is obtained as shown in Fig. 5D. Included for comparison is the EG_2 curve for the subpopulation that was just at the end of S when the thymidine-H^3 was added; this curve represents the actual distribution of G_2 durations.

B. Computer Program Characteristics

The computer program generates percentage-labeled mitoses curves in a graphical manner similar to that discussed above; the cumulative percentages of a subpopulation beginning and ending mitosis at each time unit are calculated and subtracted to yield the percentage of a subpopulation in mitosis at any time unit and the percentages are converted directly to numbers of cells. The total area under the cells-in-mitosis curve (the integral) for any one subpopulation is equal to the total number of cells in mitosis at one particular time from all subpopulations. The integral of one cells-in-mitosis curve as a function of time is the number of labeled cells in all subpopulations as a function of time after addition of thymidine-H^3. Therefore, it is not necessary to generate a series of curves offset at successive time intervals like those shown in Fig. 5C; the area under any one curve is the total number of mitoses (labeled and unlabeled) at any one time from all subpopulations, because, as may be visualized from Fig. 5C, the number of cells from various subpopulations at any one observation time, if plotted as a function of time after the end of each subpopulation's S phase, would produce the same curve as shown for any one of the subpopulations. Similarly, the integral from 0 time to time t, as a function of time after end S, calculated as a percentage of the total area, yields the simulated percentage-labeled mitoses curve. The computer program integrates the generated cells-in-mitosis curve by cumulating the numbers of cells in division at each successive time unit.

For the computer generation of a family of curves, the input variables to the computer were the length of the mitotic period relative to the median G_2 duration (equivalent to the median BM time) and the standard deviations of the BM and EM distributions. For the studies to

be mentioned below, the median G_2 duration was set at 100 time units, the median EM time was 100 + the mitotic duration, and σ for the lognormal BM and EM distributions were equal to each other.

C. Computer Simulated Percentage-Labeled Mitoses Curves

Figure 6 shows percentage-labeled mitoses curves for $\sigma = 0.20$ and several durations of mitosis plotted on log-probability coordinates. The G_2-duration distribution, applicable to all of the curves, is shown for comparison. Although the curve for the G_2-duration distribution is a

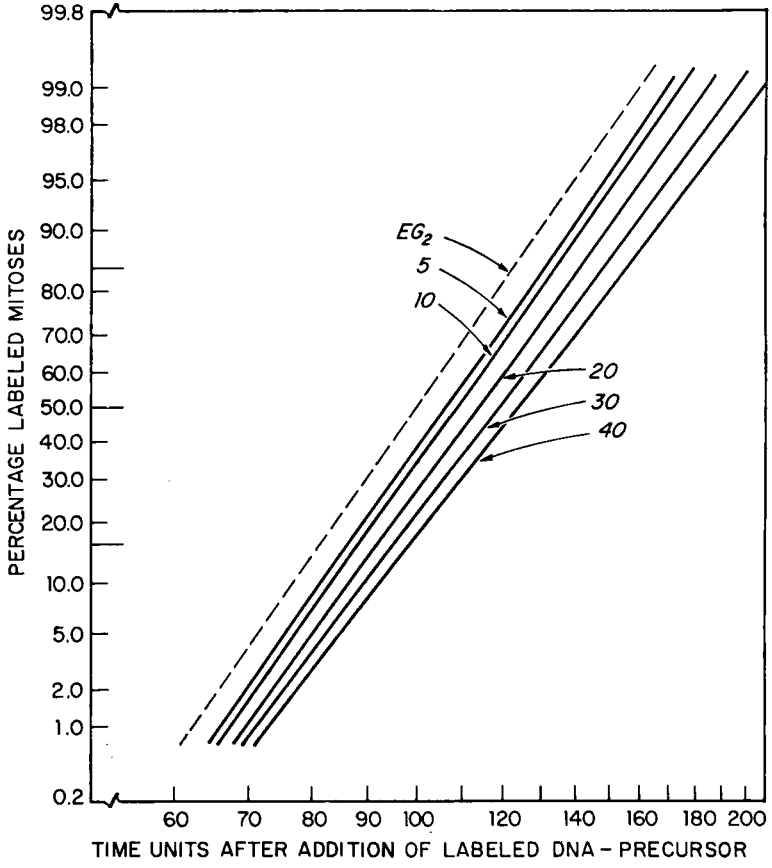


FIG. 6. Computer-generated percentage-labeled mitoses curves for hypothetical populations with a lognormal distribution of G_2-durations with $\sigma = 0.20$, and with the different mitotic durations of 5, 10, 20, 30, and 40 time units. The long tics at the 15.9, 50, and 84.1% points correspond to the mean and minus and plus one standard deviation from the mean (see text for details).

straight line (because we assumed lognormality) none of the percentage-labeled mitoses curves are absolutely straight because they result from the interaction between two lognormal distributions. However, they can be treated as though straight.

From the generated percentage-labeled mitoses curves in Fig. 6, it is apparent that the time to 50%-labeled mitoses is always greater than the median G_2 duration and that the amount by which it is greater depends upon the duration of mitosis. Families of curves with other input standard deviations show that it also depends on the standard deviation of the G_2-duration distribution and that, although the percentage-labeled mitoses curves are not exactly straight, for any one σ for the G_2-duration distribution, the trends of the labeled mitoses curves are less steeply sloped as the mitotic duration increases. Thus, there is no constant relationship between the median G_2 duration and the 50%-labeled mitoses time, or between the slope of the percentage-labeled mitoses curve and the G_2-duration curve for all σ values and mitotic durations. Furthermore, since the input mitotic durations are expressed as a percentage of the median G_2, which is an unknown in experimental studies, the computer output as it stands only demonstrates the phenomena involved but is of little practical value.

D. Application of Computer Results to Experimental Data

1. Continuous Labeling

To make the output useful for interpretation of percentage-labeled mitoses curves, a set of median G_2 times expressed as fractions of the generated 50%-labeled mitoses times were calculated using input variables of different σ values for the G_2-duration distribution and different median durations of mitosis expressed now as a percentage of the generated 50%-labeled mitoses time. Figure 7 shows the actual median G_2-duration (G_2 time) as a fraction of the 50%-labeled mitoses time plotted as a function of the calculated σ for the percentage-labeled mitoses curve [σ was calculated by Eq. (10)]. This figure can be used to determine the median G_2 duration when the 50%-labeled mitoses time, the σ value of the labeled mitoses curve, and the median duration of mitosis, expressed as a percentage of the 50%-labeled mitoses, are known. The first two variables are easily determined from the labeling technique already mentioned. The duration of mitosis can be obtained by direct observation, by application of Eq. (2) (see Section III,B), to the proportion of cells in mitosis when the generation time is known, or by a method to be

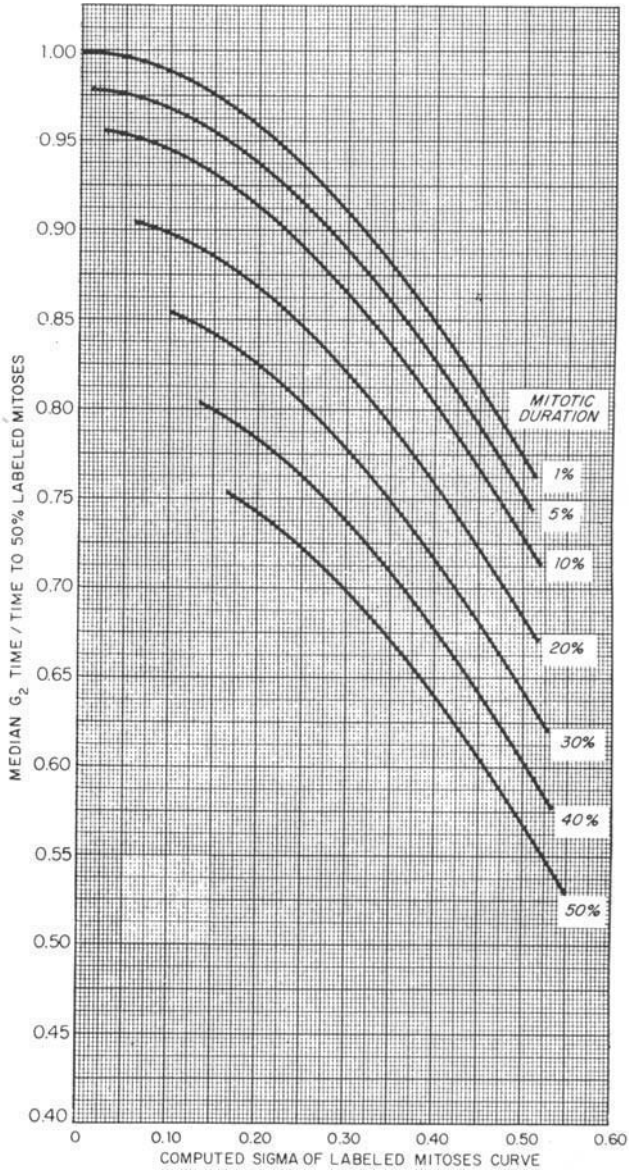


FIG. 7. Graph of computer-generated correction factors for determination of the actual G_2 from the measured time to 50%-labeled mitoses. For use the standard deviation (σ) of the percentage-labeled mitoses distribution and the mitotic duration, expressed as a percentage of the time to 50%-labeled mitoses, must be known (see text for further details).

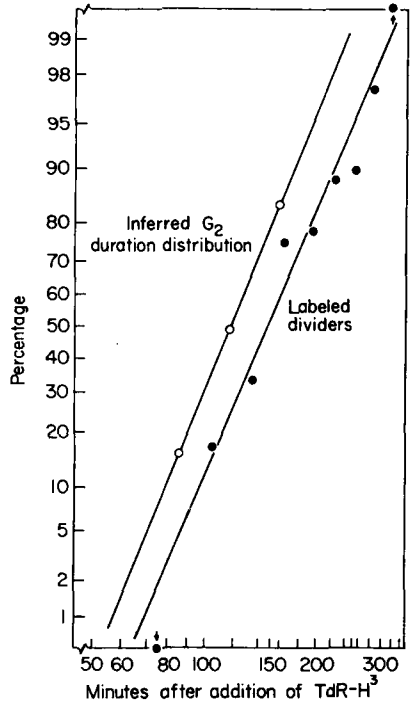


FIG. 8. Illustration of methods for determining the median G_2-duration and the G_2-duration distribution using *Tetrahymena pyriformis*. Arrows indicate points that are off-scale at 0% or 100% (see text).

presented in Section VII. The mitotic duration expressed as a percentage of the 50%-labeled mitoses time is used to enter the figure.

Some data for *Tetrahymena pyriformis* strain HSM growing at 20° in fortified proteose peptone medium (Cameron and Nachtwey, 1967) will be used to illustrate the application of Fig. 7. *Tetrahymena*, a ciliate protozoon, divides amitotically, but shows well-defined cleavage furrows for a period prior to cell separation. Therefore, we will speak of dividing cells instead of mitotic cells. The approach is equally applicable in either case. In Fig. 8 the data points, on logarithmic-probability coordinates, represent the percentage of labeled dividing cells observed at various times after the addition of thymidine-H^3. The line was computed by the maximum likelihood method (Finney, 1947), but an eye-fit giving more weight to points toward the middle of the distribution might have served as well. The 50%-labeled dividers time is 146 minutes; σ, for the labeled dividers curve is 0.32. The median division duration is 33.5 minutes (determined by observation of dividing cells) or 23% of the 50%-labeled

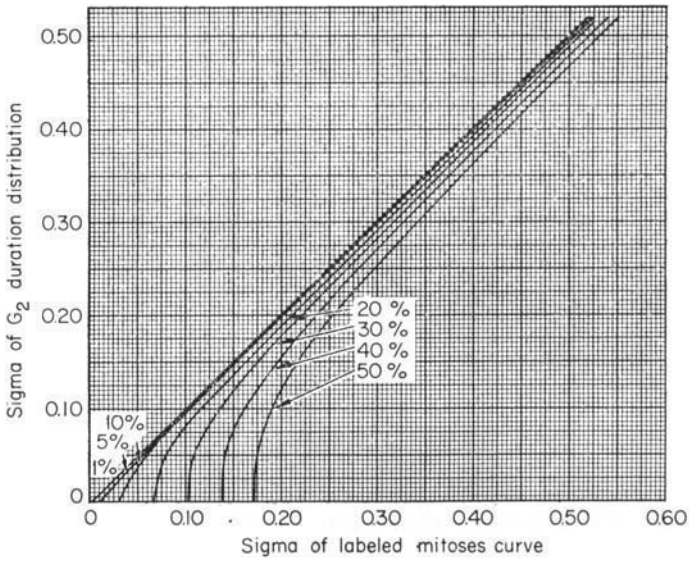


FIG. 9. Graph of computer-generated correction factors for determining the standard deviation, σ, of the G_2-duration distribution from the σ of the percentage-labeled mitoses curve when the mitotic duration is known. Numbers by each curve refer to mitotic duration, expressed as a percentage of the measured time to 50%-labeled mitoses (see text).

dividers time. With these values, Fig. 7 is entered at 0.32 on the abscissa and the ordinate values on the 20% and 30% curves determined. The ordinate value for 23%, interpolated between these two values, is 0.80. This value times 146 minutes, the measured median value from Fig. 7, is 117 minutes, the median G_2 time.

To obtain a measure of σ for the G_2-duration distribution, Fig. 9 may be used in a similar manner. This figure shows σ of the G_2-duration distribution as a function of σ of the labeled dividers curve (= labeled mitoses curve) for various division durations expressed as percentages of the 50%-labeled dividers times. The σ value for the G_2-duration distribution was interpolated to be 0.31. The time values corresponding to the 16% and 84% points, X_{16} and X_{84}, for a distribution with this σ are calculated from the following equations:

$$X_{16} = e^{(\ln X_{50})-\sigma} \tag{14}$$

$$X_{84} = e^{(\ln X_{50})+\sigma} \tag{15}$$

because $\ln X_{50} = \mu$ (the mean) for the lognormal distribution, $X_{50} = e^{\mu}$, and the 16% and 84% points are minus and plus one σ from the mean. (It may be recalled that e is the base of the natural logarithm.) For the

example, X_{16} and X_{84} were calculated to be 86 and 160 minutes, respectively, and a straight line through these points and X_{50} is shown in Fig. 8, labeled "Inferred G_2-Duration Distribution."

2. Pulse Labeling

The computer-generated relationships can also be applied to the results of pulse-labeling experiments. The inferred G_2-duration distributions can be obtained as outlined above for *Tetrahymena*. One can also obtain the inferred $S + G_2$ distribution. To obtain the latter distribution, one can use the percentage-unlabeled mitosis as a function of time and apply the same concepts as used earlier, but now assuming that the subpopulation just before the beginning of S (at the end of G_1) is synchronous at that point and that these cells are the first unlabeled mitoses to appear among a population of mitoses which up to that time was 100% labeled. One must assume that the period of availability of the thymidine-H^3 is practically negligible or subtract the time of availability from each time point. The percentage-unlabeled mitoses curve is thus comparable to the percentage-labeled mitoses curve and the same relationships apply so that the time to 50%-unlabeled mitoses multiplied by a correction factor equals the median $S + G_2$ duration.

In a pulse-labeling study of intestinal epithelium (data to be published elsewhere), it was found that the σ for the $(S + G_2)$-duration distribution is less than that for the G_2 distribution. This difference in the variances (σ^2) of the $(S + G_2)$- and G_2-duration distributions suggests that the durations of the S and the G_2 periods are not independent, i.e., if S is long, G_2 is short, and vice versa. The biological implication of such a dependency has been discussed by Sisken and Morasca (1965), who have suggested that it indicates that compensatory physiological mechanisms are involved as cells progress from phase to phase. The finding of a reduced variability in $S + G_2$ relative to G_2 in intestinal epithelium lends support to the concept (Mazia, 1961) that the preparations for division proceed on parallel pathways rather than strictly sequential ones and that the "trigger" for cell division may be related to the beginning of S and not to the end.

This brief discussion of the comparisons of the σ values serves to emphasize that information about possible mechanisms of control of cell division may be obtained from studies in which the phase-duration distributions are studied.

The reappearance of labeled mitoses (second ascending curve in pulse-labeling experiments) are difficult to interpret because of the interaction of the labeled and unlabeled mitoses from subpopulations that are by

now widely spread out from the initial point of assumed synchrony (at the time of addition of labeled DNA precursor). The second ascending curve of percentage-labeled mitoses, unless it approaches 100%, is a resultant of the second division of cells that were in S at the time of labeling and the second division of unlabeled cells that were in G_1 and G_2 or M at that time, and, also, when σ for the generation-time distribution is fairly high, some third division cells from these two groups. Thus, in those cases where the second ascending curve only reaches a peak of 50–60%, the time from the first 50%-labeled mitoses to the second, is surely an overestimate of the median generation time. In those cases, where the peak value is under 50%, extrapolation of the lower portion of the curve to 50% is likely to yield an even more invalid estimate of generation time. Unfortunately, there is no general solution to this problem at present.

VII. Methods for Determining the Durations of Mitotic Phases *in Vivo*

In many cell systems *in vivo* some cells proliferate, others differentiate, and others migrate away so that the fractions of the total population that are in the various mitotic phases may bear little relation to the durations of the phases. Unless one can distinguish the proliferating cells from the other cells, one cannot determine the durations of the mitotic phases from the fractions of cells in the various mitotic phases. The only general method of which we are aware, other than by direct observation through implanted windows and the one to be presented, that allows determination of mitotic phase durations in *in vivo* cell systems involves comparisons of frequencies of mitotic stages with and without colchicine (or other spindle poisons) (Leblond, 1959). [Odartchenko *et al.* (1964) outlined a method for the special case in which the prophase duration equals the telophase duration.]

A. Approximate Method

A new method arose from the results of the computer program outlined in Section VI,B, which showed that when the standard deviations for the G_2-duration distributions are less than 0.20, the 50%-labeled mitoses time minus one-half the median mitotic duration deviates less than 4% from the actual median G_2 duration. Therefore, when σ is less than 0.20, the following relations hold within 4%:

$$TLP_{50} = G_2 + \tfrac{1}{2}P \tag{16}$$
$$TLM_{50} = G_2 + P + \tfrac{1}{2}M \tag{17}$$
$$TLA_{50} = G_2 + P + M + \tfrac{1}{2}A \tag{18}$$
$$TLT_{50} = G_2 + P + M + A + \tfrac{1}{2}T \tag{19}$$

where TLX_{50} is the time to 50%-labeled prophases (P), metaphases (M), anaphases (A), or telophases (T), and the same letters designate the median durations of the phases. (These times are interpolated from percentage-labeled prophases, metaphases, etc., curves.) Subtracting one equation from another yields

$$TLM_{50} - TLP_{50} = \tfrac{1}{2}P + \tfrac{1}{2}M \tag{20}$$
$$TLA_{50} - TLM_{50} = \tfrac{1}{2}M + \tfrac{1}{2}A \tag{21}$$
$$TLT_{50} - TLM_{50} = \tfrac{1}{2}A + \tfrac{1}{2}T \tag{22}$$

Therefore, with this set of simple algebraic equations and the times to 50%-labeled mitotic phases, if we can determine the duration of one phase by some independent method, the others can be calculated by substitution.

The most easily distinguished mitotic phases are metaphase and anaphase. In a steady-state system or in an exponentially proliferating system in which the M + A duration is much less than the generation time, the ratio of the number of cells in anaphase to the number of cells in metaphase (irrespective of whether labeled or unlabeled) is a fairly good measure of the relative durations of anaphase and metaphase. Knowing the A/M ratio (R), one can determine the relative duration of A in terms of M [Eq. (24)], substitute for A in Eq. (21), rearrange and solve for M. In a similar manner, the other equations can be rearranged. The set of equations required for the determination of the median mitotic phase durations is as follows:

$$M = \frac{2(TLA_{50} - TLM_{50})}{1 + R} \tag{23}$$
$$A = R \times M \tag{24}$$
(R = No. of anaphases/No. of metaphases)
$$P = 2(TLM_{50} - TLP_{50}) - M \tag{25}$$
$$T = 2(TLT_{50} - TLA_{50}) - A \tag{26}$$
$$G_2 = TLP_{50} - \tfrac{1}{2}P \tag{27}$$

In many cases, distinguishing early prophase from G_2 is so uncertain that grouping G_2 and prophase together is warranted. For these cases,

$$G_2 + P = TLM_{50} - \tfrac{1}{2}M \tag{28}$$

TABLE V
APPLICATION OF APPROXIMATE METHOD OF DETERMINING MITOTIC-PHASE DURATIONS TO MOUSE COLON EPITHELIUM

Data	Results (minutes)
TLP_{50} = 66 (63–68)[a]	G_2 = 49
TLM_{50} = 92 (90–94)	P = 34
TLA_{50} = 103 (101–105)	M = 18
TLT_{50} = 119 (116–121)	A = 4
A/M ratio (R) = 0.22	T = 28
σ of percentage-labeled Anaphase = 0.18	

[a] Ninety-five percent confidence intervals in parentheses.

A significant feature of the equations presented above is that they can be applied to cells *in vivo* and it is not necessary to know the generation time of the cells or the growth fraction (fraction of the population that is actively proliferating) to determine the durations of the mitotic phases. Furthermore, if the mitotic index changes only slowly during the period of observation, the values should be fairly independent of any diurnal variation in the number of cells in mitosis. (To check this, the A/M ratio should remain fairly constant during the period of major increase in percentage-labeled mitoses, however, a perturbation that changes both the anaphase and metaphase durations simultaneously would not be detected.)

This approximate method has been applied to data obtained with mouse colon epithelium (Cameron and Nachtwey, in preparation). The results are shown in Table V.

B. Method Using Correction Factors

If σ of the distribution of the durations of G_2 + mitotic phases is between 0.20 and 0.35 or if more accurate determinations of the mitotic

TABLE VI
CORRECTION FACTORS FOR USE IN CALCULATING MITOTIC DURATIONS WITH EQS. (29)–(34)

σ[a]:	0.01	0.05	0.10	0.15	0.20	0.25	0.30	0.35
F:	1.00	1.00	0.99	0.98	0.96	0.93[b]	0.90[b]	0.87[c]

[a] Calculated σ of the percentage-labeled anaphases distribution.

[b] If the phase duration is less than $\sim \frac{1}{10}$ of TLX_{50}, add 0.01.

[c] If the phase duration is less than $\sim \frac{1}{20}$ of TLX_{50}, add 0.01; if it is greater than $\sim \frac{1}{3}$ of TLX_{50}, subtract 0.01.

phase durations are desired than available with the approximate method, the following method and equations may be applied.

Determine the σ for the percentage-labeled anaphases curve using Eq. (10). This σ should be fairly close to the σ for the distribution of durations of $G_2 + P + M$, because anaphase is usually very short compared to $G_2 + P + M$ (see Fig. 9). With this estimate of σ, enter on Table VI and determine the factor F to be used in Eqs. (29)–(34) along with the times to 50%-labeled phases and R determined by the methods given in the previous subsection.

$$M = \frac{2F(TLA_{50} - TLM_{50})}{2 - F(1 + R)} \tag{29}$$

$$A = R \times M \text{ as before} \tag{30}$$

$$P = \left(\frac{F}{2 - F}\right) \times 2(TLM_{50} - TLP_{50}) \tag{31}$$

$$T = 2(TLT_{50} - TLA_{50}) - \left(\frac{2 - F}{F}\right) A \tag{32}$$

$$G_2 = F(TLP_{50} - \tfrac{1}{2}P) \tag{33}$$

$$G_2 + P = F(TLM_{50} - \tfrac{1}{2}M) \tag{34}$$

The basis for this more accurate analytical method is somewhat complicated, so details will be presented elsewhere. Briefly, it makes use of the finding from the computer-generated curves that the ratio of the input median G_2 duration (true G_2) to the G_2 duration calculated by subtracting one-half the mitotic duration (the approximated G_2) depends only on the σ of the G_2-duration distribution for $\sigma < 0.35$ and is practically independent of the mitotic duration (up to the highest mitotic duration tested, 50% of the time to 50%-labeled mitoses). The values given in Table VI are these ratios of true G_2 to approximated G_2, which, times the approximated G_2, yields the true G_2. Therefore,

$$G_2 + P = F(TLM_{50} - \tfrac{1}{2}M) \tag{35}$$

$$G_2 + P + M = F(TLA_{50} - \tfrac{1}{2}A) \tag{36}$$

Rearranging these equations, substituting $R \times M$ for A, and subtracting one from another, yields

$$F(TLA_{50} - TLM_{50}) = M - \tfrac{1}{2}FM + \tfrac{1}{2}FRM \tag{37}$$

which rearranges to Eq. (29). In a similar fashion, the F factors are introduced into the other equations [Eqs. (30)–(34)].

The reason that Eqs. (29)–(34) are applicable only up to $\sigma = 0.35$ is that above this σ value, the F factors for different mitotic durations start

to diverge considerably (in fact, they start to diverge at $\sigma = 0.25$ but the differences are negligible), so without an estimate of the phase duration, which is the unknown we are trying to determine, the appropriate F factor cannot be selected.

C. Evaluation of the Methods

These analytical methods are only strictly valid if the distributions of G_2, $G_2 + P$, $G_2 + P + M$, $G_2 + P + M + A$ durations are lognormal and the σ's for the distributions are equal. (Preliminary results indicate that if the distributions are normal and the coefficients of variation for the distributions are equal, the analytical methods are valid to the same degree as for the lognormal situation. As yet, we have not tested the effect of having different σ's or coefficients of variation for, say, the $G_2 +$ P- and $G_2 + P + M$-duration distributions.) In an experimental situation, one may check whether these assumptions are valid by plotting the points on log-probability coordinates [or converting to probits (Table II) and plotting on semilog coordinates] and checking for linearity. The calculated σ's for the various curves of percentage-labeled mitotic phases will likely not be equal. In fact, as may be inferred from Fig. 9, the calculated σ's for the percentage-labeled anaphase and percentage labeled metaphase curves are likely to be less than for the corresponding prophase and telophase curves because the former phases are usually shorter. The inferred σ values (from Fig. 9) of the distributions of the durations of G_2, $G_2 + P$, $G_2 + P + M$, etc., should be approximately equal within experimental error.

Since the selection of F depends on the calculation of σ for the percentage-labeled anaphase curves, errors in the determination of σ will affect the results. However, as may be inferred from Table VI, F as a function of σ changes slowly, so a moderate error in determining sigma will have a small effect on F and consequently a small effect on the calculated mitotic durations.

It must be emphasized that the times to 50%-labeled pro-, meta-, ana-, and telophases (TLX_{50}) and the corresponding mitotic phase durations are in time units, e.g., minutes, and are not expressed as a percentage of TLX_{50}. This is required because the relationship upon which the method is based was established with the mitotic duration expressed as a percentage of the median G_2 duration, which was set equal to 100 for the computer generation of curves. When the median G_2 duration was set at 50 or at 200 and the duration of mitosis was expressed as a percentage of the median G_2 duration, the same F values were calculated because the input variables were in the same relative units. However, when the

duration of mitosis was expressed as a percentage of TLX_{50}, the units did not remain the same and the factor varied with the mitotic duration, which makes it less useful for our purposes.

VIII. Statistical Considerations in the Determination of Generation-Time and Phase-Duration Distributions

It must be emphasized that the medians and the sigma values determined for any distribution are subject to statistical error, which will be carried over to any inferred distributions. For this reason, a mathematical analysis of the data is recommended: maximum likelihood method (Finney, 1947), or the quicker method of Litchfield and Wilcoxon (1949). These methods allow the determination of the confidence intervals of the sigma values as well as the confidence intervals of the median values. It is important to consider the statistical variation because a measured distribution is a combination of both the biological variation that one seeks to measure, plus the sampling variation and variation introduced by technique. The sampling variation is, to an extent, corrected for in drawing the best fitting line through the series of data points, each of which is subject to error. The sampling error can be further reduced by performing the experiment several times and thus obtaining better estimates of, say, the percentage-labeled mitoses at various times. The technical variation is, however, a problem that only careful regard to experimental procedures will forestall (see Section II). Any sudden change in environment may induce delays in the progression of cells through the cell cycle. Induction of a burst of synchronous mitosis can affect the basic results that make the refined analyses useless. Such perturbations should be checked in all experiments by determining the mitotic index at all points used to construct percentage-labeled mitoses curves. If no perturbations have been introduced, the mitotic index should remain invariant; however, a perturbation that has the same proportionate effect on both the mitotic duration and the generation time would not be detected.

IX. Summary

In this chapter, we have reviewed some standard techniques for cell cycle analysis and some of the criticisms that have been leveled against certain of them. We have attempted to show that, with due regard to

limitations introduced by statistical variation and experimental technique, more information and more accurate information can be obtained from cell cycle studies (primarily experiments using radioactively labeled DNA-precursors). As a step in this direction, we have presented computer-simulated percentage-labeled mitoses curves and the relationships of the observed curves to the probable biological factors underlying them. These relationships have allowed the devising of some new analytical methods that should help in refining the analysis of empirical data and releasing more of the information therein. Applications of some of the methods have been illustrated.

The biological significance of the distribution of phase durations has been discussed. Measurement of the degree of variability of phase durations has given evidence that compensatory mechanisms may be operating in the various phases of the cell cycle. Because the understanding of distributions of phase durations and generation times are important biologically and analytically, some of the characteristics of the normal, lognormal, and reciprocal normal distributions have been reviewed.

If we have not completely succeeded in providing simple and exact methods for obtaining more information from empirical data, it is hoped that the concepts developed in this chapter may be of use in showing the complexity of cell cycle analyses and thus forestalling naive interpretations.

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Chapter 11

A Method for the Study of Cell Proliferation and Renewal in the Tissues of Mammals

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I. Introduction

Many tissues in the adult mammalian body continue to have cell proliferation throughout the life of the animal. For example, it is clearly established that the epithelium of the gut is renewing cells at a rapid rate (Bizzozero, 1894; Leblond and Stevens, 1948). Estimates are that

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cells which are born in the crypts of the small intestine require but three days to migrate to the tips of the intestinal villi where they are sluffed off into the intestinal lumen and subsequently die (Fry *et al.*, 1963). Similar phenomena occur in epidermis, hematopoietic tissues, lymphopoietic tissues, and in the seminiferous epithelium of the testis. Awareness that such a cell renewal process occurs in the body of adult vertebrates goes back to the last century when it was realized that mitotic activity in the basal layer of epidermis produces new cells at a rate faster than could be accounted for on the bases of growth of the epidermis alone (Flemming, 1884, 1885). C. P. Leblond and several of his associates have confirmed and extended the concept of renewal cell populations in a long and impressive series of reports (Leblond and Walker, 1956; Leblond *et al.*, 1959; Messier and Leblond, 1960; Leblond, 1964; *also see* Edwards and Klein, 1961).

The cytologically observable event of mitosis lasts less than 60 minutes in most mammalian tissues (Stevens-Hooper, 1961; Cameron, 1963). Thus the task of finding mitotic cells in slowly renewing tissues is difficult and sometimes impossible. It is generally conceded that cells which incorporate tritiated (H^3) thymidine into nuclear DNA are cells which are about to divide (Hughes *et al.*, 1958; Mazia, 1961). Since DNA synthesis requires between 6–8 hours in almost every mammalian cell population where it has been studied (Koburg and Maurer, 1962; Cameron and Greulich, 1963; Cameron, 1964; Pilgrim and Maurer, 1965), one can inject an organism with thymidine-H^3, kill the animal shortly thereafter, prepare the tissues for autoradiography, and expect to find seven times more nuclei labeled with thymidine-H^3 than cells which are in division. This so-called "DNA synthetic index" is therefore a more sensitive indicator of the rate of cell proliferation than is the "mitotic index." Nevertheless, even this 7-fold more sensitive indicator of proliferative activity is not good enough to study many of the slowly renewing cell populations.

If we are to study the cell renewal and growth rates of the slowly renewing tissues, it would be of great advantage to have a method which would enable us to label every cell coming into DNA synthesis during extended time periods. It is also necessary that the method of precursor administration does not disturb normal cell proliferation in the animal. A method of this type would enable us to expand and quantify knowledge of renewing cell populations in mammals and also provide an important tool to study the control of cellular growth and renewal in the tissues of the intact mammal. The present paper introduces just such an approach.

II. Assumptions in the Use of Tritiated Thymidine for Studies of Cell Renewal

The assumptions which are made in this chapter are as follows. Tritiated thymidine is a specific radioactive precursor which is incorporated into cells that are in the process of nuclear DNA synthesis. Upon introduction of the precursor into an intact mammal, the radioisotope is distributed throughout the body so that every cell which is synthesizing DNA has an opportunity to incorporate the isotope. Cells which replicate DNA proceed through the cell cycle. The precursor is stably incorporated into nuclear DNA and stays with the cell as long as it is alive. The amounts of thymidine used do not alter the cell proliferation kinetics of the experimental animal. The levels of tritiated thymidine are not toxic to the experimental animal. Every cell which synthesizes DNA during the time the animal is given thymidine-H^3 is labeled. The method of precursor administration does not, of itself, alter the cell proliferation kinetics of the animal. Most of these assumptions have been reviewed in a new book by L. E. Feinendegen (1967); the reader is referred to this excellent source for a more complete coverage.

During the course of this chapter the validity of several of these assumptions will be discussed and evaluated.

III. Choice of a Route of Tritiated Thymidine Administration

When selecting a route for precursor administration one must keep in mind that the duration of DNA synthesis is about 7 hours in almost every cell of any mammalian system where it has been tested. An exceptionally short DNA synthetic period of 4–5 hours has, however, been reported in the cells of the germinal center of rat lymphatic tissue (Fliedner *et al.*, 1964). It has also been demonstrated that unincorporated precursor (thymidine-H^3) is cleared from the blood stream in 20–50 minutes (Potter, 1959; Rubini *et al.*, 1960, 1961; Koburg, 1963; Staroscik *et al.*, 1964), and for all practical purposes a single injection of the precursor into a mammal can therefore be considered a pulse of relatively short duration (Thrasher, 1966). It follows from these facts that a mammal must be administered the precursor at least once every 4–6 hours

if every cell coming into DNA synthesis during an extended period is to be labeled.

Some of the possible routes and schedules of precursor administration are (1) an injection at least once every 4–6 hours (via intraperitoneal, intravascular, intramuscular, or subcutaneous routes), (2) a chronic and automatic infusion system (i.e., indwelling catheter and automatic pump system), and (3) administration of the precursor in the food or drinking water.

An experiment was set up to test each of these routes of precursor administration on the cell proliferation rate of mouse tissues. Use of the precursor itself was omitted from the experimental design so as to avoid the criticisms and the complications of evaluating the physiological effects of thymidine and/or the radiation toxicity of thymidine-H^3. The following section of the chapter describes this experiment.

A. A Comparison of Different Routes of Precursor Administration on the Measurement of Cell Proliferation Kinetics in Mice

Male Swiss albino mice, 75 days of age, averaging 29 gm body weight were used in this study. The mice were divided into four experimental groups 25 hours prior to being killed. The animals in all groups were given free access to mouse breeder chow and drinking water during the experiment. The mice in group I were left in their cages. This group served as the control group. The mice in group II were surgically fitted with an indwelling catheter in a manner similar to that described by Mendelsohn (1962) and were then caged individually. The mice in group III were injected with 0.2 ml of sterile saline every 4 hours. The mice in group IV were subjected to the stress of physical confinement by placing them into holes 3.0 cm in diameter, which were drilled through a piece of wood 9.0 cm thick. Numerous small holes had been drilled in the wood block to assure adequate ventilation. A mouse was confined to the hole by putting one nail across the front and two nails across the back of the hole. Pieces of sliced apple, mouse breeder chow, and water were put before the mice throughout their confinement period. The mice struggled at first then lay quietly for long periods.

Four hours before sacrifice each animal in all groups received an intraperitoneal injection of colchicine (Eli Lilly and Co.) 10 mg% solution in distilled water at a dosage of 1.0 mg/kg body weight (see Stevens-Hooper, 1961 for details of the use of colchicine). The animals were killed by a blow on the head, and the duodenum and esophagus were removed for histological preparation. The tissues were fixed in Hollande's

TABLE I

EFFECT OF DIFFERENT ROUTES OF PRECURSOR ADMINISTRATION ON THE MEASUREMENT OF CELL PROLIFERATION KINETICS IN MICE

	Group I Drinking water (control)	Group II Indwelling catheter for 25 hours	Group III Injected every 4 hours for 25 hours	Group IV Physically confined for 25 hours
Number of animals in each group:	5	4	4	4
Colchicine collected metaphases/duodenal crypt (average ± S.D.):	9.36 ± 1.39	10.30 ± 1.97[a]	9.16 ± 0.83[a]	9.47 ± 0.65[a]
Colchicine collected metaphases/1000 germinal cells of the esophageal epithelium (average ± S.D.):	34.33 ± 11.85	10.67 ± 5.30[b]	9.86 ± 4.24[b]	7.61 ± 3.82[b]

[a] Not significantly different from the drinking water control group as shown by Student's T Test.

[b] Significantly different from the drinking water control group as shown by Student's T Test ($P = <0.01$).

modification of Bouin's fluid, embedded in paraffin, and sectioned at 6 μ. Histological cross sections of the tissues were prepared, stained with Harris' hematoxylin and chromotrope, mounted, and then analyzed for the number of colchicine collected metaphase figures.

Table I lists the number of metaphases per 1000 cells in the layer of the esophageal epithelium and also the number of metaphases per crypt in the duodenum. Between 1100 and 2200 esophageal germinal cells were counted and 11–24 duodenal crypts were counted in each animal.

The results from Table I can be summarized as follows. The procedures of handling mice in groups II, III, and IV caused a significant depression of mitotic activity in the esophageal epithelium over that in the control group I. On the other hand, no significant differences in the mitotic activity of the cryptal cells of the duodenum were found between the control group and the other three groups.

It appears therefore that the rather harsh methods of handling, as carried out in procedures II, III, and IV, do effect cell proliferation kinetics, at least in the esophageal epithelium. That the stress of the handling procedures did not cause significant change in the mitotic activity of the duodenal crypts deserves brief comment. Bullough (1949) has demonstrated that epidermal mitoses are markedly reduced by starvation in mice. On the other hand, Blair and Hooper (1957) presented evidence that starvation in rats causes no significant change in mitotic activity in the duodenal crypts. Cameron (1963) has demonstrated that the cell proliferation activity of chicken-stratified squamous epithelium is depressed under the stress of starvation, whereas duodenal activity is not noticeably changed. Apparently, mitotic activity is controlled by different mechanisms in the two types of epithelial populations.

It is evident from the above experiment that administration of precursor by the drinking water is a method of choice.

B. Drinking Habits of Mice

Knowing that precursor administration via drinking water is a method of choice, it was then important to find out if the experimental animal (mouse in this case) drinks as frequently as every 4 hours. The degree of precursor distribution throughout the body, when administered via the drinking water, will be discussed later in the chapter.

The experimental design was to cage individual adult mice and to record how much water they consumed during 4-hour periods throughout the day. Calibrated 10-ml pipettes were filed down at the tip, so that the mice could drink from them without difficulty. The pipettes were filled with distilled water and stoppered closed at the top end. The mice

TABLE II
DRINKING HABITS OF MICE

Interval of day	Animal number					
	1	2	3	4	1	2
8–12 A.M.	0.82[a]	0.75	1.23	1.01	1.72	0.97
12 A.M.–4 P.M.	1.23	1.08	0.91	0.76	0.80	0.81
4–8 P.M.	1.54	1.57	0.75	0.80	0.87	0.60
8–12 P.M.	0.81	0.95	1.23	0.95	1.15	1.30
12 P M.–4 A.M.	1.11	0.77	0.90	0.80	0.93	0.77
4–8 A.M.	1.21	0.92	1.01	0.82	—	—
Total/24 hours	6.72	6.04	6.03	5.14	—	—
Average intake/ 4-hour period	1.12	1.01	1.01	0.86	1.10	0.89

[a] Milliliters of water consumed.

were allowed to adapt to this equipment several days before measurements were made. The amount of water consumed was read directly on the calibrated scale of the pipette. Control pipettes were set up to correct for evaporation.

Table II shows that mice drink at least once every 4 hours under our laboratory conditions. Observation of the animals while they were drinking indicates that little or none of the water was wasted by spillage. The amount of water consumed in any 4-hour period ranged from 0.60–1.72 ml with an average consumption of 1 ml per 4-hour period.

Thus administration of precursor via the drinking water would be frequent enough and in adequate and regular enough amounts to fulfill the requirements of a practical method of continuous precursor administration. With the above information one can design an experiment to label proliferating cells of mice as described next.

IV. Description of the Method

Adult male mice, 4 months of age, were housed, four to a cage, at 23° ± 0.5°C with 12 hours of dark and 12 hours of light. The animals were allowed to adapt to the room for a month before the experiment began. Special arrangements were made with Schwartz Bioresearch, Inc., Orangeburg, New York for the packaging and delivery of bottles of thymidine-H^3 (specific activity, 6.0 c/mmole) containing 1 mc in 100 ml

of sterile distilled water. The precursor was stored in the refrigerator until used.

Groups of mice were given drinking water which contained the radioisotope (10 μc/ml). Pairs of animals were killed after 4 hours, 1, 2, 3, 5, 7, 10, 14, 21, 30, 40, 50, 71, 90, and 146 days. Other mice were given the isotope for 30 days and then taken off the isotope and given ordinary water. The latter animals were killed at intervals of 3, 12, 36, and 60 days. Other groups of fewer animals were given different dosages of thymidine-H^3 in their drinking water (i.e., 5 μc and 100 μc/ml).

The room of residence was washed frequently and was maintained under positive atmospheric pressure and vented to the outside. The room was monitored for radioactivity. This was done both by sampling the air through a special blower filter system, and by filter paper wipes of surface areas. Counts of these samples were done in a scintillation counter. The counts were never different from the background counts. Care was taken to clean the cages and to change the drinking water at least twice a week. The latter procedure is necessary to avoid contamination of the drinking water with microorganisms which can catabolize the thymidine-H^3. Wood chips and solid waste materials from the cages were put into sealed cannisters and disposed of through a radiation safety program. The cages were washed in a specially designated "hot" sink using copious amounts of soap and water.

Tissues were fixed in Bouin-Holland's solution. Tissues were washed, dehydrated, double embedded (nitrocellulose-paraffin), sectioned at 5 μ, and prepared for liquid-emulsion autoradiography (Kodak NTB-3) (see Prescott, 1964). After exposures of from 1–39 weeks, the autoradiographs were developed (Kodak D-19), stained through the emulsion with Harris' hematoxylin and chromotrophe, cleared, and mounted for study.

V. Results and Discussion of the Method

It is not intended that this chapter cover a detailed report of the cell proliferation behavior of all of the tissues which were analyzed or studied. The detailed survey of most of the tissues of these mice will be reported elsewhere. Only those points are covered which bear directly on the usefulness of the method itself.

Labeling of diverse tissues of the body was seen as early as 4 hours after precursor ingestion. The photomicrographs in Plates I and II demonstrate the extensive and effective distribution of precursor through-

PLATES I AND II. (Figs. 1–8). The figures in these plates are autoradiograms (5-month exposure) of hematoxylin and chromotrope stained tissues of adult male mice (30 gm). In all cases the animals were given thymidine-H^3 in their drinking water at a dosage of 10 μc/ml.

FIG. 1. Plantar epidermis of the hind paw from a mouse given thymidine-H^3 for 3 days ($\times$ 520); essentially every cell in the basal layer is labeled, and some of the cells in the spinous layer are labeled. No cells in the more superficial layers are labeled at this time, however, in autoradiograms of animals left on thymidine-H^3 for 10–14 days, all of the nuclei in all of the layers were found to be labeled.

FIG. 2. Kidney cortex from a mouse given thymidine-H^3 for 21 days ($\times$ 520). Several tubules show radioactive nuclei. The glomerular capsule shows radioactive nuclei.

FIG. 3. Ventral epithelium of the tongue ($\times$ 520). The mouse was given thymidine-H^3 for 10 days; all nuclei are labeled in every layer. In an animal which was given thymidine-H^3 for 5 days all nuclei were also labeled. Care should be taken not to confuse the keratohyalin granules in the granular layer with autoradiographic reactions.

FIG. 4. Posterior pituitary from a mouse given thymidine-H^3 for 21 days ($\times$ 850). Arrows point to the labeled nuclei of two cells. Neither cell is a pituicyte. Labeled glial cells were frequently seen in various parts of the brain.

FIG. 5. Seminiferous tubules and epithelium from a mouse given thymidine-H^3 for 21 days ($\times$ 520). Spermatogonia and most maturation stages of the spermatocytes are labeled, but the layer of spermatids does not have radioactive nuclei. Sertoli cell nuclei were never radioactive.

FIG. 6. Seminiferous tubules and epithelium from mouse given thymidine-H^3 for 30 days ($\times$ 520). All of the maturation stages including mature spermatozoa are now labeled. Notice that the spermatids at the surface of the tubule lumen have radioactive nuclei.

FIGS. 7 AND 8. Epididymal tubules from mice given thymidine-H^3 for 21 and 30 days, respectively ($\times$ 520). Several epithelial nuclei in each figure show positive reactions. Notice that none of the spermatozoa that are stored in the epididymal tubules are overlaid by silver grains at 21 days but that all of the spermatozoa show a reaction after 30 days. Figures 5–8 illustrate how this technique can be used to time and follow the stages of a cell differentiation process.

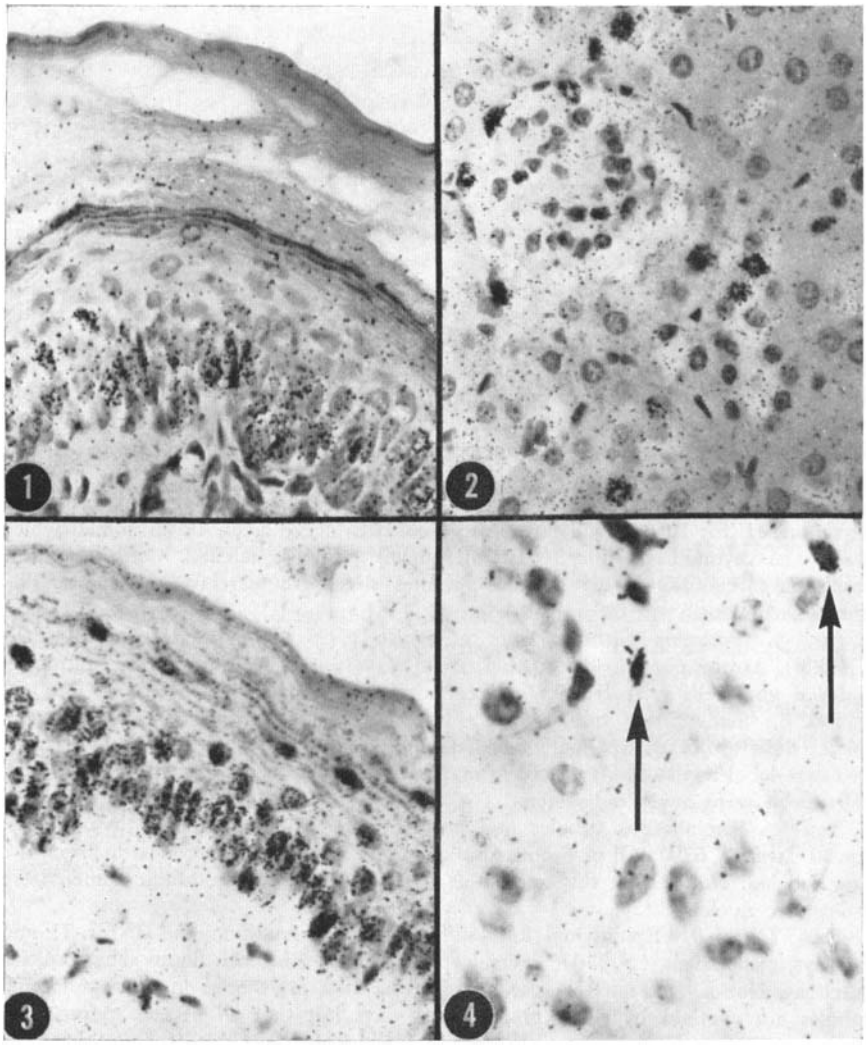


PLATE I. FIGS. 1–4. For legend see p. 269.

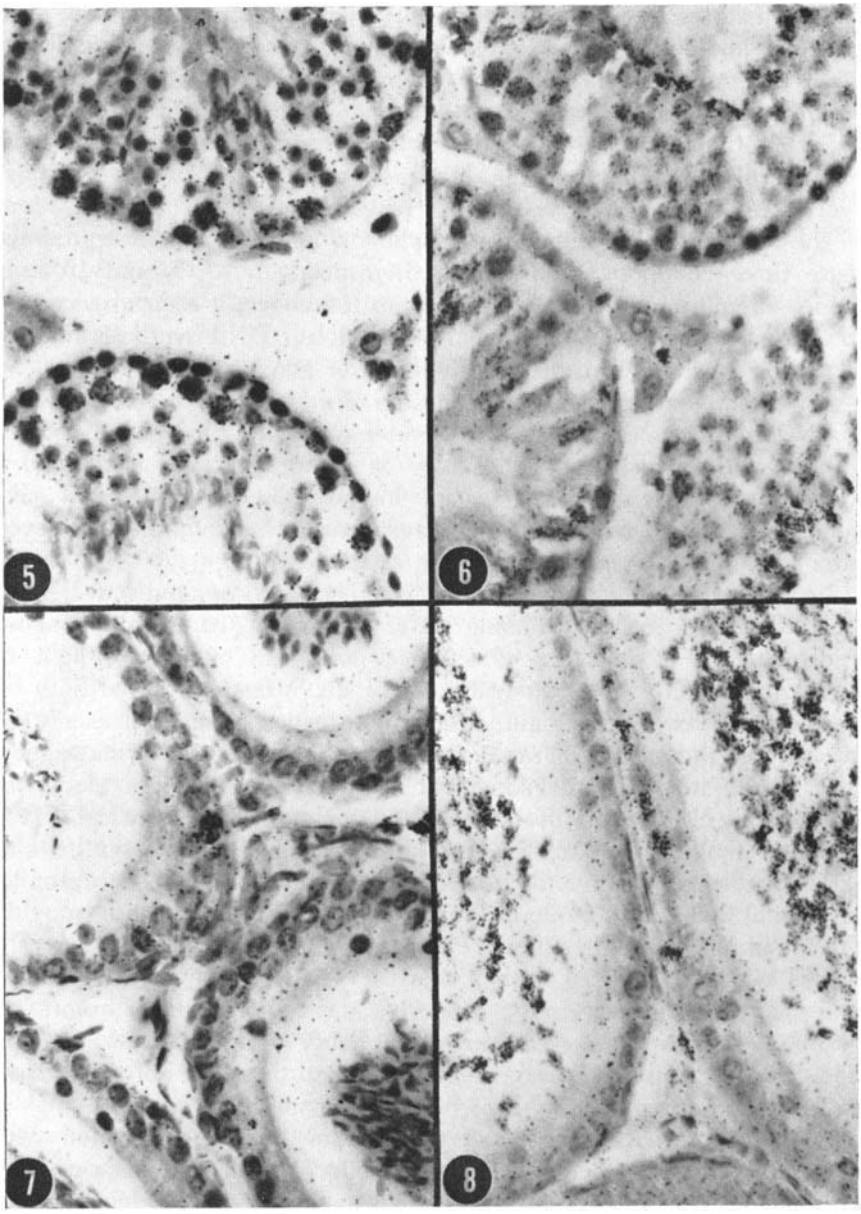


PLATE II. FIGS. 5–8. For legend see p. 269.

out the tissues of the body. Comparison of tissue autoradiographs of mice given thymidine-H^3 orally for 4 hours with those of mice which were injected intraperitoneally (2 hours before being killed) showed little or no differences in the percentage of cells which were labeled in such representative tissues as the plantar epidermis of the hind paw, the esophageal epithelium, and the duodenal epithelium. Within a few days every cell was labeled in such rapidly renewing epithelial cell populations as the small intestine villus, the esophagus, and the plantar epidermis; these three cell populations required, respectively, 3, 7, and 10 days before all cell nuclei were labeled. From the above, it seems reasonable to assume that all cells which come into nuclear DNA synthesis in mice are labeled by the oral method of precursor administration.

A 5-month autoradiographic exposure duration was necessary when the isotope dosage in the drinking water was only 10 μc/ml. Exposure durations of 2 weeks were necessary at dosages of 100 μc/ml, and 2 months were necessary at 20 μc/ml dosage. Dosages of 5 μc/ml gave labeling but the results were not good enough for quantification even after 6–9 months of autoradiographic exposure.

It is estimated that the amount of precursor incorporated into labeled cells after being on the drinking water for 1 day (10 μc/ml) is about equivalent to an injection of 0.05 μc per gram of body weight of thymidine-H^3 into the peritoneal cavity. This estimate is based both on the duration required for autoradiographic exposure as well as on the intensity of the autoradiographic reactions. Similar comparisons suggest that 100 μc/ml in the drinking water for 1 day is comparable to an intraperitoneal injection of about 0.75 μc per gram of body weight. It is apparent that the oral method of labeling cells in DNA synthesis is rather inefficient when compared to the injection method. For example, an animal that drinks 60 μc of thymidine-H^3 in 1 day is getting roughly the same amount of thymidine-H^3 incorporation into nuclear DNA as a mouse which is injected with a total of 1.5 μc of thymidine-H^3. The oral method is therefore more expensive and requires longer autoradiographic exposure times. On the other hand, very low but adequate dosages of thymidine-H^3 are actually getting to the tissues of the animal, and this minimizes the problems of radiation toxicity.

Is there any noticeable effect of radiation toxicity associated with the oral method of precursor administration? The spermatogonia of mice are known to have a very low tolerance to thymidine-H^3 (Johnson and Cronkite 1959). Injection of thymidine-H^3 at dosages as low as 1 μc per gram of body weight apparently causes a low but detectable effect on survival of spermatogonia in the testis of mice. Comparison of the data of Johnson and Cronkite with observation made on the testis of mice

kept on thymidine-H^3 in the drinking water for from 1–146 days (10 μc/ml) suggests that normal proliferation of the spermatogonia in the seminiferous tubules has not been noticeably altered by the oral method of precursor administration (see Plate II for examples of testicular tissues from animals left on the radioisotope for 21 and 30 days). Apparently, the nuclear accumulation of radioisotope per individual spermatogonia does not reach levels of detectable toxicity at dosages of 10 μc/ml in the drinking water. At present no attempt has been made to study the toxic effects of dosages above 10 μc/ml in the drinking water.

It is known that some tissues of the body show a diurnal rhythm which is manifest by a 24-hour periodical variation in the mitotic and the DNA synthetic indexes (Pilgrim *et al.*, 1963). If the animals are left on the oral precursor for at least a day, one need not be concerned with correcting for diurnal variations of this type as the method itself totals all proliferating cells over one complete diurnal cycle.

In a similar manner one need have little concern for the reutilization of catabolities of DNA labeled with thymidine-H^3 from those cells that die during the course of the study. Once again the method itself totals all proliferating and DNA-synthesizing cells and therefore any reutilization can only add to labeling of new cells that enter DNA synthesis. On the other hand, there is some reutilization of the catabolic products of orally administered thymidine-H^3. Evidence of cytoplasmic incorporation of radioactivity can be seen in the figures of Plate I. This level of cytoplasmic incorporation does not cause much of a problem when one is scoring whether a cell nucleus is labeled or is not labeled.

DNase treatment of slides before autoradiography removed essentially all of the radioactivity from the labeled nuclei of cells but left the radioactivity which was in the cytoplasm. Therefore, it appears that the cytoplasmic label is non-DNA material. In those animals which were given precursor orally for 30 days and then removed from the precursor, the amount of cytoplasmic radioactivity was reduced to background levels in most of the tissues within the first 3 days. This indicates that the cytoplasmic radioactivity is associated with nonstable and renewing materials.

Although this oral method of precursor administration should work for most species of mammals, there are some rodents (the woodchuck, the ground squirrel, and the chipmunk) that do not utilize thymidine-H^3 at all (see Adelstein *et al.*, 1964). Alternate approaches, such as the indwelling catheter, may be necessary on larger mammals. The drinking habits of the experimental animal should be established before applying the oral method to any new species or before changing the environmental conditions of an animal with known drinking habits.

VI. Possible Application of the Method for Future Studies

There are many advantages of having a method to label every cell which comes into DNA synthesis and cell division over an extended time.

The author is presently engaged in the process of reclassifying many cell populations of the adult mouse body on the basis of their proliferative behavior. It is already possible to state that stroma cells of the liver, pancreas, salivary glands, kidney, and several endocrine glands, etc., are renewing cell populations. Not only can it be stated that these are cell renewal populations in the adult animal but we can now quantify the renewal process in a precise manner.

It is hoped that future studies will include several tissues in each case so that interrelationships and levels of mitogenic control can be established. Possible experimental procedures may involve administration of hormones which act specifically on target organs and those that act generally [i.e., thyrotropin versus stomatotropin or perhaps adrenaline; for discussion of adrenaline effects see Bullough (1965)], starvation, stress of various types, specific organ regeneration, injection of specific tissue extracts, virus and/or carcinogenic administration (i.e., administration of murine leukemia virus), local tissue or organ irradiation or wounding, tissue or organ transplantations, immune reactions, aging effects, etc.; the information of levels of control which operate in the mitotic and functional homeostasis of the cell populations of the body.

The advantages of being able to "tag" proliferating cells can also be of use in following the migration, differentiation, and the fate of particular cell types. The possible problems that can be studied in this area are numerous (Plate II illustrates the usefulness of this method to time and follow stages of cell differentiation and migration in sperm development).

VII. Summary

The method discussed in this chapter can be used for labeling every cell that comes into DNA synthesis in tissue cell populations of an intact mammal (mouse). The method can be used to study slowly renewing cell populations which have not previously lent themselves to quantitative analysis of cell renewal.

Specifically, this method involves administration of thymidine-H^3 in the drinking water, followed by tissue autoradiography. As opposed to

some other methods, this method does not disturb normal cell proliferation of the animal. The dosages used in this method show no detectable radiation toxicity. Some future applications of the method are mentioned.

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Chapter 12

Isolation and Fractionation of Metaphase Chromosomes

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I. Introduction

Animal cells maintained in cell culture in the logarithmic phase of growth provide a ready source of rapidly dividing cells. By the addition of a mitotic inhibitor to these cultures, it is possible to obtain many of the cells in metaphase arrest. In this article, the procedures that have been used in this laboratory to isolate metaphase chromosomes from these cultures are described (Salzman *et al.*, 1966; Mendelsohn,

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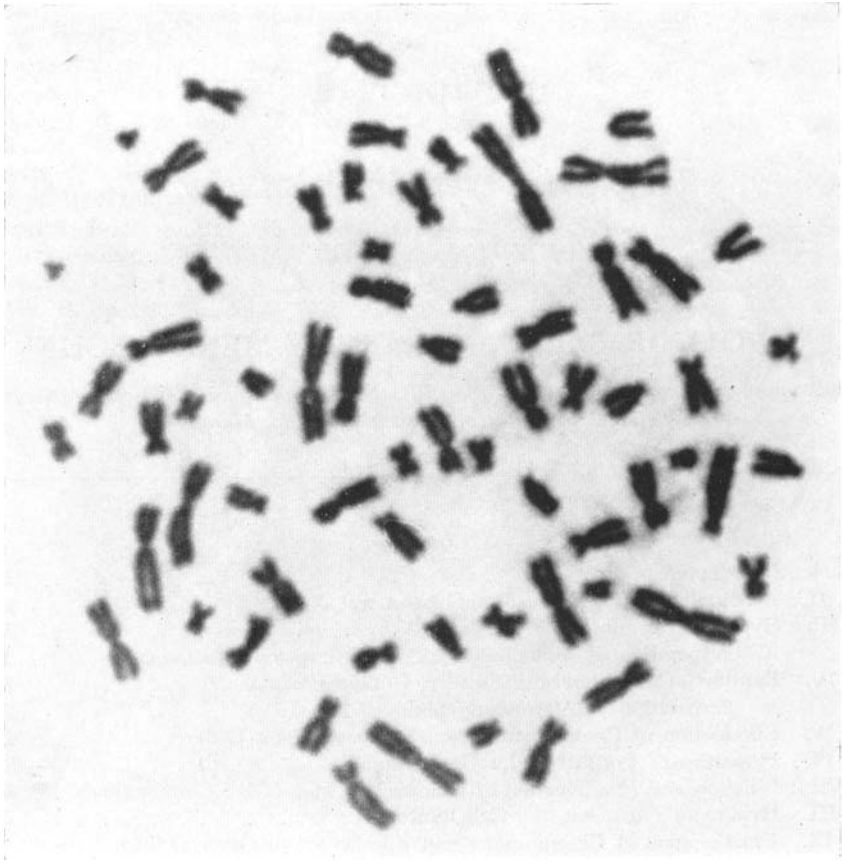


FIG. 1. Metaphase of a HeLa clonal line. Giemsa stain.

et al., 1968). Alternative procedures for the isolation of chromosomes have been reported (Chorazy *et al.*, 1963; Somers *et al.*, 1963; Lin and Chargaff, 1964; Cantor and Hearst, 1966; Maio and Schildkraut, 1967; Huberman and Attardi, 1966). The procedure that we have used to obtain distinctive size groups of metaphase Chinese hamster chromosomes is also described.

These studies were carried out with cells maintained in suspension culture. This method of cell propagation facilitates the accumulation of large numbers of cells (5×10^8 to 3×10^9) in metaphase arrest. The HeLa and the Chinese hamster (CH) cell lines have been most thoroughly investigated. HeLa cell cultures have been grown in Eagle's medium (Eagle, 1959) supplemented with 5% horse serum and 4 m*M* glutamine. CH cells were maintained in suspension culture in Hams'

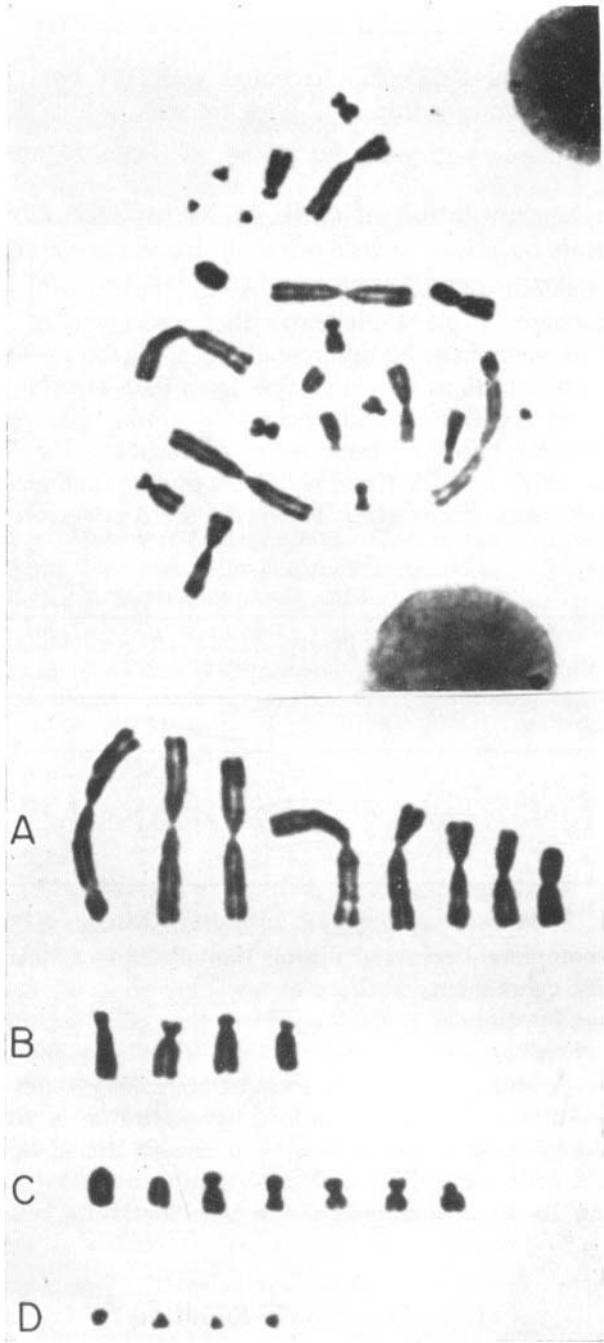


FIG. 2. Metaphase of a Chinese Hamster clonal line. Giemsa stain. In the lower part of the figure the four size groups are defined.

F-10 medium (Ham, 1963) supplemented with 10% fetal calf serum. Metaphase preparations of these cell lines are seen in Figs. 1 and 2.

II. Accumulation of Cells in Metaphase Arrest

Vinblastine sulfate (Velban, Eli Lilly & Co.) has been used to arrest cells at metaphase. Table I illustrates the percentage of HeLa cells accumulated in metaphase at different times after the addition of vinblastine. At concentrations of vinblastine from 0.01–1 μg/ml of suspension culture no significant differences are noted. The morphologic appearance of metaphase cells is altered as a function of time of exposure to vinblastine. With extended periods of exposure pronounced constriction of chromosomes is observed. However, these adverse morphologic

TABLE I
PERCENT OF HELA CELLS IN MITOSIS

Hours after addition of vinblastine	Percent of HeLa cells in mitosis at final concentrations of vinblastine (μg/ml) of:				
	5	1	0.5	0.1	0.01
0	4.0	5.9	6.5	3.9	N.D.[a]
3	9.2	11.0	12.4	9.3	10.0
6	20.5	22.7	26.2	18.8	24.4
13	35.2	47.5	43.7	42.2	54.3

[a] N.D., no data.

changes are completely reversed during the subsequent isolation procedure. For most experiments we have exposed log phase HeLa suspension cultures to vinblastine at a final concentration of 1 μg/ml for 10–14 hours since there is some suggestion that the final yield of purified chromosomes is higher using this concentration. With log phase CH suspension cultures, we have used a final concentration of vinblastine of 0.1 μg/ml and a 5-hour period of exposure. The shortened time period is used with CH cells since they have a 12-hour generation time as compared with an 18- to 20-hour generation time for HeLa cells.

III. Hypotonic Swelling

Cells that have accumulated in metaphase are collected by low speed centrifugation, then washed one time with Eagle's medium, and again

collected by centrifugation. Approximately 8×10^7 cells are suspended in 40 ml of 1% sodium citrate containing 10^{-3} *M* $MgCl_2$ and 10^{-3} *M* $CaCl_2$. The cells are dispersed by gentle pipetting and are then incubated at 37°C. A 10-minute incubation is used for CH cells and a 30-minute incubation is used for HeLa cells. At the end of this incubation, it is convenient to remove a small aliquot which is then used to determine the fraction of the population that has been obtained in metaphase arrest.

A. Preparation of Metaphase Cells for Microscopic Examination

One milliliter of methanol-glacial acetic acid (3:1, v/v) is added to an aliquot of 1.0 ml of hypotonically treated cells in order to fix the cells. After standing at room temperature for 10 minutes, the cells are centrifuged at 500 rpm (International Centrifuge, No. 269 centrifuge head) for 5 minutes. The pellet is then resuspended in 3 ml of methanol-glacial acetic acid (3:1, v/v) for an additional 5 minutes at room temperature. The cells are then centrifuged and resuspended in a small volume of fixative. A drop of this suspension is placed on a wet microscope slide which is then flamed. After drying, the slide is stained with Giemsa.

IV. Rupture of Hypotonically Swollen HeLa Cells

Cells that have been kept at 37°C in 1% sodium citrate (Section III) are collected by centrifugation at 800 rpm for 5 minutes (International Centrifuge). The cells are then resuspended in 10 ml of 2.5% citric acid-0.1 *M* sucrose (pH 2.1). They are shaken vigorously by hand for 5 minutes in a glass-stoppered centrifuge tube. The mixture is then made 0.1% with Tween-80 and shaken for an additional 5 minutes. The appearance of the mixture at this stage of the purification procedure is seen in Fig. 3. Many free chromosomes are observed. In addition, one also observes densely stained interphase nuclei, a few poorly defined masses of chromosomes from unruptured metaphase cells, and a background of scattered fine granular debris.

A. Preparation of Microscope Slides

To observe the nature of the preparation microscopically at this and at subsequent steps in the procedure, an aliquot of the mixture is removed and centrifuged at 2000 rpm for 10 minutes. Most of the supernatant fluid is removed and the pellet is resuspended in a small volume of supernatant

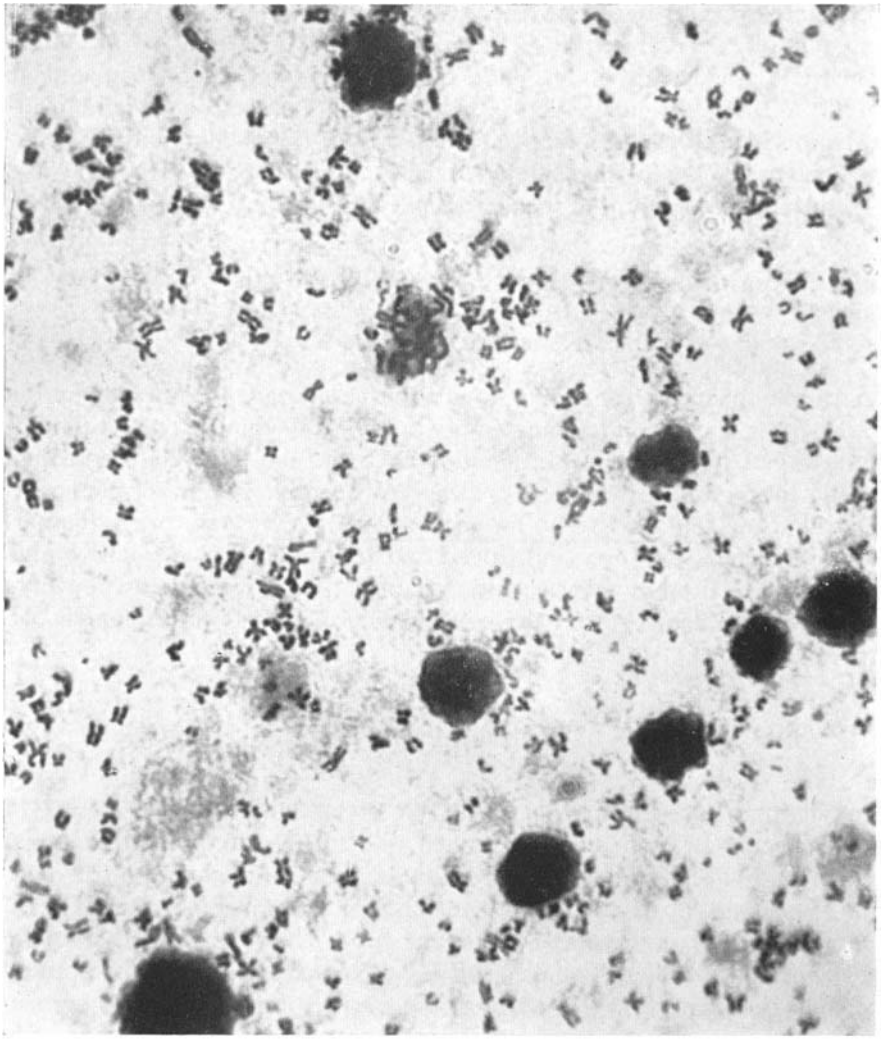


FIG. 3. Ruptured metaphase cell preparation. Cells in suspension culture were exposed to vinblastine (1 μg/ml) for 12 hours. After incubation in 1% sodium citrate and disruption of cells in 2.5% citric acid–0.1 *M* sucrose–0.1% Tween-80, slides were prepared as described in the text.

fluid. In some experiments and in certain fractions, the concentration of material is sufficiently high so that slides can be made directly without the necessity to first concentrate the material. A drop of this material is placed at one end of a microscope slide, and the drop is then drawn across the slide using the edge of a second slide to distribute the fluid.

The slide is then dried with a hair dryer and is fixed for 10 minutes in methanol-acetic acid (3:1). It is then stained with Giemsa.

V. Elimination of Contaminants from Ruptured HeLa Cells

After treatment with Tween-80 (Section IV), the disrupted cell suspension is centrifuged at 500 rpm for 5 minutes to pellet aggregated material and many of the nuclei. The supernatant fluid is then filtered through a porous stainless steel filter by gentle suction. The filter, type FCH, is obtained from the Pall Trinity Micro Corp., Cortland, New York. Contaminating nuclei are completely eliminated by this procedure. Chromosomes in the filtrate are pelleted by centrifugation at 2500 rpm for 30 minutes. The pellet is resuspended in 10 ml of 2.5% citric acid-0.1 *M* sucrose, and the centrifugation repeated. This procedure is repeated again and the final chromosomal pellet is used for analysis. The microscopic appearance of this material is seen in Fig. 4.

VI. Properties of Purified HeLa Chromosomes

The RNA, DNA, and protein content of a purified chromosomal preparation is shown in Table II and has been compared with results obtained by other workers.

TABLE II
RNA, DNA, AND PROTEIN CONTENT OF ISOLATED CHROMOSOMES AND CHROMATIN[a]

Material analyzed	Source of material				
	Chromosomes from HeLa cell[b]	Chromosomes from HeLa[c]	Chromosomes from Chinese Hamster[c]	Chromosomes from mouse ascites tumor[d]	Chromatin from pea seedling[e]
DNA	20	16	16	13.5	36.5
RNA	14	12	15	13.5	9.6
Protein (total)	66	72	69	68	47.9

[a] Expressed in percent of total mass.
[b] Prepared by present method.
[c] Prepared by the method of Maio and Schildkraut (1967).
[d] Prepared by the method of Cantor and Hearst (1966).
[e] Prepared by the method of Bonner (1965).

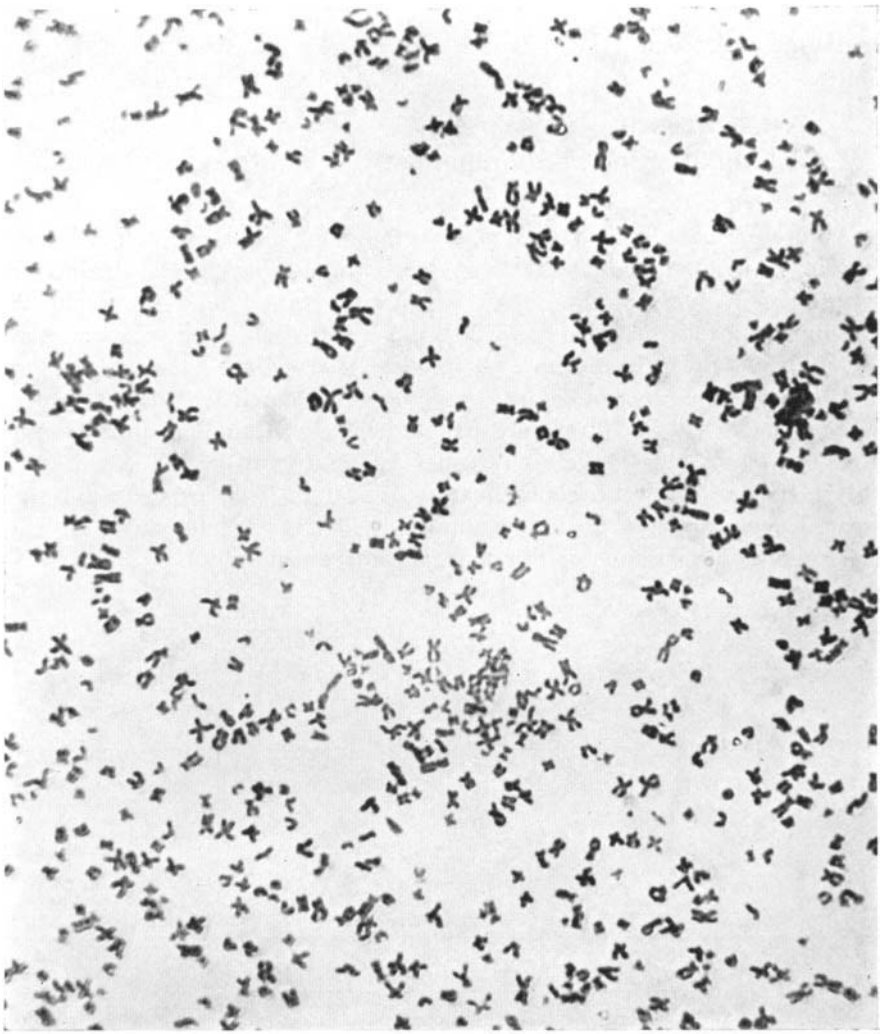


FIG. 4. Isolated chromosomes. Cells from the preparation shown in Fig. 1 were filtered through stainless steel filters. The filtrate was pelleted, resuspended in citric acid-sucrose, and repelleted. Slides were prepared from this washed pellet after resuspension in citric acid-sucrose.

The RNA that is present in purified chromosomes is 18 S and 28 S ribosomal RNA admixed with a very small quantity of 4 S transfer RNA, as judged by its distribution in a sucrose density gradient and by its nucleotide composition. Experiments have been carried out to determine whether this RNA was an intrinsic component of the chromosomes

or a contaminant that was bound to the chromosomes during the isolation procedure. The results clearly establish that ribosomal RNA associated with chromosomes is a contaminant that is derived from cellular ribosomes during purification. While our results indicate that no RNA is associated with chromosomes, we cannot rule out the possibility that species of chromosomal RNA may be lost during the isolation procedure, or that very low levels of chromosomal RNA may be present but remain undetected because of the large quantity of RNA derived from ribosomes.

In the isolation of HeLa cells, we have worked at a low pH in order to preserve well-defined chromosome morphology. However, we have observed that when microsomal fractions are prepared from HeLa cells and are suspended in solutions at pH 2–3 insoluble aggregates are obtained. This suggests the possibility that if chromosomes are purified at a pH at which ribosomes fail to aggregate, the chromosomes might be obtained free of ribosomal contaminants. The chromosome isolation procedure described by Maio and Schildkraut (1967) indicates that this is not the case. This procedure is carried out at pH 7 and yet their analysis of purified chromosomes shows the same percent contamination with ribosomal RNA.

VII. Isolation and Fractionation of Chinese Hamster (CH) Chromosomes

The procedure which is described above has been used with consistent success for the preparation of HeLa chromosomes. When studies were initiated with CH cells, certain modifications were made in that procedure. The procedure that is currently used with CH cells and also has been employed successfully with Syrian hamster cell BHK-21 and with the HeLa cells is described below.

VIII. Hypotonic Treatment and Cell Rupture

Cells are accumulated and harvested in metaphase arrest as described in Section II. Hypotonic treatment is carried out for 10 minutes at 37° in 1% sodium citrate, containing 10^{-3} *M* $MgCl_2$ and 10^{-3} *M* $CaCl_2$, and the cells are then collected by centrifugation for 5 minutes at 800 rpm. The cells are then resuspended in 10 ml of pH 3 buffer prepared in the following way. A stock solution is prepared which contains an equal

mixture of 1 *M* sodium acetate and 1 *M* HCl. The pH of this solution is adjusted to 2.2. The final buffer is prepared by diluting the stock solution 10-fold. The dilutant contains concentrations of $MgCl_2$, $CaCl_2$, and sucrose so that the final buffer contains 10^{-3} *M* $MgCl_2$, 10^{-3} *M* $CaCl_2$, and 0.1 *M* sucrose. The pH of this solution is 3.0. We prefer this buffer to 2.5% citric acid solutions, since it gives equally good cell rupture and has the advantage of a slightly higher pH. It is hoped that at this higher pH histones that are associated with chromosomes will not be solubilized. Chromosomes can be stored in pH 3 buffer at 4°C for periods as long as 2 months, and still maintain excellent morphologic appearance.

The hypotonically swollen CH cells in pH 3 buffer are disrupted by vigorous shaking for 5 minutes. The mixture is then centrifuged at 2500 rpm for 30 minutes, and the supernatant fluid discarded. The pellet is resuspended in 2 ml of pH 3 buffer and treated with 150 μg/ml RNase. [The ribonuclease (crystalline, Armour) is made as a 0.2% solution in 0.15 *M* NaCl, pH 5.0. This solution is heated at 80°C for 10 minutes prior to use (Marmur, 1961).] The mixture is incubated at 37°C for 1 hour and then a 10% solution of Tween-80 is added to give a 1% final concentration. The mixture is shaken vigorously.

IX. Fractionation of Chromosomes and Elimination of Other Cellular Constituents by Sucrose Gradient Sedimentation

In the early studies with CH cells, we used 5 μ porous steel filters to eliminate nuclear contamination. In agreement with the studies with the HeLa cell, CH metaphase chromosomes can be separated from nuclei by filtration through these filters. However, microscopic examination indicated that the filters were removing many of the longest metaphase chromosomes. It is for this reason that the filters have not been used in our studies with CH chromosomes. By using sucrose density gradients it is possible to eliminate cellular contaminants and at the same time achieve an effective fractionation of the CH chromosomes into distinctive size groups.

After the Tween-80 treatment, the chromosomes are contained in approximately 2.5 ml of pH 3 buffer containing both RNase and 1% Tween-80. An aliquot of this solution is diluted 20-fold with pH 3 buffer and its optical density at 450 mμ is measured. It should read 0.350 ± 0.050. If the optical density is higher than this value the undiluted material is adjusted with pH 3 buffer to bring a 1–20 dilution of it into this optical density range. Two milliliters of the undiluted solution

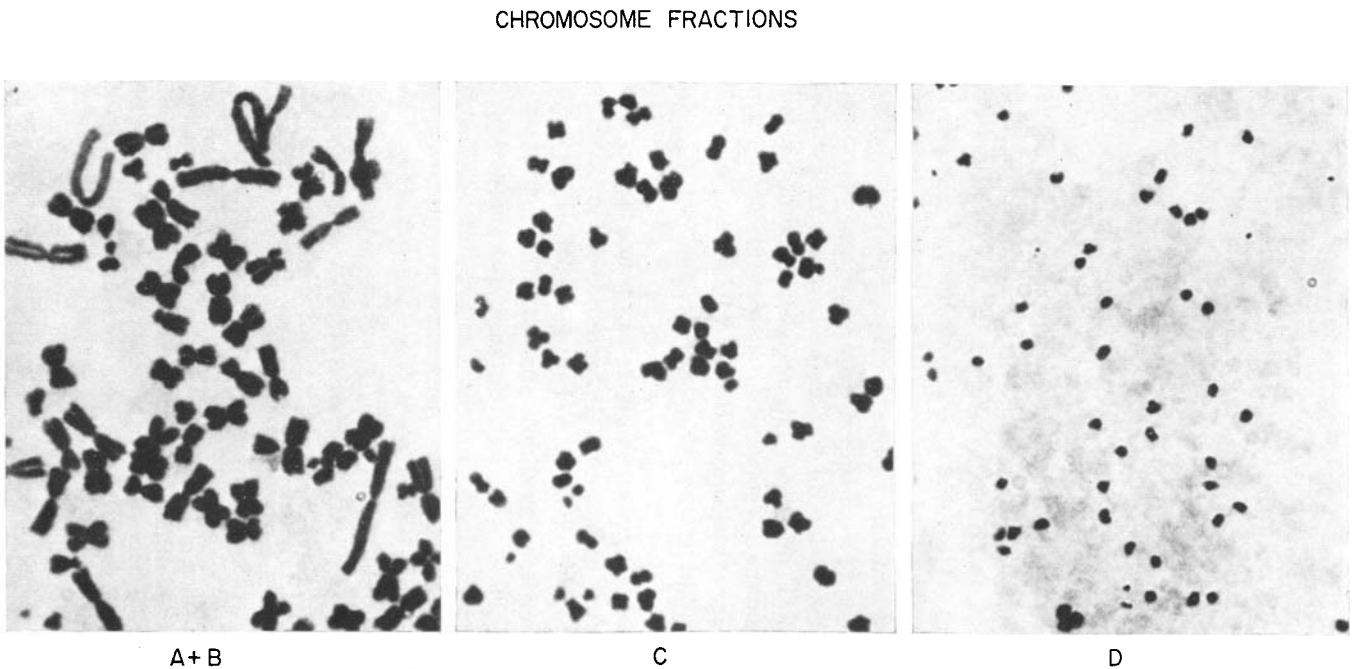


FIG. 5. Sucrose gradient separation of chromosomes into three size groups: A + B, C, and D. Fractions from the second series of gradients were pooled to produce the three final fractions pictured here. Magnification × 2100.

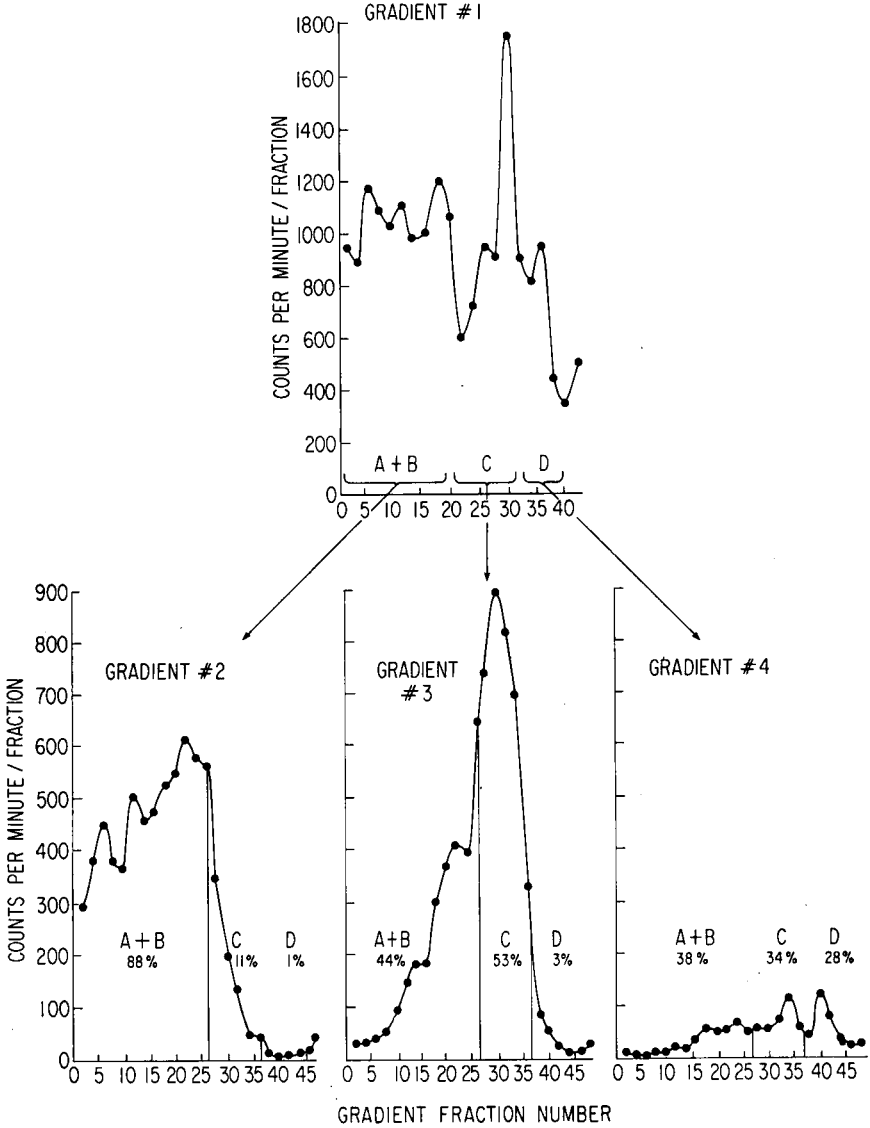


FIG. 6. Distribution of thymidine-C^{14} labeled chromosomes during separation into three size groups in sucrose gradients. Cells were grown in medium containing thymidine-C^{14} for 48 hours in order to obtain uniformly labeled DNA. Five hours after the addition of vinblastine, the cells were harvested and lysed. The fractionation was performed on 24-ml linear gradients of 10–40% sucrose in pH 3 buffer. All gradients were centrifuged under identical conditions, at 50 *g* for 40 minutes at 25°C. The four graphs show the relative chromosome contents of each fraction collected from the gradients. Those fractions from gradient 1 which were judged to

is layered onto a 24-ml 10–40% linear sucrose gradient in pH 3 buffer contained in No. 30 siliconized (Siliclad, Clay Adams, Inc.) nitrocellulose tubes. This gradient is centrifuged for 40 minutes at 25°C at 500 rpm in an International Centrifuge. The tubes are tapped by puncture near the base, with care to avoid the large pellet at the bottom which primarily contains nuclei. Two-milliliter fractions are collected and one-drop aliquots of each fraction are examined on microscope slides as previously described (See Section IV,A.) The appropriate fractions from different regions of the gradient are then combined into three pools, each containing chromosomes partially purified on the basis of size difference. In order to concentrate the three pools of chromosomes into small volumes, they are pelleted by centrifugation at 2500 rpm for 30 minutes and each pellet is resuspended in 2 ml of pH 3 buffer. They are then layered onto a series of three parallel 24-ml 10–40% sucrose gradients and centrifuged for 40 minutes. Fractions obtained from this second series of gradients are examined microscopically and scored for homogeneity with respect to one of the four size groups as defined in Fig. 2. It is found that D chromosomes can be effectively isolated from the remaining larger sized chromosomes. Fractions containing isolated C chromosomes are also obtained. A and B chromosomes can be separated from the smaller chromosomes, but are not separated from each other under these conditions.

Fractions from the second series of gradients which are homogeneous for either A + B, C, or D chromosomes are pooled to yield the final three chromosome fractions shown in Fig. 5. About half of the gradient fractions are unsuitable for this pooling step because of cross contamination between chromosome size groups.

The method of separating chromosomes on gradients is demonstrated graphically in Fig. 6. In this experiment, cells containing chromosomes labeled uniformly with thymidine-C^{14} were treated as described above. The upper graph shows the amount of TCA precipitable radioactivity in the fractions collected from the first sucrose gradient. Regions of this gradient judged to be enriched for chromosome classes A + B, C, or D are designated by the brackets above the abscissa. Fractions in these regions were pooled and concentrated in pH 3 buffer by centrifugation,

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be enriched for one or more of the four chromosome classes are indicated by the brackets. These fractions were pooled, concentrated by centrifugation, and overlayered on the designated second gradients (gradients 2, 3, and 4). Vertical lines in the plots of the second gradients between fractions 26–27 and 36–37 divide the gradients into three regions in which the predominant chromosome type is either A + B, C, or D, as judged by microscopic observation. The percent of counts contained within each region is recorded on the graphs.

and a second series of three sucrose gradient centrifugations was performed under the same conditions. The lower three graphs show the results of these gradients. It is seen that the relative positions of the A + B-rich, C-rich and D-rich pools obtained from the first gradient were maintained in the second series of gradients.

It is seen in Fig. 5 that a significant quantity of nonchromosomal material is associated with the fractionated chromosomes. This is especially the case with the D chromosomes which remain near the top of the sucrose gradient. Radioautographic studies have been performed to determine whether this contaminant nonchromosomal material contained DNA from degraded chromosomes. Cells were uniformly labeled with thymidine-H^3, and the chromosome isolation and gradient separation procedures were carried out. Radioautographs of slide preparations at all steps of the procedure showed that all of the isotope was contained in the chromosomes with no evidence of isotopically labeled DNA in the nonchromosomal material.

Each of the three chromosome fractions was analyzed for its content of DNA, RNA, and protein. The results shown in Table III are expressed as relative to the quantity of DNA, which is set at a value of

TABLE III
THE RELATIVE AMOUNTS OF DNA, RNA, AND PROTEIN IN THE DIFFERENT CHROMOSOME FRACTIONS FROM CH CELLS[a]

Fraction analyzed	Relative amount		
	DNA	RNA	Protein
A + B chromosomes	100	18	240
C chromosomes	100	79	540
D chromosomes	100	140	850

[a] Fractions were obtained by differential centrifugation in sucrose gradients. The material in the fractions was precipitated by adjusting the fractions to a final concentration of 5% TCA. Colorimetric analysis for DNA, RNA, and protein were carried out as previously described (Salzman *et al.*, 1966). The value for DNA is set at 100, and the other values are expressed relative to this value.

100. The data indicate that during the series of sucrose gradient centrifugations, contaminant RNA and protein found associated with chromosomes in the cell lysate remained high in the gradient, so that fractions enriched for larger chromosomes contained less contaminant material. The reduced amount of RNA in the A + B, fraction supports our previous finding (Salzman *et al.*, 1966) that RNA is not an intrinsic component of metaphase chromosomes.

TABLE IV
THERMAL DENATURATION TEMPERATURE (TM) AND BUOYANT DENSITY VALUES OBTAINED FROM DNA EXTRACTION[a]

DNA isolated from chromosome fraction	Thermal denaturation		Density (gm/cm³)
	Tm (°C)	Hyperchromicity	
AB	85.3 ± 0.4	38%	1.701 ± 0.0007
C	86.0	32%	1.702
D	85.7	31%*	1.701
AB + C + D	—	—	1.701

[a] Physicochemical studies on DNA extracted from the three chromosome fractions. Each recorded value (with standard deviation) is an average of determinations performed on three different DNA preparations, with the exception of a single density determination on the mixture of fractions. Thermal denaturation was performed in 0.15 *M* NaCl–0.16 *M* sodium citrate. The range over which the hyperchromic shift occurred was 12–15°C. A hyperchromic shift of 31%* was obtained on D fraction DNA only after additional purification by banding in a CsCl gradient. Densities were determined by density gradient centrifugation in CsCl.

DNA has been extracted from each of the three chromosome pools, and studies have been performed to determine certain physicochemical properties of this fractionated DNA. Table IV records the thermal denaturation temperature (Tm) and buoyant density values obtained for DNA extracted from each fraction. No significant heterogeneity was demonstrated. When equal 0.5-μg quantities of DNA isolated from the three chromosome pools were mixed together and banded by density gradient centrifugation, the densitometer tracings showed a single symmetrical peak.

X. Discussion

It should be stressed that the procedures that are described in this article are all of a provisional nature. This is not surprising if one recalls the problems that are still unresolved as to what constitutes a pure isolated nucleus. It seems likely that after additional purification procedures "pure" chromosomes will be obtained and that they will be free of RNA. The fractionated chromosomes that are now available should prove valuable as a source of three distinct genetic classes of DNA. It is hoped that the assignment of gene loci to these chromosome groups can be made using hybridization techniques. Acrylamide gel fractionation of RNA and techniques such as those described by Bautz *et al.*

(1966) for the isolation of RNA's corresponding to a single gene may provide a significant number of RNA species for such studies.

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Chapter 13

Autoradiography with the Electron Microscope: Properties of Photographic Emulsions

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I. Introduction

Autoradiography, namely the registration and localization of radiation from radionuclides by photographic emulsions, has been used in biological experimentation since the early studies of Lacassagne and Lattès (1924) who demonstrated the presence of Po^{210} in rabbit organs by this method. (For early literature see L. G. Caro, Vol. I, this treatise.)

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In recent years, a great number of compounds of biological interest labeled with low energy β-emitting radionuclides have become available and, consequently, the number of biological investigations using autoradiographic methods has increased rapidly. This is also reflected in several contributions in Vols. I and II of this treatise (Caro, 1964; Miller *et al.*, 1964; Perry, 1964; Prescott, 1964; Salpeter, 1966; Stevens, 1966).

In combination with light or electron microscopy, autoradiographic techniques are used for the localization of label within tissues and cells as well as for comparative measurements of the incorporated radioactivity. However, autoradiographic results are often unsatisfactory with regard to quantitation and reproducibility. This is partly due to the nature of the methods and can hardly be completely eliminated. For example, it is impossible to reestablish the original distribution of AgBr grains after development.

Besides these shortcomings, errors in the interpretation of autoradiographic results are often caused by insufficient consideration of the photographic elementary processes and the characteristics of the emulsions used.

In this chapter, some current techniques of autoradiography with the electron microscope will be discussed, as well as some properties of the three photographic emulsions most commonly used for this purpose, namely, Gevaert NUC 307, Ilford L4, and Kodak NTE.

II. Techniques for Autoradiography with the Electron Microscope

The preparation of labeled biological specimens for autoradiography does not differ from the usual techniques employed in electron microscopy. This means that the labeled compounds bound to the biological material must not be soluble in the solutions used for processing the specimens. The risk of chemical artifacts during long periods of exposure and during processing can be reduced by using platinum grids. The specimens, usually ultrathin sections of embedded biological material, are applied to Formvar- and carbon-coated grids. To avoid chemical interactions between section and photographic emulsion, the preparations are then coated with another carbon layer (Salpeter and Bachmann, 1964).

A convenient technique for the application of photographic emulsion to the specimen has been reported by Caro and van Tubergen (1962), and is employed in our laboratory in a slightly modified form. The emul-

sion is diluted to a suitable degree (about 1:4) with distilled water, heated to 40°C, and stirred continuously with a glass magnetic stirrer for 30 minutes. By the addition of detergent (for example Hostapon, Farbwerke Hoechst AG) (Haase and Jung, 1964), the surface tension is reduced to allow a very thin membrane of emulsion to be formed within a copper wire loop when dipped into the liquid emulsion and withdrawn slowly. The loop is fixed about 20 cm above the magnetic stirrer plate at an angle of 45° and dried for about 2 minutes in this position. Immediately after the emulsion membrane has gelled it is carefully brought in contact with the specimen by a quick breath. The zone of optimum emulsion thickness is one which contains a monolayer of AgBr grains, and its position within the loop can be found by a series of test grids from different areas of the gelled emulsion membrane. The loop should be even and either circular or oval, with a diameter of about 3 to 5 cm. An "expandable" loop has been described by Karasaki (1965). For exposure the specimens are then stored at 4°C in light tight Bakelite containers to which a small amount of Drierite is added. After an exposure time which depends on the degree of labeling (14–60 days), the preparations are developed. The problem of development will be discussed in Section III,B.

III. Theoretical Considerations

A. Resolution

In autoradiography, an electron source does not lead to a reproducible image in the optical sense, but is represented by developed AgBr grains (silver filaments) whose occurrence is only reproducible within a certain area around the source. The location of the source must, therefore, be deduced from the position of the silver filaments. Since a source emitting only one single electron cannot in practice be detected in biological material, so-called "point sources" always emit a number of electrons and are, therefore, usually represented by more than one silver filament. Autoradiographic resolution, when defined as the separation of two adjacent sources, depends on the number of silver filaments per unit area produced by the respective source. Under optimum conditions autoradiographic resolution is of the order of 0.1 μ (Caro, 1962). The following factors determine the yield of silver filaments and autoradiographic resolution: (a) grain size, grain size distribution, sensitivity, and the density of the monolayer; (b) energy and range of the radionuclides;

(c) thickness and surface quality of the biological sections and contact with the emulsions; and (d) conditions for development.

The conclusion could be drawn that resolution increases with decreasing grain size. However, as will be shown in Section IV,B, for smaller grains more than one electron is necessary to induce a latent image (Hülser and Rajewsky, 1966). It is, therefore, necessary to obtain an estimate of the probability P_t for a single AgBr grain to be hit by one electron emitted from a labeled molecule.

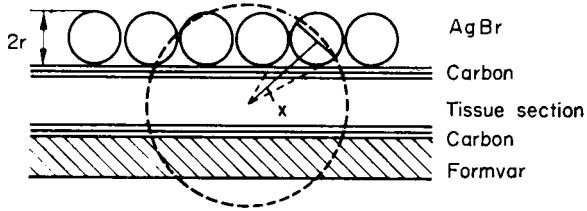


FIG. 1. The probability of latent image formation as a problem of resolution. r, Radius of AgBr grain; x, distance source–center of AgBr grain; circle area, $F_1 = \pi r^2$; sphere surface, $F_2 = 4\pi x^2$; probability for one hit, $P_t = F_1/F_2 = r^2/4x^2$; probability for latent image formation, $P_s = (r^2/4x^2)^i$; i, number of electrons necessary for the formation of one latent image.

In Fig. 1, a point source of electrons is shown, covered by a monolayer of AgBr grains. In this case, the probability for one hit is approximately given by the ratio F_1/F_2, where F_1 is the area of a circle with the radius of the AgBr grain and F_2 is the surface area of a sphere with the radius x (equal to distance from the electron source to the center of AgBr grain).

$$P_t = \frac{F_1}{F_2} = \frac{1}{4} \cdot \frac{r^2}{x^2} \tag{1}$$

However, for emulsions which require several electrons for the formation of a latent image, the probability P_s for the formation of a latent image must be taken into account. P_s is a function of the number of electrons i necessary for the production of one latent image, and of the probability for one AgBr grain to be hit by one electron.

$$P_s = \left(\frac{1}{4} \cdot \frac{r^2}{x^2}\right)^i \tag{2}$$

The validity of this equation is limited to grains in the immediate neighborhood of the source, since energy absorption by these grains can prevent latent image formation in the more distant grains (see also Caro, 1962). For good estimation of P_s, the size distribution of AgBr grains should have a small half-width value.

For thin sections and small but sensitive AgBr grains, resolution is high and increases with decreasing range of the incorporated radionuclides. In Fig. 2, the range of electrons in AgBr is demonstrated for low β-energies. Tritium (maximum energy 18.6 keV), the radionuclide with the lowest energy and range of all β-emitters, is most frequently used in electron microscopic autoradiography, followed by C^{14} (158 keV), S^{35} (167 keV), and P^{32} (1.71 MeV).

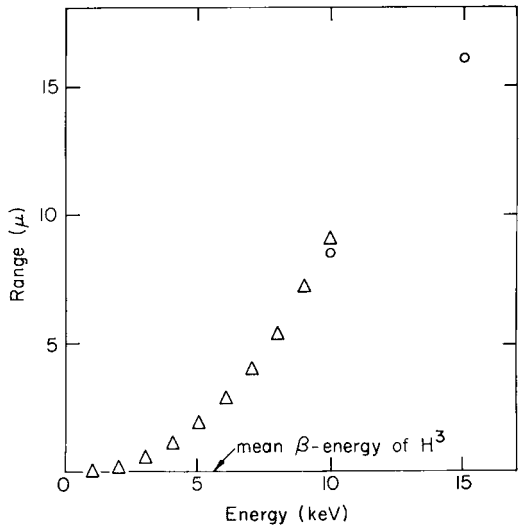


FIG. 2. Range of electrons in AgBr. △, Calculated after Feldmann (1960); ○, measured by Berger and Seltzer (1964) and Nelms (1956).

For H^3-labeled preparations, Maurer and Primbsch (1964) have shown that the yield of developed grains increases linearly with the section thickness up to a value of 0.5 to 1 μ for karyoplasm and cytoplasm, whereas for the nucleolus the linearity is only given up to about 0.1 μ. Therefore, the section thickness for microautoradiography has to be a compromise between the desire for a maximum yield of developed grains and optimum quality of the electron microscopic picture, which is satisfactory only for section thicknesses of up to 0.1 μ.

B. Development

The electron energy, absorbed by a grain, induces the formation of latent images at certain lattice sites within the AgBr crystal. Within the grain, the locus of the latent image is not necessarily identical with the site of the electron hit. The size of the latent images is of the order of the diameters of several Ag atomic diameters. Even for demonstration



FIG. 3. Ilford L4 emulsion. Irradiation with 5.7 keV electrons in the AEG "SDL" electron microscope. Developed 1 minute in Microdol X. Magnification × 53,000.

in the electron microscope, latent images have to be enlarged (i.e., developed) chemically.

In "chemical" development (see Joos and Schopper, 1958; von Angerer and Joos, 1959), the latent image is rapidly enlarged by addition of Ag which is the result of the reducing effect on AgBr by the developer. Characteristically shaped silver filaments grow out from the AgBr grain. The unexposed AgBr is subsequently removed by fixation.

In "physical" development (see Joos and Schopper, 1958; von Angerer and Joos, 1959), the unexposed AgBr is first removed by fixation, leaving only the latent images intact. These are then enlarged by a developer containing Ag^+ ions. The size of the silver spots obtained by attachment of Ag^+ ions to the latent image is, therefore, a function of the time of development. However, apparently because of the likelihood of artifacts, physical development has not been used so far in autoradiography with the electron microscope.

A third possibility for the demonstration of the latent image is the so-called "fine-grain" development (see Joos and Schopper, 1958; von Angerer and Joos, 1959), which is often misleadingly referred to as physical development. This type of development can be considered as a combination of physical and chemical development. A developer is chosen which also dissolves the AgBr and thereby produces a solution containing Ag^+ ions. In this case the latent image is not only chemically developed by reduced Ag from the same grain, but also by physical attachment of Ag^+ ions from the solution. Under suitable conditions one obtains small and characteristically "tadpole-shaped" silver filaments, in which the finer end represents the locus of the original image. Figure 3 shows such "tadpole-shaped" filaments after development in Microdol X. The developing time depends on the type of developer, but should never be less than 2 minutes at room temperature for a maximum number of latent images to be developed. After stopping the development in acetic acid for 10 seconds, the specimens are fixed for 5 minutes in Kodak AM 33 rapid fixer made up 1:7 in distilled water. The yield of developable latent images can be increased by a procedure called gold latensification (see Salpeter, 1966), where gold ions are deposited on prestages of latent images, which are normally undevelopable.

IV. Determination of the Sensitivity of Photographic Emulsions

The sensitivity of photographic emulsions for autoradiography with the electron microscope can be defined and tested in different ways

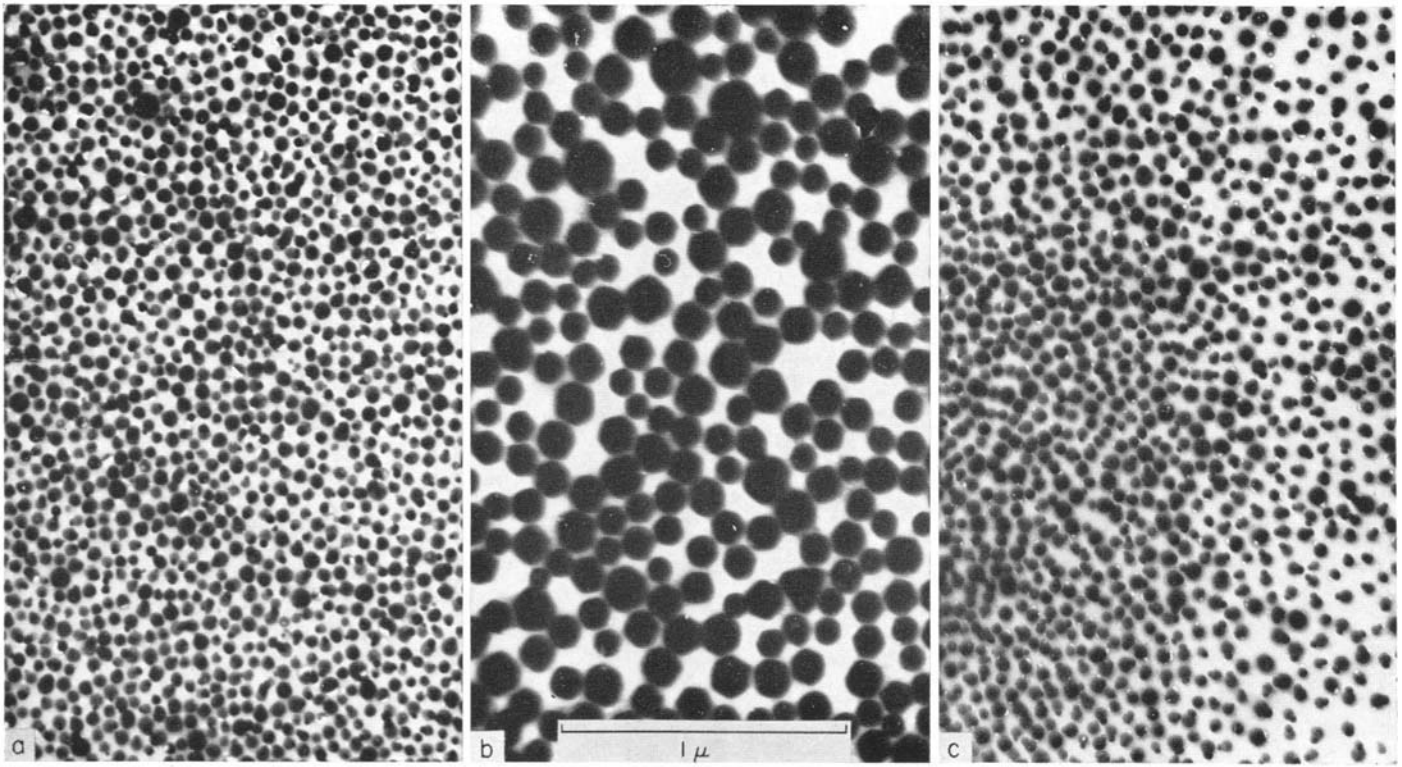


FIG. 4. Part I. See legend on opposite page.

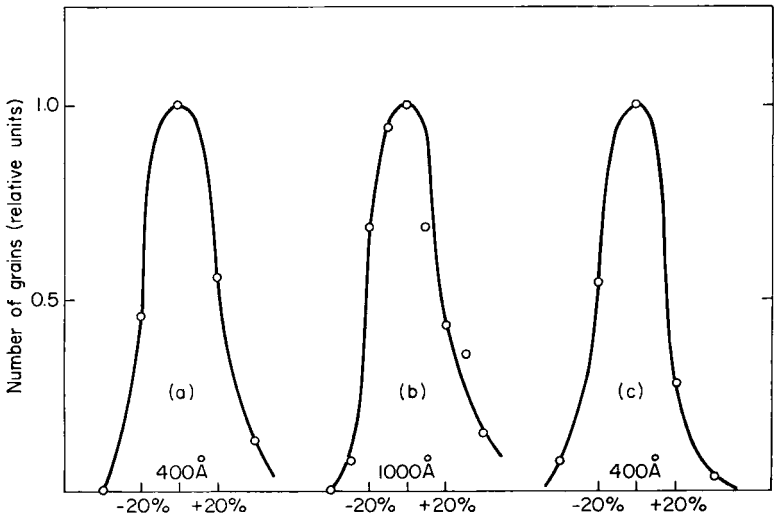


FIG. 4. Silver bromide grains of the emulsions Gevaert NUC 307 (a), Ilford L4 (b) and Kodak NTE (c), and their respective normalized grain size distribution curves. Magnification × 38,000.

(Caro, 1962; Granboulan, 1963; Caro and Schnös, 1965; Hülser and Rajewsky, 1966; Bachmann and Salpeter, 1967). Basically, it is necessary to obtain information on the electron sensitivity of single grains. This problem has been investigated for the emulsions Gevaert NUC 307, Ilford L4, and Kodak NTE. The grain size distribution curves are shown in Fig. 4. As can be seen, all three emulsions have half-width values of about 20%. Apparently, mean grain size and sensitivity of the emulsions may vary somewhat from one batch to the other; for example, the value found for the mean grain size of the Gevaert emulsion was 40% lower than that stated by the manufacturer [see also comments by Bachmann and Salpeter (1967) on the variability of different batches of Kodak NTE emulsion].

A. Methods

Monolayers of these emulsions have been prepared on Formvar- and carbon-coated platinum grids and irradiated in an AEG "SDL" electron microscope under reproducible experimental conditions. Irradiations were carried out with a homogeneous electron flux of about 2×10^{-9} A/cm^2, measured with the use of a Faraday cage. The energy of the electron beam was maintained at 5.7 keV, corresponding to the mean β-energy of tritium. A mechanical cover plate was used to perform step-

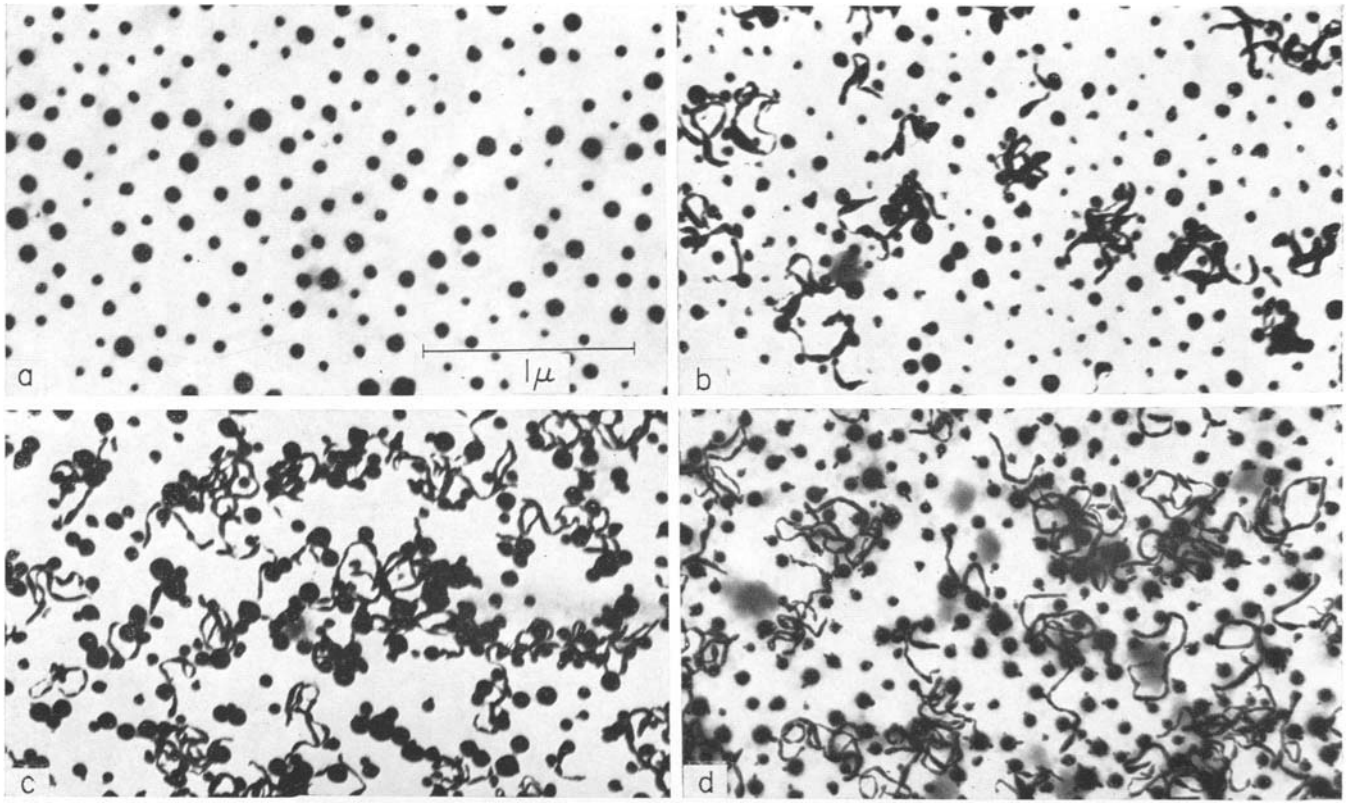


FIG. 5. Ilford L4 emulsion irradiated with 0 (a), 1 (b), 2 (c), and 20 (d) electrons per AgBr grain. Developed in Microdol X for 1 minute, stopped in acetic acid, unfixed. Similar plates were used to determine the proportion $G_N(n)$ of developed AgBr grains. Magnification $\times$ 28,000.

wise exposure of the specimens. For the sensitivity determinations, the dose to which the specimens were exposed was expressed as electrons (e) per AgBr grain (g) (e/g = N). The irradiated monolayers were developed in Microdol X (diluted 1:2 with water) at 22°C for 1 minute, stopped in 1% acetic acid for 10 seconds, rinsed in water, and left unfixed. Undeveloped and developed grains could then be distinguished and the proportion $G_N(n)$ of developed AgBr grains determined (see Fig. 5a–d).

B. Results

The evaluation of the irradiated specimens shows that even in the case of high doses ($N = 20$ e/g) a certain number of AgBr grains was not developable under the experimental conditions used (NUC 307: ≈15%; L4: ≈25%; NTE: ≈10%). Because of the short developing time not all latent images grow out completely, as it takes longer to develop the internal latent images than those on the surface of the grains. On the other hand, the time of development had to be kept short in order to obtain small silver filaments which allow a clear distinction between the single grains. The points, however, where silver filaments are just starting to grow out from the grains are clearly distinguishable. The values obtained after counting the number of developed and undeveloped grains, were normalized with respect to the percentage of nondevelopable grains in the respective emulsions.

If we assume that electrons hit AgBr grains according to a Poisson distribution

$$G_N(n) = 1 - e^{-N} \sum_{n=0}^{k-1} \frac{N^n}{n!} \tag{3}$$

we can calculate the number of grains which are hit by k or more electrons when the mean exposure is N electrons per grain. In Fig. 6 the percentage of developed AgBr grains is given on the ordinate; the number of electrons necessary for the formation of a latent image can be read from the abscissa, using the curve for the respective value of N.

According to our experiments, for the formation of one silver filament, one AgBr grain of the Ilford L4 emulsion must be hit on the average by 1 to 1.4 electrons. The corresponding figures for the Gevaert NUC 307 and Kodak NTE emulsions are 2–3 electrons and 4–5 electrons, respectively.

Integration of the curve in Fig. 2 gives the loss of energy for electrons in AgBr (see alse Pelc, 1963). From the resulting value for 5.7 keV elec-

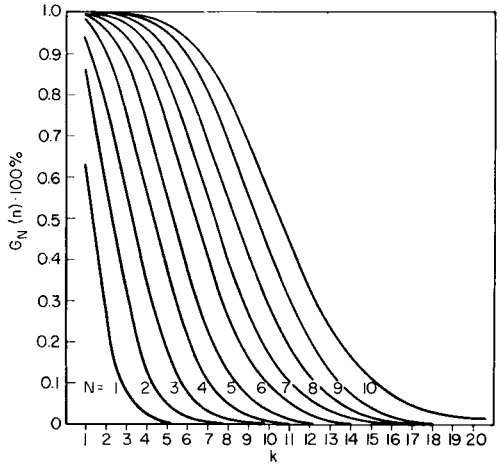


FIG. 6. Poisson distribution curves for the determination of the number of electrons necessary for the formation of one latent image. *N*, Number of electrons to which one AgBr grain was exposed; *k*, Number of electrons hitting one AgBr grain.

trons (mean β-energy of H^3) it can be estimated that the Ilford L4 and Gevaert NUC 307 emulsions absorb about 800 eV for the formation of one latent image as compared with about 1300 eV in the case of the Kodak NTE emulsion. However, it must be assumed that within the grain only part of this energy results in latent image formation (see Malinovski *et al.*, 1964).

V. Discussion

Autoradiographic resolution will, in principle, increase with the number of electrons necessary for latent image formation, thus favoring the emulsions Gevaert NUC 307 and Kodak NTE. This is, however, not a true advantage since, on the other hand, resolution also depends on the yield of developed AgBr grains, which becomes smaller the more electrons are required per AgBr grain. With emulsions requiring several electrons per AgBr grain, little labeling will be obtained. Although the Ilford L4 emulsion has about twice the mean grain size of the Gevaert NUC 307 emulsion, considerations with regard to the probability of latent image formation for one- and multiple-hit processes show that autoradiographic resolution for these emulsions is of the order of 0.1 μ in either case. The Ilford L4 emulsion is, therefore, preferred in our laboratory since with a shorter exposure time results are of comparable

quality. Furthermore, this emulsion seems to be rather constant with respect to grain size and sensitivity. The Gevaert NUC 307 emulsion is apparently subject to variations in grain size (see Section IV). Although on the basis of our findings the sensitivity of the Kodak NTE emulsion is the lowest of the three emulsions in question, results published by Bachmann and Salpeter (1967) suggest that the sensitivity of this emulsion may vary considerably.

According to our present knowledge, an overall absorption of several hundred electron volts per AgBr grain seems necessary for latent image formation by exposure to electrons when normal developing procedures are used (see Malinovski *et al.*, 1964). As mentioned in Section IV,B, not the whole energy absorbed by the AgBr grain can be attributed to latent image formation.

It is generally accepted (see von Angerer and Joos, 1959; Malinovski *et al.*, 1964) that an energy of 30 to 70 eV per AgBr grain is necessary for the formation of one latent image by photons. In the case of electrons, however, only the overall energy absorption has so far been measured. Since the specific energy absorption for photons is not comparable with the overall value for electrons, it is not possible to infer a minimum size of AgBr grains for electron exposure from the value of 30 to 70 eV for photons.

On the other hand, our results show that different numbers of electrons are necessary for latent image formation in two emulsions with almost identical grain size. This indicates that grain size is not the only critical factor for the sensitivity of photographic emulsions. The possibility should therefore be considered that in the future the presently optimum grain size for tritium autoradiography (1000 Å; Ilford L4) could be decreased by the use of suitable sensitization methods. Furthermore, techniques may be developed (as for example gold latensification) to demonstrate prestages of the latent image which remain undetected with the developers presently used.

Acknowledgment

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Chapter 14

Cytological and Cytochemical Methodology of Histones

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I. Introduction

Histones are low molecular weight basic proteins associated at one time or another during their existence with DNA (Murray, 1964). The association occurs primarily through electrostatic linkages (Mirsky and Ris, 1947). These proteins are found in the nuclei of most eukaryotic

organisms, including the higher plants and animals, protozoa, and fungi. Their presence in prokaryotic organisms, bacteria and viruses, seems unlikely (Wilkins and Zubay, 1959; Zubay and Watson, 1959), although this is in dispute (Chargaff, 1952; Chargaff and Seidel, 1949; Leaven and Cruft, 1966; Mirsky and Ris, 1951).

Since their isolation from the pus nuclei by Miescher (1897), no conclusive evidence for their function has been advanced. As early as 1950, Stedman and Stedman introduced the concept that histones control gene function. Two lines of evidence have emerged since which might support this idea; namely, (a) the inhibition of transcription *in vitro* by polycations (Allfrey *et al.*, 1963; Bonner and Huang, 1963; Frenster *et al.*, 1963; Huang and Bonner, 1962), and (b) a restricted number of changes in histone composition which are associated with developmental change (Alfert, 1956; Bloch and Hew, 1960a; Das *et al.*, 1964a; Neelin *et al.*, 1964; Messineo, 1964). From an evolutionary point of view, they are thought to play a role in the function of the more highly organized chromosomes of higher organisms (Zubay and Watson, 1959).

It is not within the framework of this chapter to present a complete survey of the field. For further information on histones, the reader is referred to Bloch (1966a), Bonner (1965), Bonner and Ts'o (1964), and Vendrely and Vendrely (1966).

II. Cytochemical Methods

The cytochemical identification of histones has presented a difficult problem because of the absence of unique constituents that would provide the basis for differentiating these basic proteins from other proteins of the cell. Histones have no known enzymatic activities and no perceptible antigenicity. Their one unique feature, lack of tryptophan, is a negative one, as is their supposed gene repression function. Nevertheless, histones have several properties, namely, basicity, acid solubility, and association with DNA, which when taken together serve to distinguish them from other proteins.

Among the first to attempt identification of histones *in situ* was Caspersson (1950). By using differences in the ultraviolet absorption spectra of ordinary and "diamino acid protein" (histone), he endeavored to localize these proteins within cells. Some of his conclusions are not in agreement with those of Mirsky and Pollister (1946) who were unable to detect significant differences in the spectral absorption of the histones and nonhistone proteins. Interestingly, however, a number of

Caspersson's conclusions have been substantiated. The implication of the nucleolus in ribosomal synthesis (Beerman, 1960; Birnstiel and Chipchase, 1963; Birnstiel *et al.*, 1963) and the presence of basic proteins within the nucleolus (Birnstiel and Chipchase, 1963; Flamm and Birnstiel, 1964) have indicated that Caspersson's original findings are in part correct. The diamino acid designated by Caspersson may be a ribosomal basic protein.

Procedures that have been used for the quantitative and qualitative analysis of proteins in general, such as the Sakaguchi and Millon reactions have also served for analysis of histones. These have been particularly useful where the histones occur to the exclusion of other proteins, as in the case of sperm cells (McLeish *et al.*, 1957) or where histones can be extracted and thereby assessed by differences in staining before and after extraction (Bloch and Godman, 1955a). Another group of techniques, Alfert and Geschwind's (1953) method and its modifications, depend on the ability of proteins which possess high isoelectric points (histone, protamines, lysozyme) to stain in alkaline pH. The purpose of this chapter is to consider such cytological methods as those that have been used effectively in localization, detection, and quantitative determination of histones and protamines.

A. Fixation

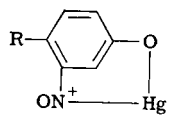
There are no set rules for the fixation of tissue for histone staining and one must find empirically the method best suited to one's particular purpose. There are certain guidelines that can be followed. Formalin is probably the most commonly used histone fixative, and is normally employed in a 10% solution (4% formaldehyde) buffered at pH 7.0 in the manner of Alfert and Geschwind (1953). Formalin immobilizes most proteins, including histones and some protamines, by reacting strongly with the lysine amino groups. Therefore, this fixative must be used with some discretion when these groups are to remain preserved, or if the tissue is to be analyzed for the presence of this amino acid. Interference by formaldehyde can be avoided by the removal of the fixative either by immersing tissue in boiling water for several minutes (Swift, 1955) or by the trichloroacetic acid (TCA) hydrolysis at 90°C used to extract nucleic acids (Spicer and Lillie, 1961). Recently, Gifford and Dengler (1966) reported that prolonged periods of washing (over 14 hours) of tissue fixed with neutral buffered formalin reduces alkaline fast green staining, and Bloch (1966b) found that overexposure to water after formalin fixation reduces histone eosinophilia. Carnoy fixative, as a rule, has a tendency to reduce both nuclear specificity and intensity, and

may result in loss of histone during staining. This fixative should be avoided unless strong protein precipitants such as picric or trichloroacetic acid are present (Bloch, 1966a). If Carnoy is used, fixation should last for only 1 or 2 hours.

Freeze-substitution (Woods and Pollister, 1954) can be employed for the fixation of tissue for histone analysis. This process, however, takes several days, as opposed to a few hours when chemical fixatives are used. This method, if used carefully, preserves the natural state of the cell while using a minimal amount of chemical fixatives. An important advantage of freeze-substitution over formalin fixative is that DNase can be used in the former case, permitting study of RNA/histone ratios. These might not otherwise be investigated because of the lability of some RNA's.

B. Millon Reaction

The original Millon's reagent (Millon, 1849) was made by digesting mercury in nitric acid and diluting the resulting solution with water. Bensley and Gersh (1933) modified the Millon reaction for histochemical use, employing the reagent for demonstration of mitochondria in freeze-dried sections. According to Gibbs (1927), the reaction occurs in two steps. First, a nitrosophenol is produced by the substitution of NO for H *ortho* or *meta* to the hydroxyl of the phenol. Second Hg^{2+} is combined by chelation into a new ring, including the nitrogen of the nitroso group. The complex is red in color. The nitrous acid and mercuric ions attach to tyrosine and tryptophan residues to form nitrosomercurial derivates of these amino acids (after Gibbs, 1927).



The Millon reaction was used principally for protein spot tests, but has been adapted for cytological and cytophotometric studies (Ansley, 1954; Bryan, 1951; Godman and Deitch, 1957; Pollister and Leuchtenberger, 1949; Rasch and Swift, 1953, 1960; Woodard, 1958; Schneider, 1945). According to Mirsky and Pollister (1946), histones are soluble in the sulfuric acid and mercuric sulfate reagent, the early method would measure only non-histone proteins. Since TCA precipitates histone, preventing leaching from fixed material, Pollister and Ris (1947) were able to devise a Millon reaction with this acid to stain total protein. The original Millon reaction could then be used to measure non-histone

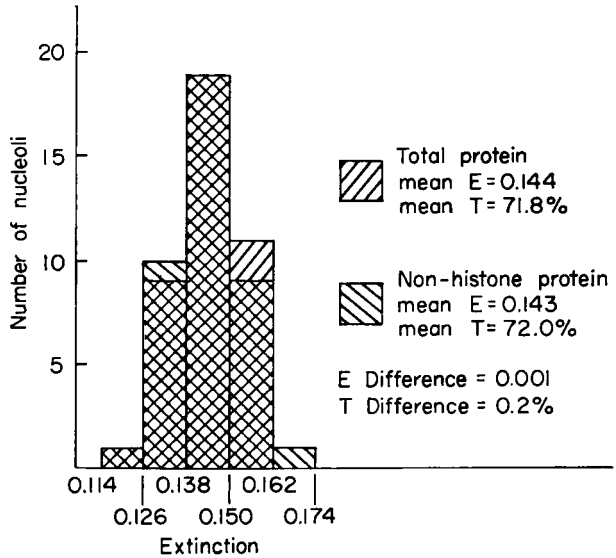


FIG. 1. Superimposed histograms of extinction values of two equal samples (40 cells) of sections of nucleoli of pollen mother cells of maize. The thickness of each nucleolus was measured, and the extinction values were corrected to a common thickness of 3 μ. Thus the extinction differences are presumably due only to variations in the amount of protein. The double-hatched blocks are regions of overlap of the histograms for total protein and for protein treated with sulfuric acid–divalent mercury reagent. Susa fixative. From Pollister and Ris (1947).

proteins. The difference between the results of these two tests (total protein and nonhistone protein) was used as a measure of histone (Fig. 1). The assay depended upon cytophotometric determination of the amount of stain.

The Millon reaction has never had much popularity in histone studies because of the relatively low absorbance of stained protein due to small amounts of tyrosine and absence of tryptophan in histone.

This reaction has been modified by Baker (1956), and according to Pearse (1960) is simple, reliable, and gives good color.

MILLON PROCEDURE AFTER BAKER (1956)

(a) Fix in neutral buffered formalin (NBF), Carnoy, or FAA.

(b) Dehydrate, embed in paraffin, section, and mount on slides.

(c) Hydrate tissue sections.

(d) Place in Coplin jar containing reagent and put in boiling water bath for 5 minutes. Preparation of reagent is as follows. Add 10 gm $HgSO_4$ to 100 ml of 10% H_2SO_4 and heat until dissolved. Make up to 200 ml. Add 0.5 ml 0.25% $NaNO_2$.

(e) Discontinue heat and allow reagent to come to ambient temperature.

(f) Remove tissue and wash three times in distilled water for 2 minutes, each wash.

(g) Dehydrate in graded series of ethanol, clear in xylene, and mount. Tyrosine-containing proteins are stained red, pink, or yellowish-red.

C. Alkaline Fast Green Reaction

Singer (1952) used acid and basic dyes over a range of pH's and showed that proteins with different isoelectric points could be distinguished on the basis of their affinities for the dyes. In 1953, Alfert and Geschwind devised a method for the *in situ* demonstration of nuclear histone based on the above premise, which permitted the selective staining of proteins having high isoelectric points. They discovered that staining with anionic dyes at rather high pH's would permit one to make use of one of the most important characteristics that differentiate histones from other proteins in the cell, their high basicity. The best test of the method lay in the facts that the fast green technique usually results in the selective staining of the nucleus, and that removal of DNA is a prerequisite for staining. This procedure is very sensitive as regards the crucial range of pH, for slight variation in the alkalinity results in changes in staining by the cell. At pH 7.9 nuclear specificity is lost, while at 8.5 nuclear staining is decreased.

The exact mechanism of fast green staining is not fully understood at this time; however, several theories have been advanced that shed light on the possible factors affecting fast green binding.

Singer (1952) suggested that staining depends principally on the overall charge of the protein rather than on the absolute amounts of the individual amino acids. The guanidine groups of arginine and ϵ-amino groups of lysine are primarily responsible for the staining by acid dye ions. Only about one-third of the terminal amino groups and less than one-tenth of the imidazole groups of histidine can be expected to bind dye (Alfert and Geschwind, 1953). Klotz (1950) has suggested that dye binding is also dependent on the number and position of carboxylate ions which may block binding of the dye.

It is generally assumed from the effect of DNase and TCA extraction that staining by alkaline fast green is due to basic groups of histones normally combined in salt linkages to phosphate esters. The basic groups are exposed by removal of DNA.

The alkaline fast green stain lacks stoichiometry; nevertheless, the staining is often proportional to the amount of histone and the tech-

nique has proven valuable in the *in situ* detection, localization, and measurement of nuclear histones as evidenced by the large number of quantitative cytophotometric analyses using the procedure.

A point of particular interest is the high degree of proportionality shown to exist between DNA and histone during chromosome duplication (Alfert, 1954; Bloch and Godman, 1955a; Rasch and Woodard, 1959). Microphotometric analysis of cells stained consecutively with Feulgen and fast green has been used for the determination of the

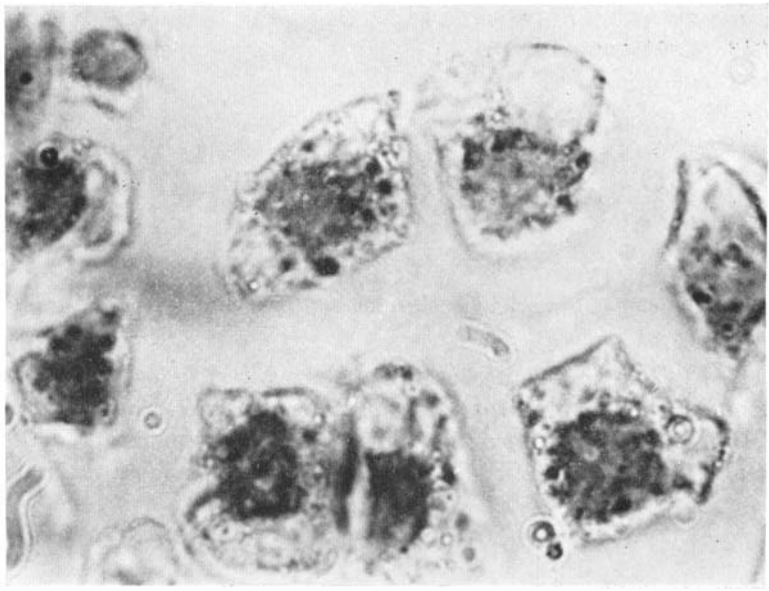


FIG. 2. Staining of nuclei of pollen mother cells with Feulgen.

ratios of nuclear DNA/histone. An inconvenience of this technique is that after Feulgen measurements, the nuclei must be restained with fast green and then relocated from photographs or maps made prior to removal of the Feulgen stain, if ratios of DNA/histone of specific nuclei are desired. Nuclei of pollen mother cells which have been stained consecutively with Feulgen and alkaline fast green are shown in Figs. 2 and 3 (Pipkin, 1968).

Using this technique, various investigators have been able to describe the time relationship of DNA and histone synthesis. The seemingly concomitant increases of both substances seen in rat liver and thyroid nuclei, and mouse kidney nuclei (Alfert and Geschwind, 1953; Bern and Alfert, 1954; Alfert *et al.*, 1956), in rat liver nuclei (Bloch and Godman,

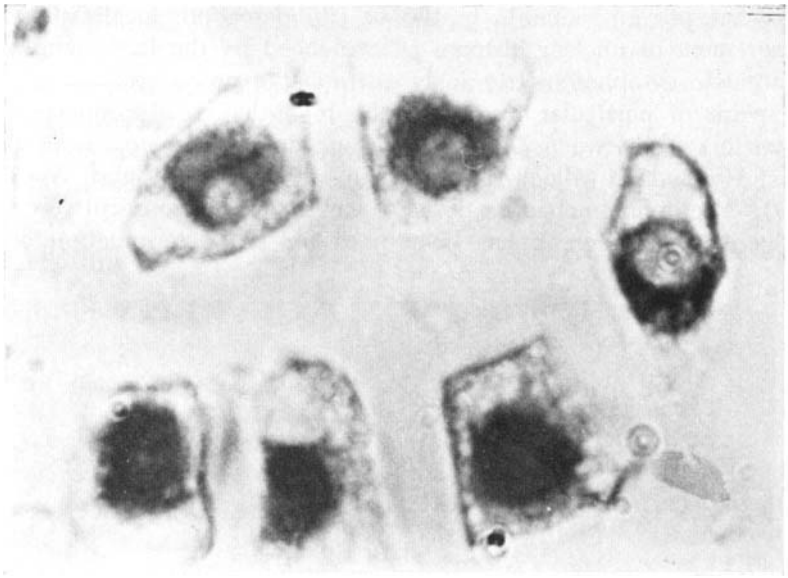


FIG. 3. Staining of nuclei of pollen mother cells with alkaline fast green.

1955a) (Fig. 4), in onion root tip nuclei (Alfert *et al.*, 1956), in the macronuclei of the protozoan *Euplotes* (Gall, 1959), in *Tetrahymena pyriformis* nuclei (Hardin *et al.*, 1967), in the X chromosome of *Rehnia spinosus* (D. P. Bloch and T. Teng, unpublished), and in the nuclei of

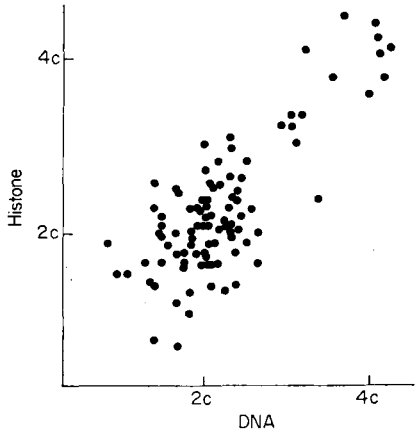


FIG. 4. Relative amounts of DNA plotted against relative amounts of histone for the same individual nuclei of 1-day-old rat liver. Logarithmic scales. From Bloch and Godman (1955a).

plant microspores and pollen grains (Pipkin, 1968) have led workers to suspect simultaneous synthesis of DNA and histone during interphase of the cell cycle.

Accounts showing deviation of DNA/histone ratios from the norm have been reported but no satisfactory explanation has as yet been given for these exceptional findings. For example, Bloch and Godman (1955b) found depressed fast green staining of cells in G_1 stages which otherwise indicated normal 2C DNA levels (Fig. 5). Umãna *et al.* (1962, 1964) found low histone staining and attributed low alkaline fast green quantities to histone that was not complexed with DNA. Dwivedi and Naylor (1968) has shown normal 2C DNA levels but depressed alkaline fast green staining in the nuclei of dormant lateral bud apices of *Tradescantia.* The author (Pipkin, 1968) found in plant microsporocytes and related tapetal nuclei the same depressed fast green levels, yet normal DNA

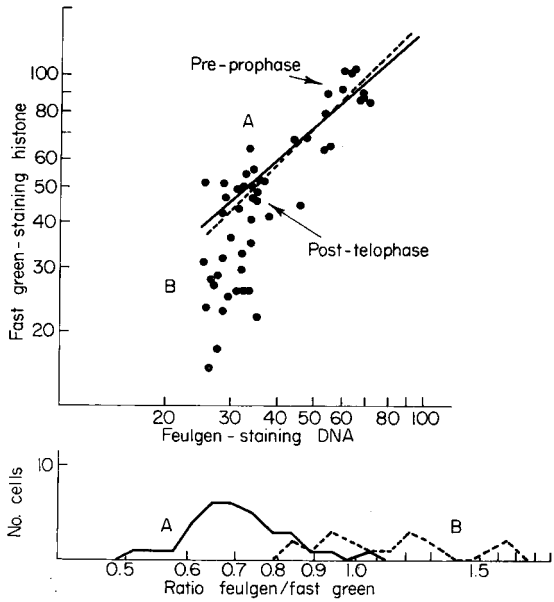


FIG. 5. *Top.* Comparison of the Feulgen staining of DNA and the fast green binding of histone in the two types of nuclei of a rat liver preparation. Among the cells involved in the duplication of chromosomal material, there is an approximately proportional increase in the DNA and the histone, as shown by the two types of stain. A fraction of the cells containing the 2 C complement of DNA show a decreased capacity for fast green binding by the histone. The broken line is the theoretical regression of fast green on Feulgen staining. *Bottom.* The frequency distribution curve of the ratios of Feulgen to fast green staining. From Bloch and Godman (1955b).

levels. Torelli *et al.* (1967) reported that during DNA replication in phytohemagglutinin-stimulated lymphocytes, alkaline fast green values were higher than would normally be expected from duplication of histone during the S period of the cell cycle.

Although alkaline fast green is usually specific for nuclear histone, accounts of cytoplasmic staining have been reported. This nonspecificity is not restricted to any particular cell type; however, the most numerous reports of cytoplasmic staining seems to be concerned with both egg cells of animals and the megaspores of plants.

Alfert and Goldstein (1955) first reported cytoplasmic staining by alkaline fast green in their studies on cultures of rapidly proliferating *Tetrahymena* cells. No suggestions as to the nature of the responsible material were given; however, it was intimated that it may be associated with RNA. Cytoplasmic staining did not occur in cells in the stationary phase. Cowden (1958) found nonspecific alkaline fast green cytoplasmic staining in slug oocytes. Taleporos (1959) reported cytoplasmic fast green staining of unfertilized sea urchin eggs. These eggs yielded high amounts of lysine and arginine in the acid hydrolyzate of acid-extractable protein leading the author to suggest the presence of cytoplasmic histone. Many of these egg cells are known to contain cytoplasmic DNA (Hoff-Jorgensen, 1954). Horn (1962) and Moore (1963) demonstrated cytoplasmic fast green staining in the yolk platelets of amphibian oocytes. Further investigation by Horn included sedimentation rates, mobilities, and amino acid composition. These results suggested that the proteins may be categorized as histones. Moore found that after TCA extraction, fast green stains the large yolk platelets of the vegetative hemisphere intensely and the small yolk platelets of the animal hemisphere lightly or not at all. Pritchard (1964), in a study of embryos of the plant *Stellaria media,* demonstrated alkaline fast green staining in the cytoplasm of endosperm and embryonic cells which he attributed to the presence of extranuclear histone. The parallel distribution of histone and RNA in the embryo and endosperm was suggested. Setterfield *et al.* (1960) reported cytoplasmic fast green staining in pea seedlings. Whitfield and Youdale (1965) found similar staining in mouse fibroblasts which were not dehydrated in either ethanol or xylene after staining. The authors interpreted this observation as indicating the existence of basic protein associated with cytoplasmic ribosomal aggregates. The stain was normally removed during dehydration. Cytoplasmic acidophilia was considered indicative of histone accumulation prior to its migration into the nucleus. Agrell (1961) has suggested that in rapidly dividing cells of *Tetrahymena pyriformis* and in early embryos of the sea urchin *Psammechinus miliaris* cytoplasmic RNA may be associated with basic protein. Butler *et al.*

(1960) and Phillips (1962) have pointed out that rat liver microsomes contain basic proteins that have a similar composition to nuclear histone. It is interesting that Bloch and Brack (1964) were unable to stain cytoplasmic histones with alkaline fast green (their presence inferred from the results of autoradiographic studies) until migration into the nucleus and complexing with DNA rendered them stainable. Gifford and Dengler (1966) demonstrated cytoplasmic basic protein staining in onion root cells with both alkaline fast green and ammoniacal silver. Davenport (1967) has recently investigated alkaline fast green staining in the cytoplasm of the pre-yolk oocyte and in the yolk platelets of mature eggs. It is pointed out that the two principle yolk proteins phosreitin and lipovitellin may be responsible for cytoplasmic staining. However, the electrophoretic complexity seems to indicate that this staining is due to something other than these proteins. These investigators favor the view that cytoplasmic staining in amphibian oocytes result from the structural proteins of ribosomes where the concentrations of these organelles are sufficiently high to permit detection by fast green staining.

More recently, Bloch (1966b) and Chevaillier (1967) have shown the existence of cytoplasmic basic proteins in the spermatozoa of certain crustacea. Its significance is unknown.

1. Alkaline Fast Green Procedure after Alfert and Geschwind (1953)

(a) Fix tissue in 10% NBF. Extensive washing when formalin fixation is used may result in loss of labile histones and should be avoided (Gifford and Dengler, 1966; Bloch, 1966a).

(b) Dehydrate, embed in paraffin, section, and mount on slides.

(c) Tissues are immersed for 15 minutes in a 5% TCA solution in a boiling water bath to remove nucleic acids. Temperature should range from 90°–100°C during the 15 minutes.

(d) Immerse in three changes of 70% ethanol, 10 minutes each, to remove TCA.

(e) Stain for 30 minutes in a 0.1% aqueous solution of fast green FCF. Adjust pH to 8.0–8.1 with minimum amount of NaOH. Use stain immediately.

(f) Wash 5 minutes in distilled water.

(g) Dehydrate in a graded series of ethanol, clear in xylene, and mount. [See Whitfield and Youdale's (1965, p. 14) comments on effects of dehydration on cytoplasmic staining.]

Bloch (1966b) has reported specific staining of histone by buffering aqueous eosin Y and fast green between pH's of 8.2 and 8.3 with tris-HCl buffer (see p. 338).

2. Feulgen-Fast Green Procedure after Bloch and Godman (1955a)

(a) Fix in 10% NBF.

(b) Dehydrate, embed in paraffin, section, and mount on slides.

(c) Hydrolyze tissue for 25 minutes in 1 *N* TCA at 60°C.

(d) Immerse in Schiff's reagent for 1 hour at room temperature. Preparation is as follows: 5 gm basic fuchsin dye (Allied Chemicals), 11 gm potassium metabisulfite and 500 ml of 1 *N* TCA. Shake overnight in dark. Add 3 gm activated charcoal (Norite), shake for ½ hour, and filter. Filtrate should be either colorless or light straw yellow. After Schiff's reagent is placed in a Coplin jar, it should be used the same day. The reagent will last for several months in an opaque bottle under refrigeration.

(e) Drain and rinse in three changes of freshly prepared metabisulfite solution. Preparation is as follows: 1 part of 1 *N* TCA to 1 part 10% metabisulfite and 100 parts water.

(f) Rinse in distilled water.

(g) Dehydrate in a graded series of ethanol, clear in xylene, and mount.

(h) Hydrolyze tissue in a 5% TCA solution in a boiling water bath to remove nucleic acids and Feulgen staining. The remaining steps are identical to the alkaline fast green procedure as described above.

3. Picric Acid-Schiff Procedure after Dwivedi and Bloch (Unpublished)

(a) Fix tissue in 10% NBF.

(b) Dehydrate, embed in paraffin, section and mount on slides.

(c) Hydrolyze sections in saturated aqueous solution of picric acid at 60°C for 20–30 minutes. (Hydrolysis for as short periods as 10 minutes is sufficient to produce good color, and hydrolysis periods for as long as 60 minutes do not seem to produce any appreciable decrease in color intensity.)

(d) Rinse in distilled water and stain for 1 to 2 hours in picric acid-Schiff reagent, prepared as follows: Pour 100 ml of boiling distilled water over 0.5 gm of basic fuchsin. Shake and let cool to 50°–60°C and filter. To the filtrate, add a mixture of 2.29 gm of picric acid in 5 ml of 10% potassium metabisulfite. Shake thoroughly and let stand in cold for 12–18 hours. Excess picric acid will setttle to the bottom. Add 0.5 gm activated charcoal and filter through Whatman No. 1 filter paper. The picric acid-Schiff reagent should be a yellowish solution free from any tinge of pink.

(e) Pass slides through three changes of 10 minutes each of potassium

metabisulfite which is prepared as follows: Mix 5 ml of saturated picric acid and 5 ml of 10% potassium metabisulfite and add to 90 ml of water.

(f) Pass slides through running tap water for 5 minutes then through a brief rinse in distilled water. Remove picric acid by a brief rinse in acidified acetone (see Section II,j).

(g) Rinse in distilled water, dehydrate through ethanol-xylene series and mount.

This procedure can be followed by any of the histone staining procedures utilizing acidic stains at alkaline pH.

D. Naphthol Yellow S Reaction

Naphthol yellow S (NYS) is another acid dye which, by bonding to the cationic ϵ-amino groups of lysine, the guanidyl groups of arginine, and the imidazole groups of histidine, can be used effectively for staining of proteins. This stain as used by Deitch (1955, 1966) binds stoichiometrically; however, unlike fast green used at high pH's, it does not react with histone specifically and will stain nonbasic proteins as well.

The greater affinity of NYS, as compared to the anionic dyes fast green and Beibrich scarlet, for protein basic groups has been demonstrated by Deitch (1966). Various dilutions of these three dyes were made from identical concentrations and applied to albumin models. After 1 hour of staining, the same intensity of NYS staining was found over a range of 100-fold dilution. Such dilutions of fast green and Biebrich scarlet resulted in decreased staining.

The estimation of DNA and protein is made possible by employing Feulgen counterstained with NYS. As shown in Fig. 6, no overlap occurs between the NYS and Feulgen spectral absorption curves in the region of maximum absorption (435 mμ for NYS and 575 mμ for Feulgen). Separation of the curves provides for convenient microphotometric measurement of DNA and protein in the same nuclei (Deitch, 1955, 1966).

It is significant that Deitch (1966) discovered that the hydrolysis in 1 *N* HCl commonly used in the Feulgen reaction causes about 10% loss of NYS staining, suggesting that histone loss occurs during acid hydrolysis. On the other hand, DNase or TCA pretreatment results in a 20–30% gain in NYS intensity. These data indicate that enzymatic and TCA hydrolysis may remove DNA which masks some of the protein basic groups, thus preventing them from binding the dye.

Deitch (1955) suggests that NYS binds stoichiometrically to the dibasic amino acid residues of protein. By using albumin and casein models, it was learned that mild acetylation (acetic anhydride at 5° or 25°C), which blocks lysine basic groups, reduces NYS binding by about

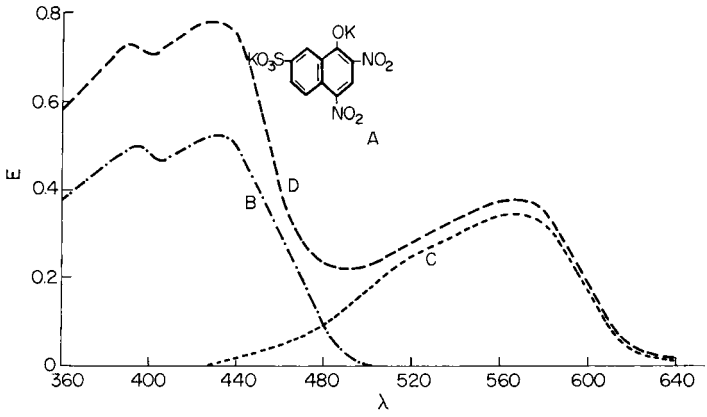


FIG. 6. Spectral absorption curves of Naphthol Yellow S, Feulgen, and the combined stains. *A*, Structural formula of Naphthol Yellow S. *B*, Absorption curve of Carnoy fixed salamander (*Necturus*) liver cytoplasm stained with Naphthol Yellow S. *C*, Absorption curve of salamander liver nucleus stained with the Feulgen reaction. *D*, Absorption curve of salamander liver nucleus stained with the Feulgen reaction and counterstained with Naphthol Yellow S. From Deitch (1955).

45%. This staining reduction is fairly proportional to the percentage of lysine comprising the total dibasic amino acids of the purified proteins.

Various workers, using albumin models, have shown that when protein is acetylated under these conditions, the ninhydrin-Schiff and the chloramine T-Schiff reactions for the detection of amines give negative results (Barka and Anderson, 1963; Burstone, 1955). This mild acetylation has no detectable effect on the staining intensity of the Sakaguchi reaction. Hence, Deitch concluded that NYS binding becomes specific for arginine and histidine after such pretreatment. However, the contribution of histidine is slight. Deitch (1966) has suggested the possibility of describing the lysine to arginine ratio of proteins by measuring NYS binding prior to and after acetylation.

NAPHTHOL YELLOW S PROCEDURE AFTER DEITCH (1955)

(a) Fix in alcoholic fixatives, such as Carnoy, or FAA. If formalin is used, remove before staining by immersing slides in boiling water.

(b) Dehydrate, embed in paraffin, section, and mount on slides.

(c) Stain sections for 15 minutes at room temperature in 1% NYS dissolved in 1% acetic acid. The pH is approximately 2.7.[1]

[1] Deitch (1955) found that material exposed to a very dilute aqueous solution of NYS (0.001%), which was not differentiated after staining, gave extinction values comparable with the standard staining method described above. This suggests that lower dye concentration and shorter periods of differentiation may be used.

(d) Remove slides without blotting to a differentiating solution containing 50 ml of 1% acetic acid. Leave for 15–24 hours at room temperature. After 10 slides have been differentiated, discard the solution.

(e) Blot and pass directly through pure tertiary butanol, xylene, and mount.

E. Sakaguchi Reaction

The Sakaguchi reaction is a highly specific colorimetric test for the guanidyl group of arginine and has been developed by several workers for its *in situ* localization (Baker, 1944, 1947; Carver *et al.*, 1953; McLeish and Sheratt, 1958; Thomas, 1946; Warren and McManus, 1951). The color intensity in some of these reactions is not very reproducible. The colored products act as pH indicators. They are red at high pH, and turn orange and finally yellow with increasing acidity. Another disadvantage of the technique as used by the above workers is the distortion of tissue morphology that commonly results from the extreme alkalinity of the reaction. The alkalinity may also result in loss of sections from the slide (Deitch, 1961). Like the NYS reaction, the Sakaguchi reaction is not specific for histones but will stain the arginine residue of nonbasic protein as well.

Several theories for staining have been suggested. According to Serra (1944), the Sakaguchi reaction is specific for guanidine derivatives in which a hydrogen atom of one amino group is substituted by an alkyl or acyl radical. Thomas (1946), on the other hand, believes that guanidine derivatives in which a hydrogen atom of one or both amino groups was substituted would react, but that those in which both hydrogen atoms of a single amino group were substituted would not. In practice, arginine is the only amino acid which stains in biological material with the Sakaguchi reaction (Pearse, 1960). Sakaguchi (1950) states that this reaction depends on the coupling of α-naphthol or 8-hydroxyquinoline and by hypochlorite or hypobromite to the quanidyl group of arginine in the presence of a high concentration of sodium or potassium hydroxide. Bhattacharya (1960) has suggested that the colored product is a quinone imine.

McLeish and Sheratt (1958) have improved the Sakaguchi reagent somewhat by substituting 2,4-dichloro-α-naphthol which gives a more intense red color than α-naphthol or 8-hydroxyquinoline. These workers reported that their preparations only faded 2% in the first 8 hours after the reaction occurred. Nevertheless, 25% of the color was lost after 3 days. It was necessary to mount these preparations without dehydration, in a mixture of glycerol and sodium hydroxide, which preserved their

color. However, one major difficulty arises in that the tissue treated by this procedure is highly refractile owing to the discrepancy between the refractive indices of the specimen and of the glycerol-NaOH medium. Pollister and Ornstein (1955) state that such differences in refractive indices result in nonspecific light losses and consequently hinder light absorption measurements. Therefore, the preparations are not ideally suited for quantitative microphotometric analysis.

An improved Sakaguchi reaction has been devised by Messineo (1966). Here, the reaction permits the determination of the free base arginine and the arginine residue of proteins. Interference by other amino acids which he shows does occur, has been eliminated. The innovations include the use of 100 mg of 2,4-dichloro-α-naphthol in 100 ml of 5.0 *N* KOH and 180 ml of absolute ethanol. This is brought to full color by the addition of a small amount of NaOCl and the further addition of 2 moles KI per mole of naphthol, which stabilizes the reaction.

Deitch (1961) has substituted barium hydroxide for sodium hydroxide in a Sakaguchi reagent containing NaOCl and 2,4-dichloro-α-naphthol. There is no apparent tissue disruption or loss, and the addition of an organic amine to the media prevents color fading during dehydration and mounting. These reaction conditions are found to give an intense staining which is reproducible, and there is no significant loss of stain after several weeks. Deitch further states that tissue treated in this manner is suitable for quantitative microphotometric analysis of arginine, since the reaction was shown to be stoichiometric. Deitch found it advisable to leave the tissue in the Sakaguchi reagent for 10 minutes. No fading was noted after a period of several weeks. However, if the slides remained in the reaction bath for only 5 minutes, the tissue was seen to lose approximately 10% of its stain intensity within 1 week.

The Sakaguchi reaction has been used by various workers (Bloch, 1966b; Bloch and Brack, 1964; Deitch and Terner, 1965; Godman and Deitch, 1957; Hoover and Thomas, 1951; McLeish, 1959; McLeish and Sheratt, 1958; McLeish *et al.*, 1957; Rasch and Woodard, 1959).

Sakaguchi Procedure after Deitch (1961)

(a) Tissue may be fixed in 10% NBF, Carnoy, or any fixative which does not destroy the arginine group.

(b) Dehydrate, embed in paraffin, section, and mount on slides.

(c) Place tissue sections for 10 to 15 minutes at room temperature in a solution containing 5 parts of barium hydroxide, 1 part 2,4-dichloro-α-naphthol (Eastman Organic Chemicals), and 1 part sodium hypochlorite added in that order. Preparation is as follows: 4% barium hydroxide, 1% sodium hypochlorite (prepare by diluting a commercial preparation of

fresh Clorox 1:5, and 1.5% 2,4-dichloro-α-naphthol in tertiary butanol). Use Sakaguchi reagent *immediately*.

(d) Flood slides with a 1% urea solution allowing the $BaCO_3$ scum to flow over the edge of the Coplin jar. The scum should not come into contact with the tissue.

(e) Rapidly transfer into three changes of tertiary butanol containing 5% tri-*N*-butylamine (Eastman Organic Chemicals) moving them vigorously for 5 seconds.

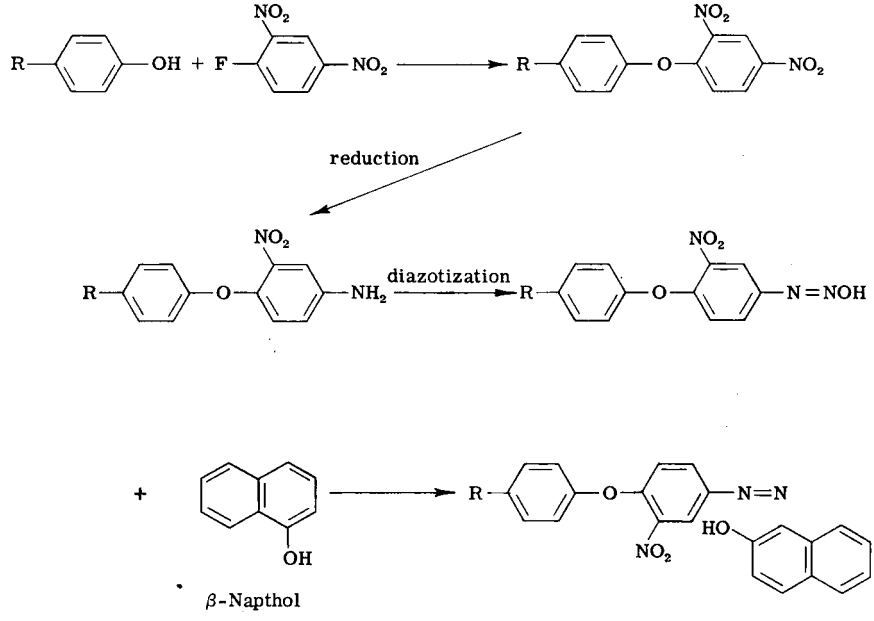
(f) Transfer rapidly into two 30- to 60-minute changes of xylene containing 5% tri-*N*-butylamine.

(g) Mount directly.

F. Dinitrofluorobenzene Reaction

Dinitrofluorobenzene (DNFB) is another useful protein stain that can be used for the detection and localization of certain amino groups of proteins. According to Sanger (1945, 1950) DNFB reacts with tyrosine, histidine, SH groups, the free terminal amino groups of proteins, and to some extent with the ϵ-amino groups of lysine.

Danielli (1953) found that the intensity of this color reaction was too weak for the usual cytological observations. He found that the intensity can be greatly enhanced by the reduction of the nitro groups of



the reagent to amino groups, followed by diazotization of these, and finally by linkage to a phenol or to aniline. The result is the production of an azo dye linked by covalent bonds to the group reacting with the stain.

Danielli used 1-amino-8-naphthol-3:6-disulfonic acid as his final coupling agent. The result is a rich reddish-purple color soluble in neither alcohol nor xylene. The reaction is thought to proceed as follows (from Pearse, 1960):

Zerlotti and Milton (1962) used DNFB without the coupling procedure for the *in situ* demonstration of proteins in connective tissue and epithelial structures. According to these authors, the reagent reacts primarily with the ϵ-amino groups of lysine in most tissues.

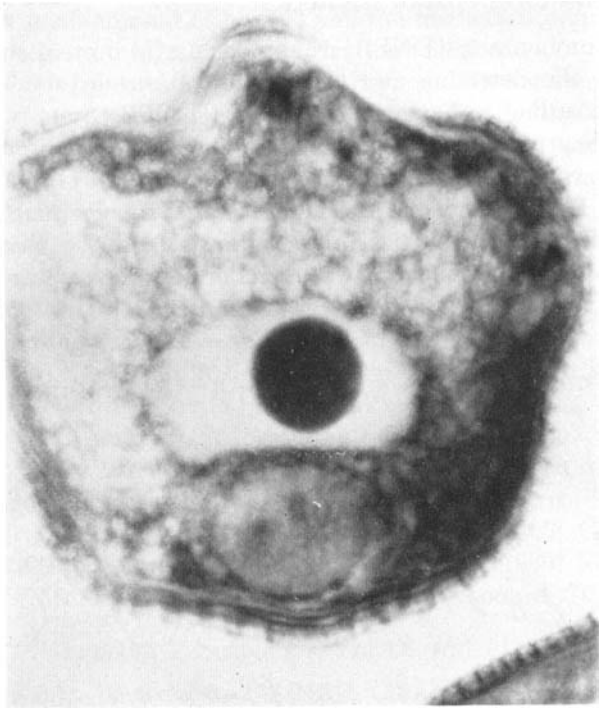


FIG. 7. Staining of a pollen grain with DNFB.

Bloch (1966b) and the author (Pipkin, 1968) found that the DNFB reaction can be modified for microphotometric determinations. The staining of a pollen grain with DNFB alone (Fig. 7) gave adequate dye intensity for photometric analysis obviating the necessity for enhancement of dye coupling (Pipkin, 1968).

1. DINITROFLUOROBENZENE PROCEDURE AFTER DANIELLI (1953)

(a) Fix in Carnoy or FAA. If NBF is used, the formaldehyde must be removed prior to staining.

(b) Dehydrate, embed in paraffin, section, and mount on slides.

(c) Place tissue for 1 hour at room temperature in a mixture of 0.5 ml DNFB and 1 gm sodium bicarbonate in 100 ml of 70% ethanol. Use this solution within several hours.

(d) Differentiate in three changes of 70% ethanol.

(e) Immerse for 2 minutes in a solution of chromous chloride in 0.1 *N* HCl (reduces nitro group of bound DNFB).

(f) Rinse in two changes of 0.1 *N* HCl for 4 minutes.

(g) Transfer to a nitrous acid solution for 5 minutes at 0°C prepared by dissolving 0.5 gm of sodium nitrite in 100 ml of 0.1 *N* HCl (diazotizes amino groups).

(h) Immerse for 15 minutes at room temperature in a solution containing α-naphthol or β-naphthylamine.

(i) Dehydrate in ethanol, clear in xylene, and mount.

2. DINITROFLUOROBENZENE PROCEDURE AFTER BLOCH (1966b)

(a) When NBF is used for fixation, immerse tissue in boiling water for a few minutes to remove the formaldehyde; however, this is unnecessary if DNA is removed by acid hydrolysis.

(b) Hydrolyze 15 minutes in 5% TCA in a 90°–100°C water bath to remove the nucleic acids.[2]

(c) Rinse in 70% ethanol in three 10-minute changes to remove the TCA.

(d) Stain in DNFB solution for 30 minutes at room temperature. Preparation is as follows: 0.60 ml DNFB in 26 ml of ethanol, 4.0 ml 1 *M* sodium bicarbonate, and 20.0 ml of distilled water. Use the reagent within several hours.

(e) Rinse at least ½ hour in each of several changes of 70% ethanol, and 1 change each of 95% ethanol, absolute ethanol, and ether.

(f) Clear in xylene directly, and mount.

G. Dinitrofluorobenzene-Sakaguchi Reaction

Histones owe their basicity primarily to the amino acids lysine and arginine. Generally speaking the most commonly occurring histones contain greater amounts of lysine than arginine. Nevertheless, a variety of

[2] Optional. DNFB binding by protein is relatively unaffected by the presence of nucleic acids.

L/A ratios are found, ranging from zero in many animal sperm, to 20 or more in "very lysine rich" histones. Usually the term "lysine rich" refers to a protein which has considerably more lysine than arginine. It includes the "very lysine rich" F-1A fraction of Hnilica *et al.* (1962) and the "slightly lysine rich" histones (Phillips, 1962; Murray, 1964) whose L/A ratio $\simeq 2$. "Arginine rich," on the other hand, describes the histone whose (Arg/Lys $\geqq 1$) (Hnilica *et al.*, 1962) and also the protamines or protamine-like histones of sperm.

Increasing curiosity and the realization of the possible vital functions histones may play in genetic regulatory systems have stimulated investigators to seek various techniques that describe changes in histone composition during cell development.

A double stain for the comparison of (lysine and tyrosine)/arginine ratios has been devised by Bloch (1966b), using a modified DNFB reaction, followed by a modified Sakaguchi reaction. By comparing the extinctions, microphotometrically, at 400 mμ (for DNFB) and 520 mμ (for Sakaguchi), the relative ratios of (lysine and tyrosine)/arginine of nuclei can be estimated. One advantage of this technique is that ratios of nuclei can be compared directly, thus circumventing the necessity of

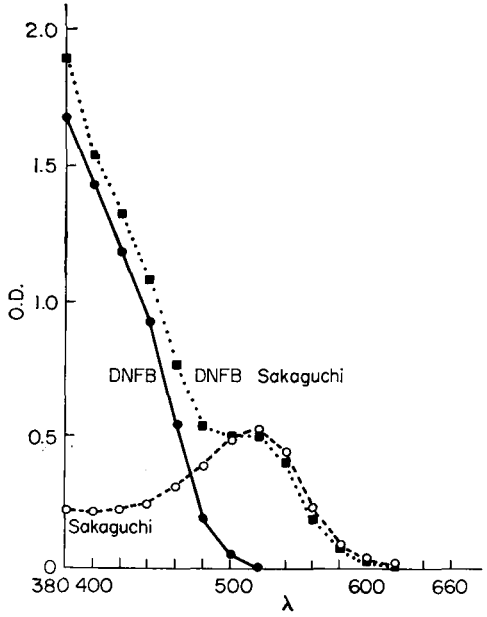


FIG. 8. Spectral absorption of three samples of bovine serum albumin stained with dinitrofluorobenzene (DNFB), the Sakaguchi stain, and the combined stains, the Sakaguchi following the DNFB. From Bloch (1966a).

photographing, mapping, and relocating nuclei in making the two measurements. [See Fig. 8 for the absorption spectral curve of DNFB and Sakaguchi reaction (Bloch, 1966a).] The method's chief disadvantage stems from the variable reactivity of the respective groups and the consequent inability to relate the extinction ratios to the amino acid ratios with a high degree of accuracy.

DINITROFLUOROBENZENE-SAKAGUCHI PROCEDURE AFTER BLOCH AND BRACK (1964)

(a) Fix tissue in ethanol or NBF.

(b) Dehydrate, embed in paraffin, section, and mount on slides.

(c) Hydrolyze tissue 15 minutes in 5% TCA at 90° to 100°C to remove the nucleic acids and the bound formalin.

(d) Rinse in three 10-minute changes of 70% ethanol to remove TCA.

(e) Stain in DNFB solution for 30 minutes at room temperature. The reagent should be used within several hours.

(f) Rinse at least ½ hour in each of several changes of 70% ethanol, and 1 change each of 95% ethanol, absolute ethanol, and ether.

(g) Rehydrate.

(h) Stain for 6 minutes at room temperature in Sakaguchi reagent. Preparation is as follows: 1 part 1.5% 2,4 dichloro-α-naphthol in *t*-butanol, 5 parts saturated $Ba(OH)_2$, 1 part 1% NaOCl (Clorox diluted 1:5), mixed in the order indicated. Reagent must be used *immediately.*

(i) Rinse in 1% urea in saturated $Ba(OH)_2$. Avoid $BaCO_3$ scum as mentioned in the description of the Sakaguchi reaction.

(j) Rinse in saturated $Ba(OH)_2$.

(k) Rinse in ¼ saturated $Ba(OH)_2$, not more than 3 seconds.

(l) Dehydrate by agitating in freshly distilled aniline and remove to fresh aniline when tissue becomes transparent. The aniline closely matches the refractive index of the stained tissue.

H. Sakaguchi-Feulgen Reaction

Attempts to measure the relative arginine/DNA ratios have been made by first staining with the Sakaguchi reagent and then with the Feulgen reagent and determining the relative ratios of the extinctions at 430 and 570 mμ (Bloch and Brack, 1964). Absorption spectra of nuclei with the Sakaguchi and Feulgen reaction are shown in Fig. 9.

SAKAGUCHI-FEULGEN PROCEDURE AFTER BLOCH AND BRACK (1964)

(a) Fix in 10% NBF, or Carnoy.

(b) Dehydrate, embed in paraffin, section, and mount on slides.

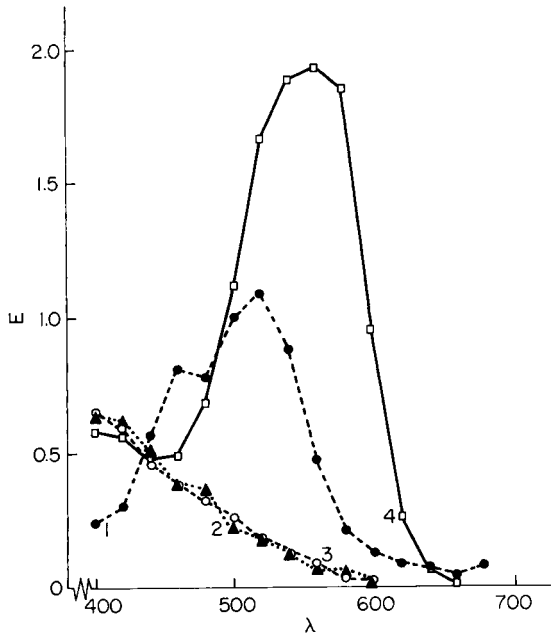


FIG. 9. Absorption spectra of nuclei stained with the Sakaguchi and Feulgen reactions. Curve 1: ●, Sakaguchi stain. Curve 2: ▲, Sakaguchi stain followed by treatment with cold acid. Curve 3: ○, Sakaguchi stain followed by acid hydrolysis used for the Feulgen reaction. Curve 4: □, Sakaguchi and Feulgen stain. A single grasshopper spermatid nucleus was used in obtaining all four curves. From Bloch and Brack (1964).

(c) Stain tissue for 6 minutes at room temperature in Sakaguchi reagent without prior hydrolysis.

(d) Rinse in 1% urea in saturated $Ba(OH)_2$.

(e) Hydrolyze at 60°C for 25 minutes in 1 *N* TCA.

(f) Immerse in Schiff's reagent for 1 hour at room temperature.

(g) Drain and rinse in 3 changes of freshly prepared metabisulfite solution.

(h) Rinse in running water.

(i) Dehydrate in a graded series of ethanol, clear in xylene, and mount.

I. Van Slyke Reaction and Acetic Anhydride Reaction

The staining of sperm histones and protamines during histone transitions has been described in a number of organisms. The classic case is the differentiating spermatid nucleus. As early as 1897, Miescher's pioneering studies revealed that the nuclei of immature salmon testes contain

nucleohistones while ripe salmon sperm contain nucleoprotamines. The changes in the chemical constitution of the chromatin occurring during sperm formation can also be revealed by special cytochemical tests that are essentially modifications of Alfert and Geschwind's (1953) original method.

Deamination by the Van Slyke reaction blocks the amino groups of lysine and results in the loss of alkaline fast green stainability of typical histones that owe much of their basicity to lysine. If a transition to a "very arginine rich" histone occurs,[3] the nuclei will stain with alkaline fast green after either acetylation or deamination (Alfert, 1956). Das *et al.* (1964a) determined that during spermatogenesis in *Drosophila melanogaster*, the developmental stages from the spermatogonia up to but not including the mature spermatozoa contain histones typical of somatic tissue, since they do not stain with fast green or bromphenol blue after deamination. The spermatozoa stain after deamination, suggesting transition during sperm maturation, and indicating that the basic protein of the mature functional spermatozoa is a "very arginine rich" histone, and not a simple protamine nor a typical histone.

1. Deamination Procedure after Van Slyke (1911) for Use with Acid Dye Staining

Hydrolyze in TCA as for fast green staining, rinse in 70% ethanol as usual, then immerse tissue in three successive 15-minute changes at 0°C in freshly prepared nitrous acid solution. Preparation is as follows: Add equal parts of 10% sodium nitrite and 10% acetic acid. Rinse, and stain with alkaline fast green.

2. Acetylation Procedure after Monné and Slaughterback (1951)

Hydrolyze in TCA as above, rinse in 70% ethanol, then dehydrate slides in ethanol and transfer to a solution containing 1% glacial acetic acid in pure acetic anhydride at room temperature and leave for 1 hour. Rinse in absolute ethanol, rehydrate, and resume staining.

J. Picric Acid Bromphenol Blue and Picric Acid Eosin Reactions

Certain histones and the protamines have characteristically low molecular weights and are easily lost from the nuclei as a consequence of the

[3] The term "very arginine rich" is used here in the sense that "very lysine rich" has been used to describe the F1 histone. "Very arginine rich" histones include the simple protamines, such as salmine, and the more complex protamine-like histones found in sperm of many mammals.

removal of DNA by standard HCl and TCA extraction procedures. Since these basic proteins are no longer present to be stained with the dye, some modifications that ensure their retention are necessary for their demonstration. Examples are the picric acid bromphenol blue and picric acid eosin Y procedures (Bloch and Hew, 1960a). The essential features of these two methods comprise the use of an acid and of dyes which will form precipitates with histones and protamines. Picric acid hydrolysis (6–12 hours), which stabilizes protamines, completely removes the DNA and exposes the basic groups of the protein to binding with the acid dyes bromphenol blue or eosin Y. Consequently, staining of nuclei with these methods, and failure of the nuclei to stain with alkaline fast green, reflects the existence of labile histones and protamines.

Representative examples of protamine-containing nuclei are the salmon sperm and the fully mature snail spermatophore sperm. Alfert (1956) found that in the salmon there is a change from a histone-type nuclear protein (fast green positive) to a protamine (fast green negative) which occurs at a late stage in spermatogenesis. Bloch and Hew (1960a), studying spermatogenesis in the snail *Helix aspersa,* showed that deamination causes complete loss of fast green staining by somatic cells, spermatocytes, and early spermatids whose protein contains appreciable lysine. Fully developed spermatophore sperm do not stain with alkaline fast green. Staining of the mature sperm with bromphenol blue and eosin Y after picric acid hydrolysis indicates the existence of a labile protein. Lack of affect of deamination shows these to be protamines.

Examples of labile histones whose compositions have not been ascertained (other than that they are not "very arginine rich") have been found in certain cells during embryonic development. These have been termed "cleavage histones." Das *et al.* (1964b) reported that during early embryonic development of *Drosophila melanogaster* the nuclei contain in sequence two kinds of chromosomal proteins. The cleavage nuclei until just before the blastoderm stage contain a juvenile histone which is bromphenol blue positive yet fast green negative. Immediately prior to blastulation, at the close of the period of synchronized cleavage mitosis, the typical fast green positive histone appears. In another paper, dealing with *Helix aspersa,* Bloch and Hew (1960b) noticed that after fertilization the protamine of the sperm is lost and is replaced by faintly basic histones. As in the case above, this histone differs from the adult histone by its inability to bind fast green, and from the protamines by its lack of affinity for eosin Y and weak stainability with bromophenol blue. Bloch called these "cleavage histones." They were also found in both pronuclei. Later in development the nuclei undergo a transition after which they contain histone indistinguishable from those of the adult.

The changes in composition from a typical histone to one that is very rich in arginine is fairly common in sperm cells (d'Alcontres, 1953; Dallam and Thomas, 1953; Daly *et al.*, 1951; Das *et al.*, 1964a; Hamer, 1955; Hultin and Herne, 1949; Rosedale, 1945; Vendrely *et al.*, 1957).

Ringertz and Zetterberg (1966), in a number of experiments employing tissue as well as model systems, have shown the specificity and quantitative features of alkaline bromphenol blue for the demonstration of histones and protamines. Microspectrophotometric analysis of bromphenol blue-stained material is possible after the removal of DNA by picric acid. If the amount of picric acid which remains bound to the material is calculated from the extinction at 370 mμ, then the amount of dye bound in single cells can be determined with a high degree of accuracy. Results obtained from model experiments suggested that the dye binds to basic proteins such as histones, protamines, and to some extent lysozyme. The authors further suggested that there is some indication that picric acid may block dye binding. An overall stoichiometric relationship is found between the quantity of basic polypeptide and the amount of dye bound. However, the authors warn that this does not suggest that each basic group binds with one molecule of bromphenol blue.

1. Picric Acid Bromphenol Blue Procedure after Bloch and Hew (1960a)

(a) Hydrolyze for at least 6 hours at 60°C in saturated picric acid solution.

(b) Rinse in water.[4]

(c) Stain overnight in a 0.1% solution of bromphenol blue brought to pH 2.3 with a minimum of HCl.

(d) Rinse for 5 minutes in 95% alkaline ethanol. Preparation is as follows: Wave a glass stirring rod, which has been previously wetted with water, over concentrated ammonium hydroxide, and then stir the ethanol with the same rod.

(e) Bring slide directly to a fresh Coplin jar of 95% ethanol leaving for a minimum of 12 hours.

(f) Dehydrate through a graded series of ethanol, clear in xylene, and mount.

2. Picric Acid Eosin Y Procedure after Bloch and Hew (1960a)

(a) Hydrolyze for at least 6 hours at 60°C in saturated picric acid solution.

[4] Picric acid can be removed rapidly from tissue by rinsing briefly in acetone containing 1% concentrated hydrochloric acid (v/v).

TABLE I

COMPARATIVE INTENSITY OF STAINING WITH FAST GREEN AND BIEBRICH SCARLET IN VARIOUS SITES[a]

	Fast green FCF alone						Biebrich scarlet alone						
	Fixation												
	Buffered $HgCl_2$ at pH:			Neutral formalin at pH:			Buffered $HgCl_2$ at pH:			Neutral formalin at pH:			F. green + B. scarlet with buffered $HgCl_2$ fixation at pH:
Histologic structure	6	8	9.5	6	8.0 or 9.5	TCA then F. Gr. at 8.0 or 9.5	6	8	9.5	6	8.0 or 9.5	TCA then B. Scar. at 8.0 or 9.5	9.5
Chromatin	0	1	1	0	0	3	0–1	0–1	4	0	0–1	3–4	4OrR[b]
Nucleoli[c]	0	0	0–1	0	0	0	0–1	0–1	4	0	0–1	0–1	4OrR
Seminal vesicle fluid	4	3	3	3	0	3	4	3	3	3	0	4	4P
Spermatozoon heads	0	2	3	0	0	3-4	2	3	4	0	0–2	3–4	4PR
Granules in eosinophilic leukocytes[d]	4	4	4	4	4	4	4	4	4	4	4	4	4P
Granules in Paneth cells[e]	4	4	4				4	4	4				4P
Keratohyaline granules in proventriculus[e]	1	0	2				1	0	3				4PR
Stratum lucidum proventriculus	3	0	1				3	1	3				4PR

Arterial elastica	2	2	2	2	2		2	2	2	2	2		2PR
Erythrocytes	1–3	0	0	3	0	0	3	0	0	3	0	0	+
Zymogen granules pancreas	3	0	0–1	3	0		3	0	0–1	3	0		0-PR
Chromidial substance pancreas	0	1	1				0	1	1				0-Or
Muscle, collagen													GC

[a] This staining of chromidial substance and zymogen granules differs significantly from that of chromatin and nucleoli in that it occurs also at pH 8.0 and is pink to red rather than orange-red. Likewise, the fast green dye fails to visualize sites rich in RNA, except that it visualizes similarly the chromidial substance in pancreatic acinar cells after chloroform-methanol or Carnoy fixation.

Characterization of acidophilic components. Somatic nuclei, in tissues fixed with solutions containing formaldehyde, lack reactivity toward Biebrich scarlet at any pH level. From Spicer (1962).

[b] G = green, C = gray, Or = orange, P = purple, R = red, 0 = unstained. Numbers indicate staining intensity.

[c] Best visualized in cerebellar Purkinje cells of guinea pig.

[d] Applies particularly to rat tissues.

[e] In the mouse.

(b) Rinse in water.[4]

(c) Stain overnight in a 0.1% solution of eosin Y buffered at pH 8.1–8.3 with tris-HCl buffer. Solution should be used the same day.

(d) Rinse in distilled water.

(e) Dehydrate in a graded series of ethanol, clear in xylene, and mount.

K. Biebrich Scarlet Reaction

The acid dye Biebrich scarlet has the ability of staining protein metachromatically between the pH range of 6.0 to 10.5. At pH 6.0, generalized staining is common, but as pH levels are increased specific nuclear staining is observed, presumably because of the relative basicity of the nuclear

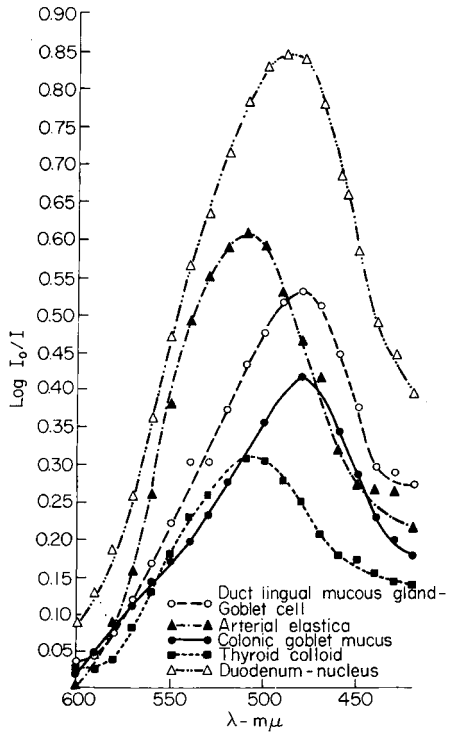


FIG. 10. Absorption curves of tissues stained with alkaline Biebrich scarlet. Spectra are illustrated for rat lingual duct goblet cell mucus, rat elastic tissue, rat colonic goblet mucus, mouse thyroid colloid, and mouse duodenal nucleus. Tissues were fixed in Carnoy fluid except in the case of the duodenal nucleus which was fixed 40 minutes with 2% calcium acetate, 10% formalin. From Douglas *et al.* (1966).

protein (Spicer and Lillie, 1961). The colors of various tissues after exposure to alkaline Biebrich scarlet are shown in Table I (see pp. 332–333).

Biebrich scarlet has several advantages over alkaline fast green. First, histones and protamines can be stained at higher pH's with Biebrich scarlet, permitting staining without prior extraction of DNA; and second, they can be distinguished by their varied affinities for the dye over the range of pH's used. According to Spicer, susceptibility of the protein staining to blockage by formaldehyde indicates staining by lysine. Chemical blockage by acetylation or deamination has a similar result, therefore, the type of basic protein can be determined. The differences in absorption maxima of the stain substantiates the authors' belief that variation in coloration occurs among various sites in the tissue (Douglas *et al.*, 1966). (See Fig. 10 for absorption curves of various tissues stained with alkaline Biebrich scarlet.)

Certain potentialities exist which may prove advantageous over the more widely used histone stains. One is the possibility of differentiating between different proteins metachromatically.

The basis for the metachromatic staining of basic proteins by this dye is not presently understood, but Douglas *et al.* (1966) stress the possibility of order in the spacing of basic groups in protein imposed by anionic substance associated with the protein.

BIEBRICH SCARLET PROCEDURE AFTER SPICER AND LILLIE (1961)

(a) Tissue must be fixed for 24 hours in two changes of 6% mercuric chloride with 1.3 gm of sodium acetate per 100 ml of water.

(b) Dehydrate, embed in paraffin, and section. Rinse tissue in running water. Remove mercuric chloride with iodide followed by thiosulfate (see Jones, 1961 for details).

(c) Stain from ½–1½ hours in a 0.04% aqueous solution of Biebrich scarlet (C.I 280; National Aniline Division) in McIlvanine-Lillie phosphate buffer at pH 6.0. Dye solutions must be made on the same day as used.

(d) Dehydrate through a graded series of ethanol, clear in xylene, and mount.

L. Ammoniacal Silver Reaction

Ammoniacal silver was first described as a selective stain for nuclear chromatin by Black and Speer (1958). In 1960, nuclear histone specificity was suggested by Black *et al.*, after using the stain on a variety of tissues from mammals, fowl, invertebrates, and plants. Ammoniacal silver (A-S) staining was considered selective at least for chromatin and

perhaps histone since the cytoplasm stained faintly or not at all, and nucleoli always remained unstained.

The idea of specificity of A-S for histone was further supported by Black and Ansley's (1964) finding that the bands of giant salivary chromosomes of diptera stain distinctly with A-S in a manner corresponding to that seen with Feulgen and fast green methods and that interband areas indicated no affinity for either dye. Black and Ansley also found that extraction with cold HCl reduced or destroyed A-S staining while Feulgen staining was unaffected. Thus, ammoniacal silver and Feulgen can be used in sequence.

The mechanism of staining is unknown. The fast green reaction requires acid extraction of DNA before staining, while A-S staining only needs formalin treatment. Therefore, Black and Ansley suggest that alkaline fast green combines with bonds of histone molecules which normally associate with DNA, while the A-S reaction involves histone groups not so bound.

Of particular interest is the fact that the A-S reaction varies in color, from yellowish red to black. This change in tinctorial properties of the nucleus could be correlated with presumed change in function of lymphocytes stimulated by antigen injection (Black and Ansley, 1965b). Staining of electrophoretically separable histones with A-S revealed that yellowish staining is associated with lysine-rich histones, while the blackish staining is indicative of arginine-rich histones (Black *et al.*, 1963). These authors originally concluded from these staining studies that activation of the lymphocytes is accompanied by loss in lysine histone. Again pure lysine and lysine-rich fractions stained yellowish, while pure arginine and arginine-rich fractions stained black. The variation in color of both nuclei and histone models may reflect the differences in the availability of ϵ-amino groups of lysine and the quanido groups of arginine for dye binding. This argument is further substantiated by the fact that nitrous acid, which commonly blocks the ϵ-amino group of lysine, obliterates yellow A-S staining while altering the black staining much less.

Black and Ansley (1965c) discuss the possibility of using A-S as a quantitative histone stain. Microspectrophotometric measurements of A-S stained mouse nuclei were made at 480 mμ, representing the total stain, and at 700 mμ, representing the black stain indicative of arginine staining. Such measurements showed that tetanus toxin administration caused a loss in yellow staining (lysine-rich histone) and a loss in fast green staining of lymphocyte nuclei, while Feulgen measurements were unaffected. Extinction spectra of various A-S stained cells are shown in Fig. 11.

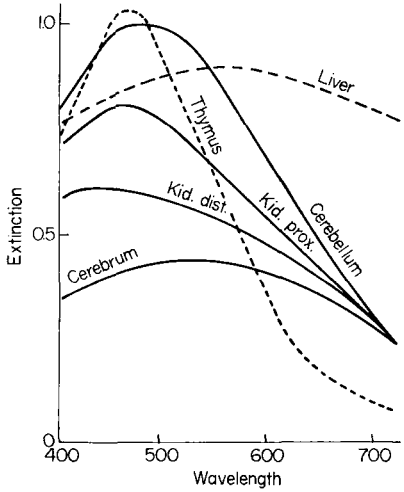


FIG. 11. Extinction spectra of various A-S-stained cells of CF-1 mouse. The thymocyte gives a well-defined peak at 480 mμ in contrast to the black staining neurons and hepatocytes, which have a slight maximum at 560 mμ. Kidney tubule cells present spectra which are intermediate between these two extremes. From Black and Ansley (1965a).

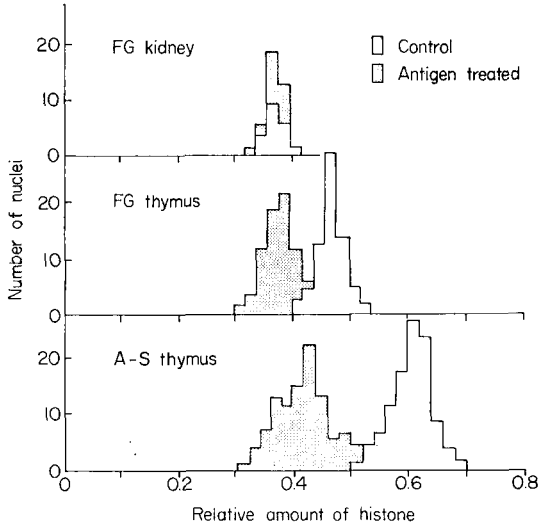


FIG. 12. Frequency distribution of relative amount of ammoniacal-silver (A-S) and fast green (FG) stainable histone in thymocytes and renal tubular cells. An antigen-induced decrease in thymic histone is revealed by both A-S and FG staining procedures. Such changes contrast with the lack of altteration in the nucleohistone of renal cells. These data are representative of the findings during the period 1 to 5 hours after antigen injection. From Black and Ansley (1965b).

Black and Ansley (1965b) have shown that histograms of amounts of A-S stain in cells reveal meaningful patterns as cell function changes, indicating an importance of this stain in cytophotometric analysis (Fig. 12). Furthermore, the stain has one advantage over most of the other histone dyes, including alkaline fast green, for it can be used both for photometric determinations of histone quantities and for changes in histone composition.

AMMONIACAL-SILVER PROCEDURE AFTER BLACK AND ANSLEY (1965a)

(a) Use air-dried smears which are immersed in 10% acetate-neutralized formalin for 1 hour at room temperature. Preparation is as follows: Add 200 gm. of sodium acetate to 1 liter of formaldehyde and dilute 10:1 with tap water. The pH of the solution is 7.1.

(b) Wash tissue in five changes of distilled water.

(c) Stain in ammoniacal-silver for 10 seconds. Preparation is as follows: Add dropwise 10% silver nitrate to 4 ml of concentrated ammonium hydroxide until a persistent turbidity is obtained.

(d) Wash again in five changes of distilled water.

(e) Immerse in 3% formalin for 2 minutes.

(f) Wash, dehydrate through a graded series of ethanol, clear in xylene, and mount.

M. Eosin Y-Fast Green Reaction

Bloch (1966b) discovered that the basic proteins of different nuclei of a given tissue react in a variety of ways when exposed to a mixture of eosin and fast green at alkaline pH. This technique has the capacity of distinguishing subtle variations existing among the cells of otherwise apparently homogeneous populations. Bloch equates the phenomenon to chromatography, "where substances that differ little, may, under appropriate conditions, exhibit large differences in their binding capacities."

TABLE II

DRY MASSES OF NUCLEI *in situ* AND OF ISOLATED NUCLEI IN SMEARS OF *Xenopus* ERYTHROCYTES[a]

	Dry mass $\times 10^{-11}$ g	FG-E color[b]	Composition[c]
Nuclei *in situ*	5.10 ± .22	Pink-purple	Rel. rich in lysine
Isolated nuclei	2.38 ± .06	Lavender-green	Rel. rich in arginine

[a] From Bloch (1966b).

[b] Color based on staining with fast green eosin.

[c] Compositions as determined by DNFB-Sakaguchi staining.

He has suggested after a study of eosin-fast green staining of different cell types that RNA biosynthesis levels are reflected by eosinophilic staining capacities of the nuclei. By way of example, in the polychromotophilic erythroblasts of the mouse and guinea pig that are notably low in RNA synthesis, the nuclei stain eosinophilic (pink). In the developing sperm of grasshopper, just prior to elongation, the nucleus stains purple, hence, is classified as eosinophilic. RNA synthesis is known to be negligible in the X chromosome of the grasshopper and it was found to be eosinophilic.

It is interesting that the altering of the metabolic level of nondifferentiating cell nuclei can be correlated with eosinophilia. The acinar cells of mouse pancreas normally bind fast green; after starving the animals for several days many of the acinar cells become eosinophilic. After refeeding, the cells bind fast green.

Mitotic and meiotic metaphases and later division stages were usually found to be eosinophilic.

A direct correlation was shown between loss of protein from nuclei during preparation and staining and loss of eosinophilia. DNFB-Sakaguchi staining of erythrocyte nuclei of blood smears indicated that the eosinophilic protein loss can be associated with a decrease in the lysine-rich histone (Table II).

Bloch validates this staining technique in defending it against the argument of artifact resulting from fixation or penetration by the following criteria—cells of close proximity have different nuclear staining reactions. The near "purity" in reaction of many cells, the scarcity of intermediately stained cells, and lack of color gradient with respect to cell orientation are all indicative of inherent differences in the cell as a cause of the type of staining reaction.

These findings of Bloch indicate that eosinophilia is characteristic of cell nuclei in which RNA synthesis is terminated as a consequence of development (spermatids and erythroblasts), or where it is intermittently halted, as during the metaphase and telophase stages of mitosis and meiosis, and also perhaps, where a temporary superabundance of RNA occurs.

Eosin Y-Fast Green Procedure after Bloch (1966b)

(a) Fix in tissue in 10% NBF.

(b) Use paraffin sections.[5] Hydrolyze tissue 15 minutes in 5% TCA, 90°–100°C to remove nucleic acids.

(c) Impregnate with celloidin by running rapidly through higher alcohols, then into methyl benzoate, and finally into 1% celloidin in methyl benzoate for ½ hour. Rinse in methyl benzoate, alcohols, and bring to cold 70% alcohol.

(d) Three 10-minute rinses in 70% ethanol from 0° to 5°C removes TCA.

(e) One-half hour in 0.1% fast green FCF from 0°–5°C, buffered at pH 8.2 to 8.3 with tris-HCl buffer whose final concentration is 0.07 *M*.

(f) Two hours from 0°–5°C in a mixture containing 0.05% fast green FCF and 0.05% eosin Y in the above buffer.

(g) Differentiate 10 minutes in buffer, 0°–5°C.

(h) Ten minutes in 70% ethanol, 0°–5°C to remove nonspecific acid dye.

(i) Ten minutes in 95% ethanol, 0°–5°C.

(j) Further dehydration in 100% ethanol, clear in xylene, and mount.

N. Feulgen-Eosin Y-Fast Green Reaction

The author (Pipkin, 1968) has found that a Feulgen-eosin-fast green sequence can be employed which is useful in comparing DNA levels with those in the same nuclei. More consistent DNA C levels are obtained when the Schiff's reaction and subsequent metabisulfite rinses are carried out at reduced rather than ambient temperature. Figures 13 and 14, re-

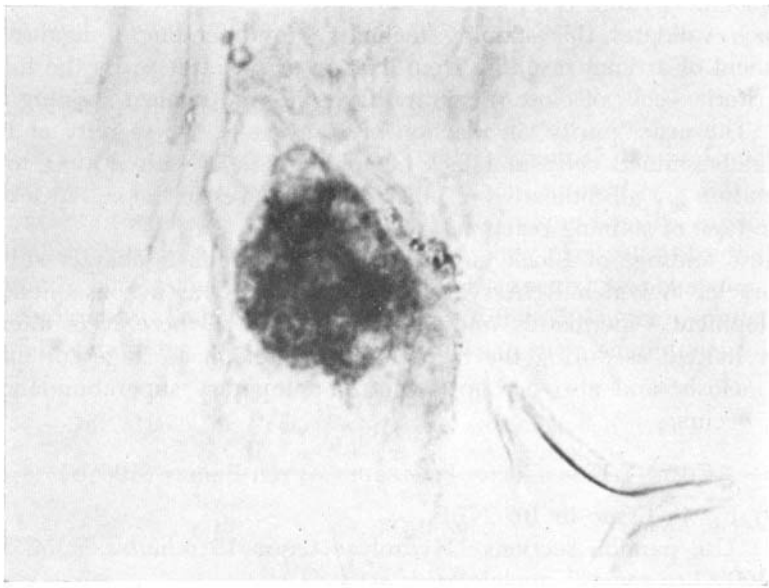


FIG. 13. Staining of a tapetal nucleus after the Feulgen reaction.

[5] Eosinophilic histones are often very labile, and may be lost by leaching during drying of the paraffin sections onto the slides. Drying is hastened by draining slides, after spreading the sections, then blotting by rolling with a test tube covered with dampened filter paper.

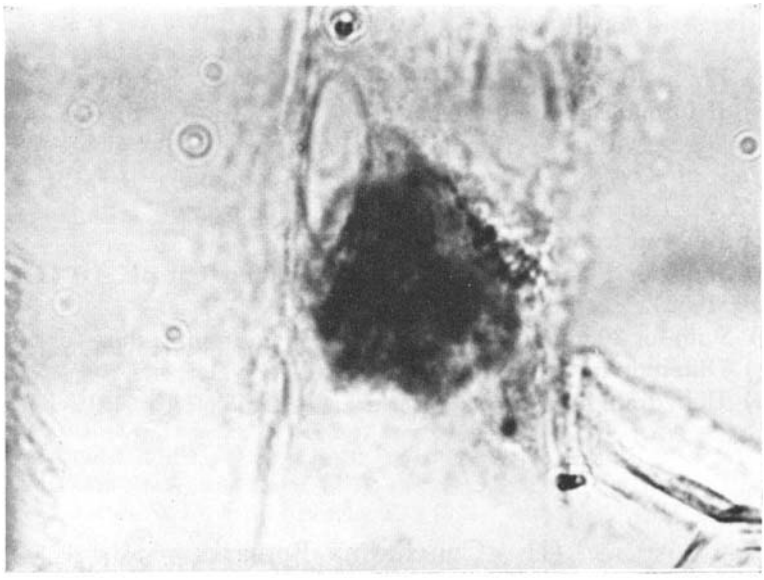


FIG. 14. Staining of the identical tapetal nucleus after the eosin-fast green reaction.

spectively, show a tapetal nucleus which has been stained first with Feulgen and second with eosin-fast green (Pipkin, 1968).

FEULGEN-EOSIN-FAST GREEN PROCEDURE

(a) Fix in 10% NBF.

(b) Either smears or paraffin sections can be used. Hydrolyze at 60°C for 25 minutes in 1 *N* TCA.

(c) Immerse in Schiff's reagent for 2 to 3 hours (a longer period of staining is necessary because of reduced temperature) from 0°–5°C.

(d) Drain and rinse in 3 changes of freshly prepared bisulfite solution from 0°–5°C.

(e) Immerse in 3 changes 15 minutes each in distilled water 0°–5°C.

(f) Three 10-minute rinses in 70% ethanol from 0°–5°C.

(g) Hydrolyze tissue 15 minutes in 5% TCA from 90° to 100°C which will remove nucleic acids and Feulgen stain. The remaining steps are identical to the eosin Y-fast green procedure as seen above.

O. Azure B-Eosin Y Reaction

Cytoplasmic RNA and nuclear histone can be stained by an azure B-eosin Y double stain. The cytoplasm stains blue while the nucleus stains orange and there is usually little indication of nuclear basophilia.

Azure B-Eosin Y Procedure

(a) Fix in 10% NBF or in Carnoy.

(b) If formalin is used, remove it by brief exposure to boiling water.

(c) Digest with deoxyribonuclease. Preparation is as follows: 1 mg/ml solution of crystalline deoxyribonuclease containing 0.003 *M* $MgCl_2$, pH 6.5, for 1 hour at 37°C (Swift, 1955, see below).

(d) Rinse in water.

(e) Stain for 1 hour with 0.1% azure B buffered at pH 4.0 (Flax and Himes, 1952).

(f) Stain for 30 minutes with eosin Y as in eosin procedure for histones.

(g) Differentiate for 5 minutes in distilled water.

(h) Dehydrate in a graded series of ethanol, clear in xylene, and mount.

III. Concluding Remarks

The techniques of cytochemical identification of histone and basic protein described herein have been used successfully by different workers and represent the most commonly used methods. However, some of these techniques have not been widely employed and may be inadequate for certain studies; therefore, the author suggests the use of more standard procedures (e.g., alkaline fast green, Sakaguchi reaction, NYS, and picric acid bromphenol blue or eosin Y) in conjunction with the less explored methods. It must be remembered that relatively few dyes are absolutely specific for nuclear histones (most stain basic proteins in general as well) and these usually sacrifice stoichiometry for specificity. The problem of specific staining of nuclear histone depends on the type of tissue used, consequently, the investigator may find it necessary to modify various techniques to achieve the desired results. Nevertheless, the quantitative, as well as the qualitative evaluations of histone staining, can be made; and the DNA-histone relationship has been satisfactorily investigated by appropriate cytochemical staining techniques. As in all such approaches, interpretation must be tempered by judgment.

The ultimate characterization of the proteins and the description of their role in the cell will require more exact analysis than can be accomplished by staining methods. However, in absence of definitive methods for chemical extraction and isolation of histones, and of methods which provide homogeneous populations of cells in defined developmental or physiological states as sources of histones, the staining methods

serve well as a means of characterization compatible with localization within cells and tissues.

ACKNOWLEDGMENT

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Chapter 15

Mitotic Cells as a Source of Synchronized Cultures[1]

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I. Introduction

A. Mitosis and Synchrony

Mitotic cells are of great interest in their own right as members of the most distinctive phase of the cellular life cycle and, as such, have been

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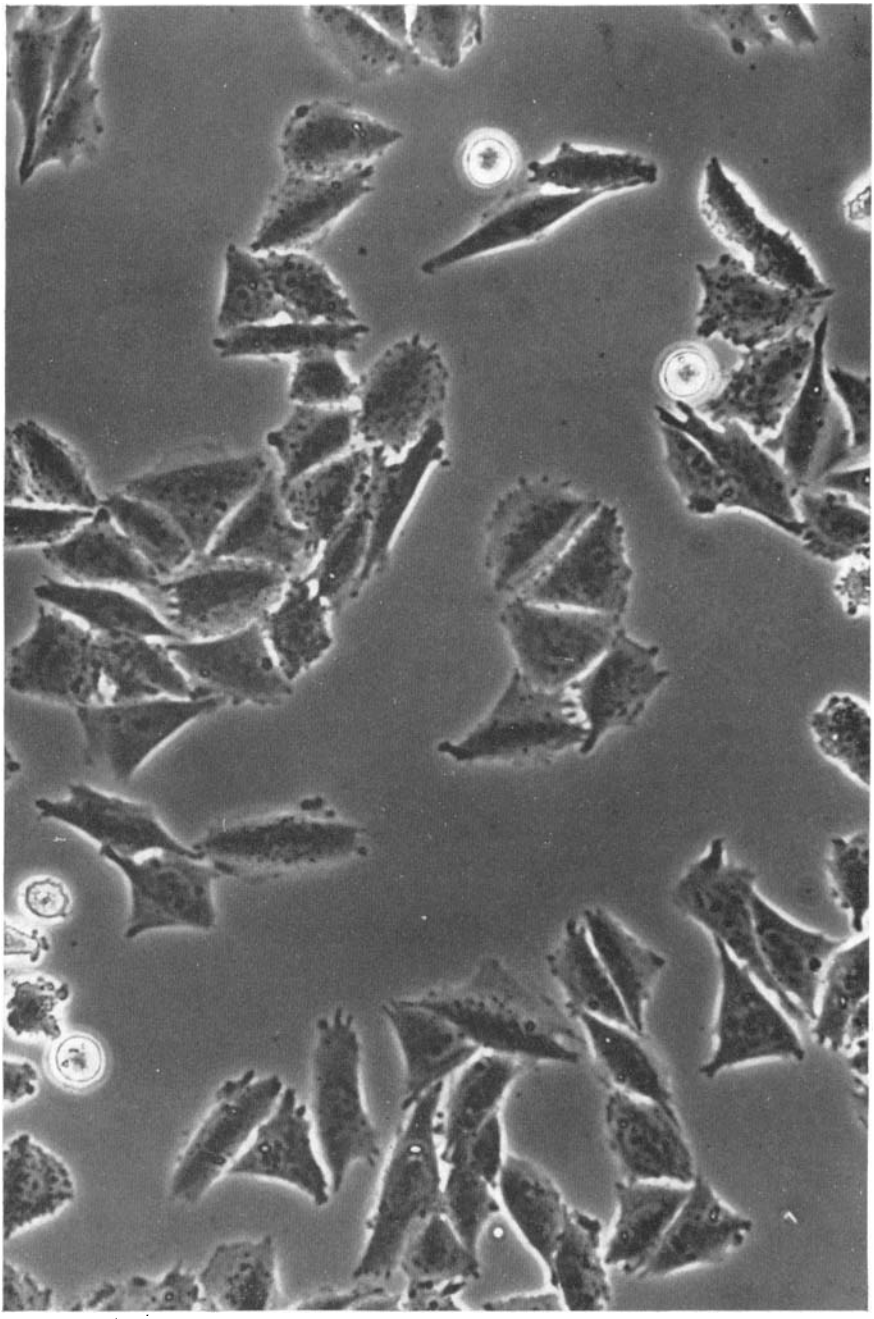


FIG. 1. Monolayer of Chinese hamster cells grown in low-calcium F-10 medium to approximately the density used for mechanical detachment of mitotic cells, showing the typical change in morphology between interphase and mitotic cells.

intensively studied for many years. This chapter, however, is concerned with an entirely different view: namely, the preparation and utilization of these cells as a source of synchronized cultures with which to investigate the biochemistry of "interphase." It is now realized that the spectacular events of mitosis can occur only after extensive preparations during interphase, not the least important of which is the replication of the genome during the DNA-synthetic period S. The preparations in question are biochemical rather than morphological and seem to occur in a sequence which is at least partly ordered in time. Biochemical studies of interphase usually require populations rather than single cells, and the populations must be "synchronized." The production of these populations in which most of the cells are at a similar stage of biochemical development has thus become an important technique of contemporary cell physiology.

The utility of synchronized cultures in the study of the biochemistry of several phases of the cellular life cycle of mammalian cells has been long recognized, and numerous methods have been developed to produce such cultures. Most of these methods require that the population be perturbed (e.g., by chemical inhibition, heat or cold shock, etc.), and it then becomes difficult to prove that the biochemical balance (Anderson *et al.*, 1967) of the culture has not also been altered by the treatment. The ideal synchronization method would be one which is purely selective and which could be applied to a growing population without affecting the growth rate. A number of procedures which approximate these requirements have been described using sedimentation (Sinclair and Bishop, 1965) or electronic selection (Fulwyler, 1965) to isolate groups of cells of a limited volume range. The resolution of such methods is limited, and cells are selected on the basis of volume or density rather than on the basis of age. By far the most attractive method is due to Tolmach; it is based on the fact that some types of cells when growing in a monolayer culture are tightly bound to glass during interphase but abruptly loosen their hold and assume a spherical shape after they enter mitosis. Division occurs while in the loosely bound state, and later, in early G_1, the daughters reattach themselves and spread out into the typical interphase morphology (Fig. 1). During the loosely bound period, these cells can be selectively removed by gently washing or shaking the monolayer with culture medium. There need be no interference with normal growth, and the separated cells can be selected exclusively from a very narrow fraction of the life cycle (namely, about two-thirds of late metaphase). In its simplest form, the method is capable of yielding some 10^5–10^6 cells in a total volume of 25 to 100 ml with an initial mitotic fraction of 0.85 to 0.95, essentially all metaphases.

B. Historical Development

The striking change in the attachment of many monolayer cells which occurs during mitosis was described by Axelrad and McCulloch (1958). Use of this phenomenon as a method of isolating mitotic cells was first proposed and developed by Tolmach and his collaborators (Terasima and Tolmach, 1961; Terasima and Tolmach, 1963), and it seems appropriate, therefore, to refer to it as the Tolmach method. As originally described for HeLa S-3 cells (Terasima and Tolmach, 1961, 1963), the method was purely selective and provided a yield of about 1% of the cells growing on the monolayer, or about one-half of the total mitotic cells. Trypsinized cells from an exponentially growing monolayer culture were planted in 100-mm plastic culture dishes ($1–2 \times 10^6$/dish) and incubated overnight. The growth medium was replaced with fresh medium about 4 hours before harvest. The separation of mitotic cells was effected by gently pipetting 5–7 ml of fresh medium over the culture several times. The separated cells ($1–5 \times 10^4$) were then replanted in monolayer for subsequent use. The collected cells were 80–90% mitotic, and 70% divided within 1 hour after collection (90% within 2 hours).

Sinclair and Morton (1963) found this procedure unsuccessful when applied to Chinese hamster lung cells, presumably because these cells were very firmly bound. Their modified procedure required cooling to 4°C, reincubation, and finally gentle mechanical shaking with a 0.03 trypsin solution. Up to 75% of the cells were mitotic.

Robbins and Marcus (1964) demonstrated the importance of calcium ion concentration and were able to greatly increase the total yield of cells by harvesting from Blake bottles containing about 4×10^7 cells each. They used HeLa S-3 cells grown in Eagle's medium with NaCl substituted for $CaCl_2$ in osmotic equivalence, the only calcium coming from the 7% fetal calf serum supplement. [Only for the determination of plating efficiency was it necessary to raise the calcium level; other growth characteristics were normal, but Stubblefield *et al.* (1967) have suggested the possibility of metabolic alterations by the low calcium concentration.] Cells were removed by a single manual shake and were filtered through a nylon mesh to remove any interphase clumps. Up to 95% mitotic cells were obtained, a series of 20 confluent bottles giving an average yield of 30 to 40 mg of cells. Harvest could be repeated every 30 minutes.

Production of large quantities of mitotic HeLa cells (maximum 4×10^6/hour) was achieved by Lindahl and Sörenby (1966) using a rather complicated mechanical system with continuous removal from a rotating bottle monolayer by mechanical shaking and continuous low-

temperature centrifugation. Interphase cells constituted 5 or 6% of the populations. The distribution of the mitotic cells between prophase, metaphase, and anaphase plus telophase varied considerably, depending on conditions, as did the mitotic fraction. The mitotic fraction was rather slow in dropping when separated cells were incubated in Petri dishes, 4 hours being required for the fraction to fall from 0.90 to 0.05, an effect which the authors ascribed, in part, to the great genetic heterogeneity of the HeLa culture.

C. Variations in Methodology

An outstanding advantage of the original Tolmach method is the absence of interference with the normal growth pattern of the culture; no inhibitors are added and selection can be accomplished from cultures which are demonstrably in the state of exponential growth. This feature has been abandoned in some modifications because of the desire to increase the yield of mitotic cells. This can be accomplished by using vinblastine sulfate to accumulate mitotic cells to concentrations far above the normal 2 or 3%, at which point they are then shaken off (Robbins and Marcus, 1963; Johnson and Holland, 1965). Colcemid has also been used for the same purpose (Stubblefield *et al.*, 1967). These procedures are not further considered here, as they lack the elegance of the simple selection methods. Because of the large deviations from normal cellular growth conditions, we feel they should be considered in a class with the other methods of enforced synchrony such as thymidine block and thermal shock. Such methods are indeed useful and necessary on occasion but share the major problem of requiring explicit proof that normal patterns and mechanisms of growth and division have not been altered and, thus, can be recommended only as a last resort.

The mitotic selection method can also be applied in an inverted sense; that is, the experimental material can be the cells remaining on the monolayer rather than those removed (Belli, 1965; Pfeiffer and Tolmach, 1967). In principle, one can accumulate larger populations in this manner with both boundaries sharply defined. In practice, the gain in population size is not impressive, it is obtained only by a corresponding loss in temporal resolution, and the inevitable dispersion during manipulation further reduces the attractiveness of these procedures. Once again, the temptation to resort to chemical blockade (e.g., vinblastine) proves difficult to resist (Pfeiffer and Tolmach, 1967). The objection is here mitigated by the fact that one can utilize those cells which have not yet been blocked, but the onus of demonstrating that these cells were unaffected by the drug remains as a significant limitation.

The procedure we have used for separation of mitotic cells [both Chinese hamster ovary (CHO) and HeLa] is described in detail below. It eschews the use of any chemical inhibitors, and every effort is made to maintain the population in continued exponential growth and biochemical balance throughout the procedure. No trypsinization or preliminary cold shock is required. A mitotic fraction of 0.85 to 0.95 is attained with yields of 2×10^5 cells/harvest. The mitotic fraction drops to zero in 20 minutes or less, indicating a very narrow "age" range. Harvesting can be repeated at 10-minute intervals to increase the yield at the expense of an equal broadening of the initial population width. Where larger populations of narrow width are required, the population can be chilled after separation and held in mitosis for periods of up to 6 hours. This treatment potentially compromises the "normality" of the cultures but to a considerably lesser degree than any alternatives. No drugs are used, and the population arrested is extremely homogeneous and is held in a state in which biochemical activity is at a minimum (Prescott and Bender, 1962). Careful measurements indicate that, upon rewarming, the cells resume growth and division at their normal rate with no evidence of dispersion during the cooling period. With this technique, populations of approximately 5×10^7 cells have been prepared.

We believe a significant factor in the successful development and use of the method to be the routine use of electronic cell volume spectrometry to monitor the behavior of the cultures and the efficacy of the separations. By providing an immediate measurement of the quality of a preparation, the volume spectrum permits easy testing of the effects of modifications of the procedure and gives prompt warning of any deterioration in the technique.

II. Equipment and Procedures

A. Cell Cultivation

The cells used for these studies were derived from the CHO line established by Tjio and Puck (1958) and from both wild type and S-3 clones of HeLa. Hamster cells were cultivated both as monolayers and in suspension in calcium-free F-10 medium (Ham, 1963) supplemented with calf (10%) and fetal calf (5%) sera, penicillin (100 units/ml), and streptomycin (100 γ/ml). The calcium present in the serum was sufficient for rapid attachment to glass and permitted the routine alternation between suspension and monolayer culture conditions in the same

medium without adaptation of the cells. Similar conditions were maintained for the HeLa cell lines except that calcium-free Eagle's medium containing 5% calf serum and antibiotics was used. Suspension cultures were grown in the conventional manner in jacketed spinner flasks (Bellco, Inc., Vineland, New Jersey) at 37°C.

B. Preparations for Mitotic Scoring

Aliquots containing 5 ml of cell suspension are centrifuged for 5 minutes at 800 to 1000 rpm, the medium is decanted, and the cell pellet is resuspended by dropwise addition of 5 ml of cold 0.1 *M* sucrose with continuous gentle agitation. Hamster cells are allowed to swell for 1.5 minutes and HeLa 3.0 minutes at 0°C (ice bath), and the cell suspension is centrifuged 3 minutes at 800 to 1000 rpm. The sucrose is decanted, and cold acetic acid-methanol (1:3) is added dropwise with continuous agitation to a final volume of 5 ml. This procedure is facilitated by leaving approximately 0.2 ml of sucrose behind, dispersing the cells as completely as possible, and mixing the first few drops of fixative thoroughly. The cells are stable for prolonged periods (at least 72 hours) in fixative, and all samples of an experiment may be collected and worked up together. Following centrifugation of the fixed cells at 400 to 500 rpm, the pellet is resuspended in 0.1 ml of acetic acid-methanol solution, applied to a slide, spread by gently blowing on the droplet, and air-dried. Cells are stained with Jenner-Giemsa stain (Humason, 1967) and scored for mitotic figures. Five hundred to 1000 cells are scored routinely, depending on the experimental design.

C. The Cell Spectrometer

Cell volume spectrometry is accomplished using the Coulter aperture (Coulter, 1956) as a volume sensor. Details of the cell spectrometer have been previously presented, together with experimental data verifying that the measured spectra correspond closely with the true volume distribution spectra of the cell cultures (Anderson and Petersen, 1967). The only source of serious error encountered was in the determination of the volumes of pairs of sister cells which had completed mitosis but had not yet separated physically. The measured volume of such a pair of tangent spheres was 20% less than the true volume (Anderson *et al.*, 1967). The number of cells in this state can generally be kept sufficiently small to make this error negligible.

The apparatus used is conventional except for special attention to matching electrical and hydraulic parameters to ensure minimum dis-

tortion of spectral shape (Kubitschek, 1962). Typical apertures are 100 μ in diameter by 200 μ in length. Transistorized electronics were designed and built in this Laboratory to match a modified 400-channel pulse-height analyzer.[2]

The components of our system include a low-impedance preamplifier (less than 1000 ohms input impedance; 300,000 ohms transimpedance) and an amplifier consisting of two conventional feedback stages, each with maximum gain of 100 and with bandwidth-restricting networks between stages. Integrating and differentiating time constants of 5 and 50 μseconds, respectively, were selected empirically on the basis of best signal-to-noise ratio. The multichannel pulse-height analyzer (RIDL, Melrose Park, Illinois) was modified to give proper analysis of the comparatively slow pulses. The input time constants to the lower level discriminator of the ADC were increased, and the duration of the reset trigger pair was lengthened.

Spectra are routinely analyzed in 100-channel quadrants of the analyzer which provide adequate resolution and dynamic range except for unusually broad spectra. (Logarithmic channel widths would be preferable but are not currently available.) Spectra are recorded by photogaphing the (linear) oscilloscope display of the analyzer and also on punched paper tape, which can be converted to punched cards for filing and computer processing. Double logarithmic graphs usually provide the most convenient presentation of the spectra and are plotted by computer.

Stability of the system is checked with a precision pulse generator and by calibration with a freshly prepared saline suspension of ragweed pollen. Fluctuations due to temperature changes are controlled by housing the instrument in a constant-temperature room maintained at $22 \pm 1°C$.

Cell suspensions are diluted, if necessary, with isotonic saline to concentrations not exceeding 10^4/ml to minimize spectrum distortion. Spectra are time-invariant for periods which are long compared to the interval required to make a measurement, but they are not indefinitely stable. Usually, 10^5 cells are measured, giving a count of approximately 5000 in the modal channel (usually near channel 30). This gives a statistical precision of $\pm 2\%$ in a theoretical electronic volume resolution of about 3% at peak amplitude. The ends of the spectra contain fewer cells and, therefore, have a large statistical uncertainty and may, in addition, be distorted by the presence of noncellular objects such as dust and debris.

[2] We are indebted to R. D. Hiebert of the LASL Electronics Group for his competent work in designing and building the transistorized electronics.

D. The Oscillating Mechanical Shaker

The apparatus used to standardize the mechanical force employed to detach mitotic cells is a Precision equipoise reciprocating shaking machine (Precision Scientific Co., Chicago, Illinois) operated at 265 strokes/minute with a 2⅜ inch stroke amplitude. The carrier of the instrument is compartmentalized so that from 1 to 6 Blake bottles can be shaken simultaneously with comparable force and geometry. The bottles are loaded with their long axes perpendicular to the direction of motion and with the monolayer horizontal and down so that the medium washes across the monolayer from side to side. A simple switch and timer are used to govern the duration of agitation. All operations are carried out in a warm room at 37°C to avoid thermal shock.

III. Selection of Mitotic Populations

A. Model of the Selection Process

A simplified picture of the operation of the mitotic selection process is given in Fig. 2. The diagrams are plots of $N(\tau)$, the number of cells of age τ, versus age for a small portion of the life cycle spanning the M/G_1 boundary. Termination of mitosis and separation into daughter cells are both assumed to occur at this point, which is assigned age zero.[3] Ages are measured in both directions from this boundary and are considered negative in the period preceding division. Figure 2A shows the age distribution of an unperturbed, exponentially growing population with $N(\tau)$ declining (exponentially) with increasing age and doubling abruptly at $\tau = 0$ when division occurs. The age at which the cells are released from attachment is τ_R (observed to be within the mitotic period M), and the age at which the daughter cells attach again (in early G_1) is τ_A. The population in the shaded area between these two ages is the population which will be released the first time the monolayer is shaken. It will clearly not be 100% mitotic, since some of the loosely bound cells are in G_1. During the time following separation, the remaining population will age and hence pass from left to right across the diagram. (Dis-

[3] The end of mitosis and separation of daughter pairs are not coincident; the microscopic disappearance of recognizable telophases precedes separation detected by the Coulter counter by approximately 10 minutes in the Chinese hamster cell (Anderson *et al.*, 1967; unpublished data).

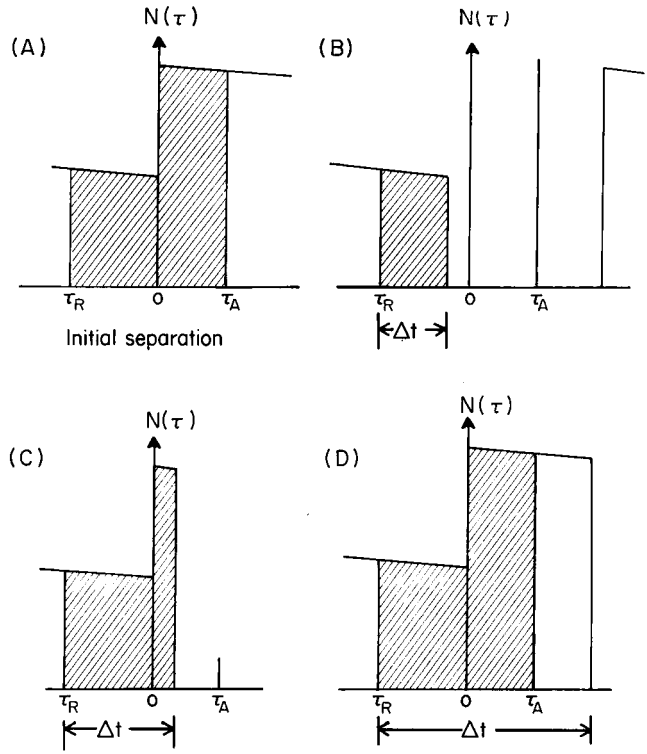


FIG. 2. Schematic diagram of the selection process depicting the population removed by shaking (shaded areas) in terms of time between separation (Δt) and the points in the life cycle when cells become easily detached (τ_R) and tightly reattached (τ_A).

persion is neglected in this simplified approximation). The composition of the population removed in later separations will depend on the duration, Δt, of the time between the two separations. If, as shown in Fig. 2B, Δt, is less than τ_R, no cells will have reached the end of mitosis, and the population removed (shaded area) will be 100% mitotic. Figure 2C shows the case in which Δt slightly exceeds τ_R; the cells to the right of $\tau = 0$ are nonmitotic, having divided and entered G_1. As Δt is further lengthened, the number of such cells removed will increase until $\Delta t = \tau_R + \tau_A$. Beyond this time, the cells reattach and the composition and number of the separated population remain constant with increasing Δt (Fig. 2D).

Experimental results appear consistent with the model. Thus, in Fig. 3A the mitotic fractions of separated populations are plotted against the time between successive separations. (These data were taken after the

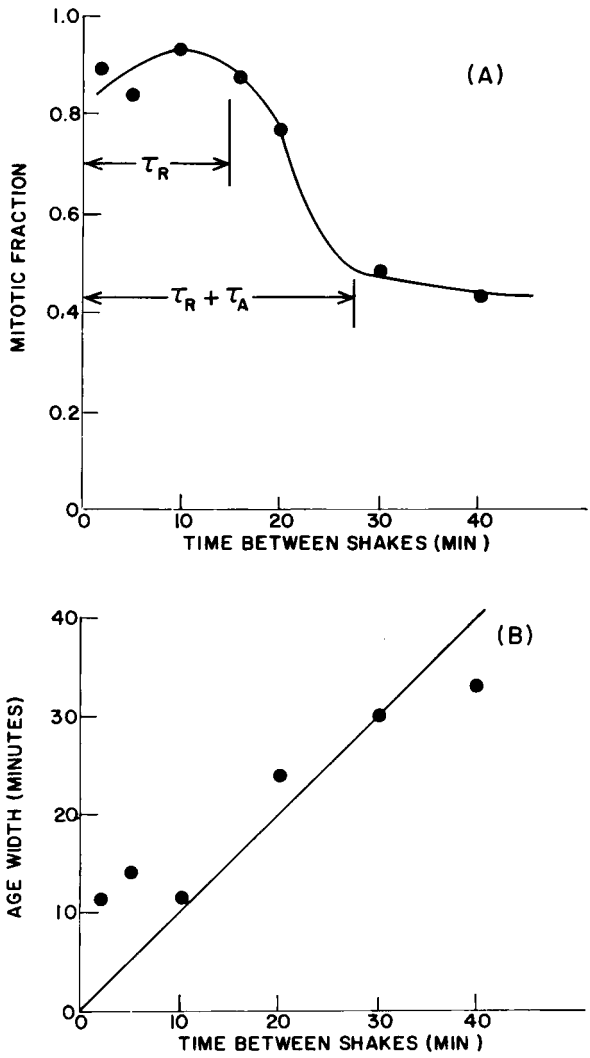


FIG. 3. Curves showing the effect of the interval between successive separations on (A) the mitotic fraction, and (B) the age width of the separated populations (see text).

monolayer had been processed a few times in order to reach equilibrium under the cycle time used.) It appears that the mitotic fraction remains high to Δt of about 15 minutes, then falls rapidly but remains constant when Δt exceeds 25 to 30 minutes. A similar result is obtained for the age width of these populations (measured as discussed later) shown in

Fig. 3B. The solid line represents age width equal to Δt. The points all lie above the line, an effect which would result if 100% of the loose cells were not removed at each cycle, a few remaining to broaden the population on the following separation. There is a suggestion that the width does not continue to increase proportionally for time beyond 30 minutes. Note also that age width appears to be independent of Δt when the latter is less than 10 minutes.

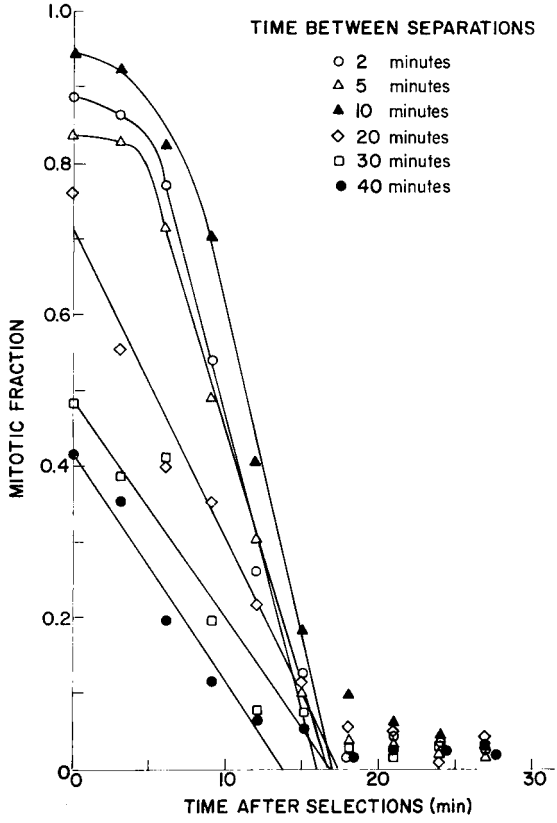


FIG. 4. Curves depicting the residual mitotic fraction as a function of time in suspension culture following selection at varying separation intervals.

Accurate estimates of τ_R and τ_A and closer numerical checks on the model can be obtained from the following studies. Figure 4 shows the mitotic fraction as a function of time for several populations of separated cells which were placed in suspension culture immediately after separation. The populations were selected using different values of the time Δt between separations and were isolated only after a number of cycles had

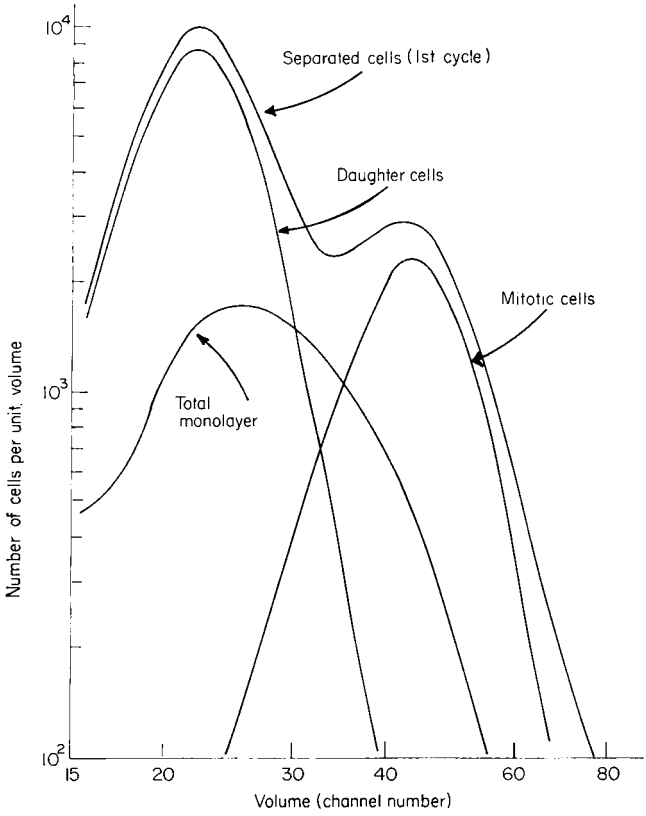


FIG. 5. Analysis of the volume distribution spectrum of a population of separated cells into three components: mitotic (undivided) cells, daughter cells, and unfractionated monolayer cells of all ages.

equilibrated the monolayer to these conditions. (The data for zero time are those of Fig. 3A.) In all cases, the mitotic fraction falls rapidly with time but, in spite of the differences of the initial values, reaches zero for all populations at about 15 minutes. This is the time required for the earliest M cells to reach division, i.e., it is τ_R of Fig. 2 and should be indeed independent of Δt according to the model. The value of τ_A can be estimated by turning to data obtained on the very first extraction of the monolayer, that is, under conditions for which Fig. 2A represents the age distribution of the separated cells. If the relative number of cells in late M and early G_2 can be determined, then the relative age widths of the corresponding shaded areas of the figure can be established. If only these two components were present in the first extraction, the answer would be obtained directly from the mitotic fraction. However, other

interphase cells may also be removed, and this third component must also be considered. Its volume spectrum would presumably be more or less that of the total monolayer. Figure 5 shows the analysis of data on the basis of this assumption. Four volume distribution spectra (obtained with a Coulter counter and 100-channel analyzer) are displayed here on a double logarithmic plot. This scale preserves the shape of the spectra independent of cell number and of calibration of the volume scale, so that normalization of different spectra can be accomplished by simple translation of the plots; replotting is not necessary. The upper curve is the spectrum of the cell population obtained by the first separation from a monolayer. The lowest curve is the spectrum of a total monolayer culture which was removed by trypsinization and resuspended for the determination. The curve labeled "mitotic cells" is the spectrum of the population obtained on the seventh shake (mitotic fraction 0.92), after a small correction was made for the presence of a few percent of early G_1 cells. The curve labeled "daughter cells" is the spectrum of newly divided cells which have just entered G_1. In this case, this spectrum was obtained by shifting the spectrum of mitotic cells a factor of two downward on the volume scale. We have previously demonstrated (Anderson *et al.*, 1967; and unpublished data), by measuring the spectrum of mitotic populations as a function of time, that the spectrum of cells after division is essentially identical with that of mitotic cells (i.e., sister cells have nearly identical volumes, the dispersion being small compared with the coefficient of variation of the parent population). The relative amplitudes of the three lower spectra were adjusted by successive approximations so that their sum equaled the upper curve. An excellent fit was easily obtained over virtually the entire volume range (the summation begins falling below the separated cell spectrum at volumes above 50). The relative areas under the curves are M = 28%, G_1 = 50%, and random population = 22%. This corresponds to a mitotic fraction of 0.28, which is close to the value of 0.32 observed. The ratio of G_1 to M cells is 1.78 (similar to the value of 1.70 obtained if the presence of interphase cells is neglected). The G_1 cells are twice as numerous per unit age increment as the M cells so that their relative abundance of 1.78 times implies they are derived from an age band 0.89 times that of the mitotic cells. This is the ratio of τ_A to τ_R. If the latter is 15 minutes, as indicated by Fig. 4, then τ_A must be about 13 minutes for these conditions. These numbers are compatible with the more crude estimates from Fig. 3.

Continued separations after the first should yield populations which are entirely mitotic, provided the time between separations, Δt, is kept less than τ_R. This condition is indeed attained after a few cycles. Once the steady state of maximum mitotic fraction is reached, one can calcu-

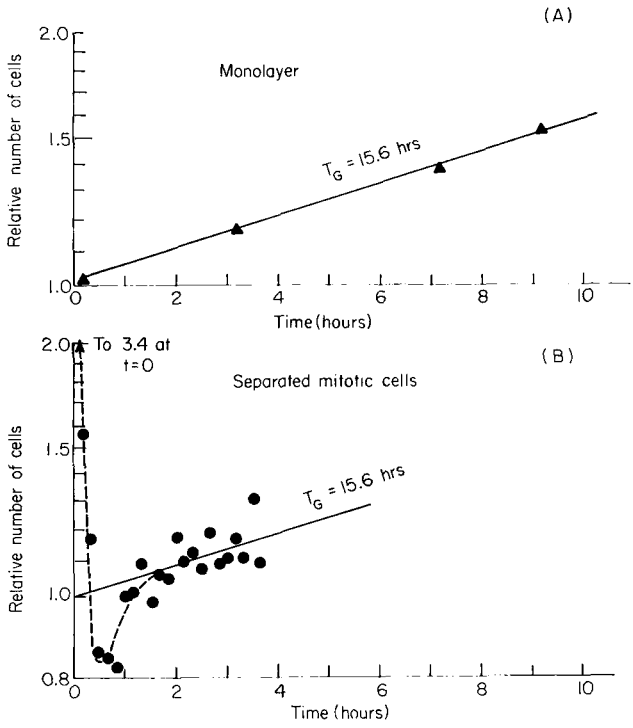


FIG. 6. Growth rate of a cell population determined by (A) trypsinization and Coulter counting of replicate cultures, and (B) Coulter counting of successive detached crops of cells from a single monolayer.

late the expected yield in relation to the yield of the first shake on the basis of the values of Δt and $\tau_R + \tau_A$. Thus, for $\Delta t = 10$ minutes and the values of τ_R and τ_A obtained above and remembering that the age density of cells in G_1 is twice that of cells in M, the relative yield should be $10/(15 + 2 \times 13) = 0.24$. The observed yield ratio was 4,900/16,900 $= 0.29$, in satisfactory agreement.

These results suggest that the model illustrated in Fig. 2 is adequate to account numerically for the behavior of the system. That exponential growth is maintained during the operation is suggested by the following results. Figure 6A shows that the rate of increase of the total monolayer population, determined by trypsinization of replicate cultures and Coulter counting of the resulting cell suspension, gives a generation time of 15.6 hours which is not incompatible with the rate of increase of successive milkings of one of the replicates (Fig. 6B). The monolayer is in exponential growth, and the separation process has not detectably disturbed this growth. The rather large variation in yield of mitotic cells

is evident in the data, as well as an "undershoot" of the yield of the fourth to sixth separations, the origin of which is not understood.

The observed yield of cells in a separation is nearly that expected. Thus, a CHO culture with a generation time of 15 hours and a mitotic index of 3.7%, corresponding to a mitotic period of 48 minutes, gave a yield of 1.8×10^5 mitotic cells from a monolayer of 2.5×10^7 cells. The time between separations was 10 minutes so that the fraction of cells available for removal would be 10/48 times the mitotic fraction, or 0.008. This is close to the yield actually observed of 0.007.

The model outlined above predicts a number of numerical consequences when the method is operating at maximum efficiency. These are

1. An initial "conditioning" will be required (i.e., a series of extractions to remove the loosely bound G_1 cells and raise the mitotic fractions to a maximum).
2. The age width, $\Delta\tau$, of the selected population should approach the cycle time, Δt.
3. The fractional yield after equilibrium is established should be (cycle time $\times$ mitotic index)/mitotic time.
4. The yield should rise with the same doubling time as that of the original monolayer culture.
5. The mitotic fraction should approach 1.0 if Δt is less than τ_R.

B. Conditions Affecting Yield and Quality

In maximizing the yield and quality of the separation, one can manipulate a number of variable factors: biochemical, biological, and mechanical. Little has been done with the first category other than regulation of the calcium content of the growth medium, as suggested by Robbins and Marcus (1964). An aspect of the second (i.e., selection of subclones) will be discussed in the next section. The remaining variables are those which control the shearing force which removes cells and the frequency and duration of the shear. The optimum values of these are likely to depend on local conditions: the cell line in use, the growth medium and supplements, the surface characteristics of the culture flasks, the cleaning procedures used, etc. While we will later specify in detail the procedure which we have found to be generally effective, it is appropriate here to mention a few generalities which may be helpful in a search for optimal conditions. The objective is, of course, to maximize the mitotic fraction and thus obtain a population with a demonstrably narrow age distribution. However, volume distribution spectra can be determined in a very few minutes and are thus more immediately avail-

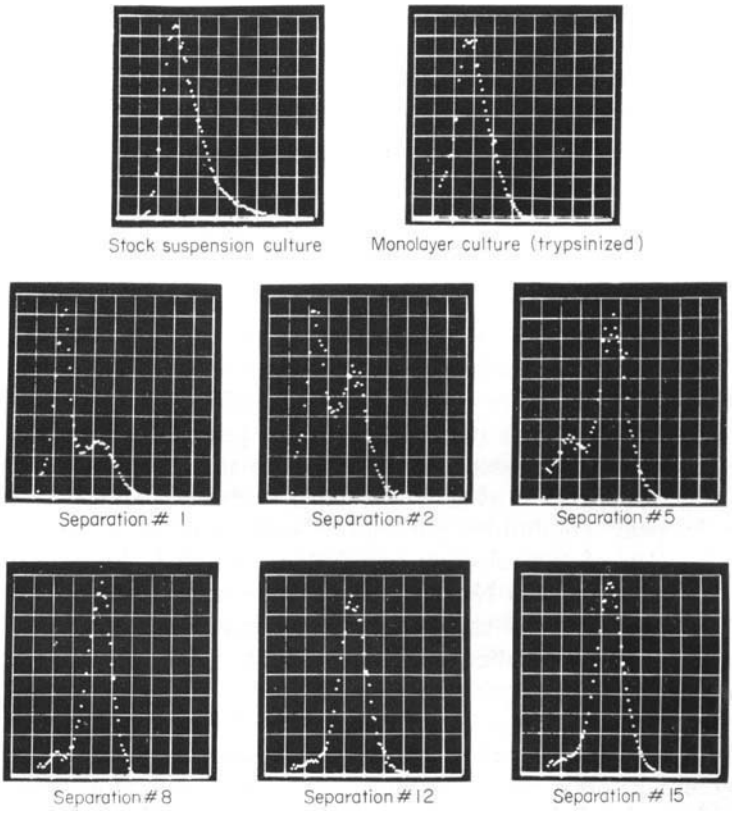


FIG. 7. Size distribution spectra of Chinese hamster cells determined with the Coulter spectrometer (see text for details).

able as criteria. We will digress briefly, therefore, to illustrate this use of volume spectrometry.

Monitoring of the progress of a separation procedure by means of the cell volume distribution spectra is illustrated in Fig. 7. The graphs are Polaroid photographs of the oscilloscope display of the output of the 100-channel pulse-height analyzer. The abscissa is channel number, corresponding closely to a linear volume scale, and the ordinate is the number of events (cells) observed to fall within each channel. The first spectrum is that of a stock suspension culture in exponential growth; the second spectrum is that of a monolayer culture plated from the suspension stock about one generation time(15 hours) previously. The monolayer was gently trypsinized to release all the cells for volume measurement. Both spectra have modal values in channel 26 and are nearly identical except that the monolayer contains somewhat fewer large cells (which

may be unseparated pairs in the suspension culture) and a slight excess of very small objects (which perhaps may be some cell debris resulting from trypsinization). The shape of these spectra is characteristic of balanced, exponential growth as we have shown by theoretical analysis (Bell and Anderson, 1967; Anderson and Petersen, 1967; Bell, 1968).

The six spectra of Fig. 7 are the volume distributions of the cell populations separated from the monolayer by successive applications of the shaking procedure at 10-minute intervals, the number of the cycle being indicated below each graph. The first two separations show a preponderance of small cells whose modal volume corresponds to channel 22 on the volume scale. These can be identified as early G_1 cells which have just divided and have not yet reattached to the Blake bottle. Since the cycle time is less than τ_R, the remaining cells are not able to reach division between separations and this population is rapidly depleted in subsequent spectra relative to the mitotic cells which correspond to the peak in channel 44 (i.e., at twice the volume). The change in peak amplitudes parallels a corresponding change in the mitotic fraction of the selection population, as noted above in Section III,A. Beyond separation No. 8, the amplitude of the G_1 peak becomes negligible. These results can be obtained immediately (within a few minutes of each separation) and provide a very valuable measure of the quality of the separation. Any failure of the operation, whether due to a poorly growing initial culture, to inadequate attachment of interphase cells, inappropriate schedule of shaking frequency or intensity etc., is immediately apparent in the spectra and, furthermore, the effects of changes in the shaking technique can be assessed without delay. When spectra such as those of the bottom line are obtained, one can be confident that the mitotic fraction is greater than 0.9.

One can thus use the relative areas under the two volume peaks to estimate the probable mitotic fraction of the population. Since the peaks have virtually the same shape and the one at small volume is half the width of the other, its relative area is half its relative amplitude. Thus, the estimated mitotic fraction, MF, is given by:

$$\mathrm{MF} = \frac{A_L + A_S/2}{A_L}$$

where A_L and A_S are the amplitudes of the large and small volume peaks, respectively. This relation will be quite inaccurate, however, if there is significant contamination with random interphase cells which have a different spectral distribution. In the latter case, the ratio of the modal volumes of the peaks may differ significantly from 2.0. A three-component analysis (cf. Section III,A, Fig. 5) would be necessary for any accurate

calculation, but the poor quality of the preparation would be evident by inspection.

To return to the monitoring of the separation process, if the shearing force is too large or too prolonged, interphase cells may be indiscriminately detached, increasing the relative amplitude of the peak of smaller volume or, in extreme cases, concealing the bimodal structure completely. Under these conditions, continued shaking will not improve the situation and indeed may cause it to deteriorate. If insufficient force is used not all the mitotic cells will be removed, and those still attached can complete mitosis and may appear in later separations as G_1 cells in the small volume peak. In the cases of both too much and too little force, the smaller volume peak will be enhanced and it may not be easy to decide which alternative is correct. If the number of cells removed by successive separations is roughly constant, one can perhaps decide on the basis of the yield being obtained. If it exceeds expectation, the force is too vigorous; if it is too small, then the force is inadequate. (A microscopic inspection of the separated population for large confluent sheets can also be useful evidence, indicating that the trouble lies in a poor or variable attachment of the culture.) A spectrum with the modal volume and characteristic shape of the total monolayer culture is, of course, an unambiguous indication that the removal process is completely nonspecific either because of too great a shearing force or because of inadequate difference in the attachment strength of the mitotic cells of the culture.

In preliminary experiments, a cycle time which is a fraction (one-third to one-quarter) of the duration of the mitotic period should be tried. The primary effects of varying this time should be those predicted by the elementary model, but there may be secondary effects, especially when it becomes very short. Thus, if interphase cells are partially loosened by the shaking process but are able to re-establish their bond to the substrate between cycles, they may not be removed. However, if the interval between separations becomes shorter than the "recovery time," the cell may eventually be detached. No quantitative data are available on this possibility, but we have observed that frequent repetition of short-duration stresses fails to accomplish the desired end. Mitotic fractions do not rise, and contamination with interphase cells persists.

A final point affecting both yield and quality of the selected populations is cell density of the monolayers at the time of harvest. Yield and mitotic index comparisons in Table I show that the best compromise between yield and a high mitotic fraction for our Chinese hamster line is obtained from bottles seeded to contain approximately 1.8×10^7 cells/Blake bottle at the time of harvest.

TABLE I

THE EFFECT OF CELL DENSITY ON THE YIELD OF MITOTIC CELLS FROM MONOLAYERS HARVESTED BY REPEATED DETACHMENT

Number of cells per monolayer	Cells per bottle recovered from 7th harvest[a]	Mitotic fraction
1.8×10^7	1.2×10^5	0.89
2.5×10^7	1.8×10^5	0.95
2.9×10^7	2.0×10^5	0.87
3.8×10^7	2.1×10^5	0.76

[a] Materials from the first 6 detachment cycles was discarded. Cells in the 7th supernatant were counted in the Coulter counter without dilution.

C. Selection of Suitable Subclones

The ideal culture for selective detachment of mitotic cells would be one in which there was a large and abrupt decrease in the tightness of binding to the culture vessel coincident with entry into mitosis. Details of the reattachment process are much less critical since, by milking at frequent intervals, one can eliminate all cells from this region. (However, if reattachment proceeds gradually over too long an age span, the difficulty might develop that an excessive number of cycles would be required before all these cells were removed.) The differential nature of the separation process is clearly based on the contrast of binding strength across τ_R, as well as on the relation between this binding and the magnitude of the shearing forces which can be developed by the separation process.

We have observed variations in the morphology of some clones of our Chinese hamster line, and one might expect as a corollary that binding strength would be subject to similar variation. We have investigated, therefore, a series of subclones isolated from the CHO line (Tobey *et al.*, 1967). Some of these failed to show satisfactory behavior when separations were attempted; the mitotic fractions were low and failed to rise on continued shaking. Three of the better colones are shown in Fig. 8, in which mitotic fraction is plotted as a function of time after the separated cells were returned to suspension culture. There is a slight difference in τ_R as determined by extrapolating the linear portion of the curve to zero mitotic fraction; this parameter varies from about 17 to 25 minutes for these clones under these conditions. However, the slopes are the same, indicating comparable age widths of about 12 minutes for all three. The maximum mitotic fraction was significantly higher for clones T_0 and T_4 than it was for the C_{10} clone. Similar results have been obtained with wild type and S-3 subclones of HeLa, and the selection of

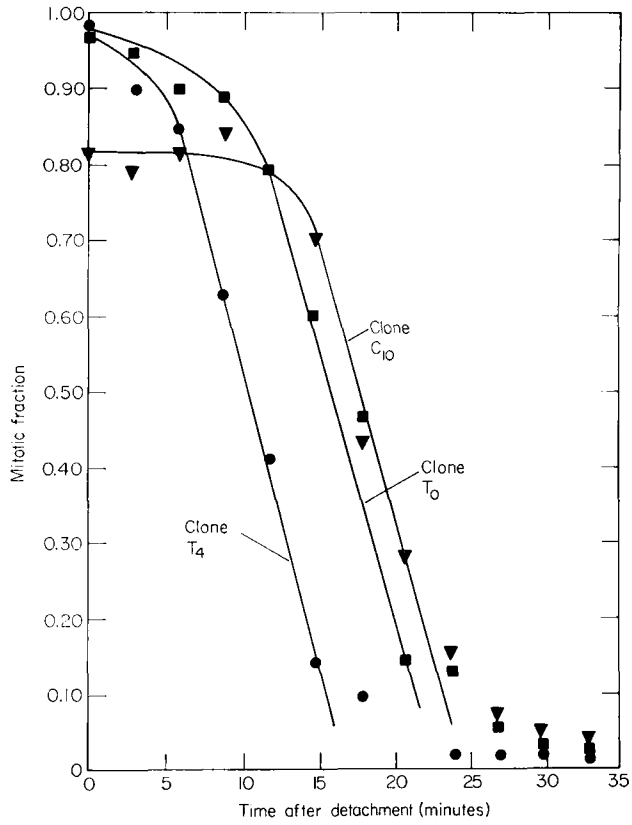


FIG. 8. Curves showing the residual mitotic fraction as a function of time in suspension culture following mechanical separation from monolayers. The codes T_0, T_4, and C_{10} designate three subclones of the Chinese hamster line selected because their differential detachment properties were superior to those of the parent cell line (Tjio and Puck, 1958).

subclones which routinely yield mitotic fractions in excess of 0.9 would appear to be possible, in principle, for most mammalian cell lines which can be grown as monolayers.

D. Cell Selection Protocol

On the basis of the foregoing considerations, a protocol has been developed for the consistent selection of highly phased populations. The protocol is presented here with the idea that it may serve as a starting point, and provisions must be made to tailor the basic technique to the peculiar properties of an individual culture.

1. Cells should be seeded to provide approximately 1.5–2.5 $\times$ 10^7 cells/Blake bottle at the time the harvesting procedure begins. New Pyrex Blake bottles (A. H. Thomas, Philadelphia, Pennsylvania) were found to be initially unsuitable for selection of mitotic cells after routine cleaning; agitation removed not only mitotic cells but large sheets of interphase cells as well. However, these bottles can be "seasoned" by repeated use for monolayer cultivation and, after cultures have been grown to confluency in them 5 or 6 times, some subtle change in the glass occurs which enhances the differential attachment properties of interphase and mitotic cells and makes selective detachment practical. Most of the cell lines employed have been found to attach completely within about 2 hours so that cells can be seeded at final density several hours before harvest. However, we find it convenient to seed at approximately half the final density in the evening for harvest the following morning.

2. The medium used for harvesting cells is adjusted to approximately pH 6.8 with CO_2 to compensate for degassing during agitation and decanting. This is most conveniently accomplished by introducing a piece of dry ice about the size of a pea prior to membrane sterilization of the complete medium in liter quantities. During harvest this rather acid medium becomes slightly more alkaline, and the initial pH of the selected population is generally quite satisfactory. If pH adjustment is omitted, cultures started too alkaline may fail to grow or may grow at a sharply reduced rate. For the standard Blake bottle, a volume of 25 ml provides the correct hydrodynamic shear for selectively detaching mitotic cells. Larger or smaller volumes, either because of excessive foaming or damping the oscillation, have proved less satisfactory. Immediately after agitation and decanting, a fresh 25-ml aliquot of the acid medium is replaced using care not to pour the fresh medium over the surface of the monolayer.

3. For reasons already discussed in detail, monolayers are initially unsuitable for mitotic selection because of loosely attached early G_1 cells, as well as some other floating cells and debris. These contaminants are stripped by a series of preliminary agitations, during which the supernatants are immediately monitored with the spectrometer. The harvest cycle, Δt should be shorter than τ_R if use of mitotic fraction as a measure of quality is desired. In the case of the CHO cell a 10-minute harvest interval has been most successful, with material from the first six detachment cycles being discarded. During the stripping process, the monolayers are shaken for 3 seconds. When the quality of the product ceases to improve with successive shakes (usually about cycle No. 6), the duration of agitation is reduced to 1 second. As shown in Fig. 6B, the yield per harvest rises over several hours, and each 10-minute cycle provides a new crop of highly phased cells—all late metaphases. The most de-

sirable condition is one in which the harvested material is used immediately and not artificially delayed for the purpose of pooling successive crops. In the absence of an enforced delay, there is no reason to suspect significant biochemical perturbation. However, if large numbers of cells are required without the age broadening which results from sequential harvesting, cells collected over several hours can be pooled by immediately chilling the supernatant from each cycle in an ice bath. Upon warming, cells held for long periods (8 hours for hamster cells) resume division without detectable delay, as evidenced by the comparable mitotic fraction curves for chilled and nonchilled cells (Fig. 9).

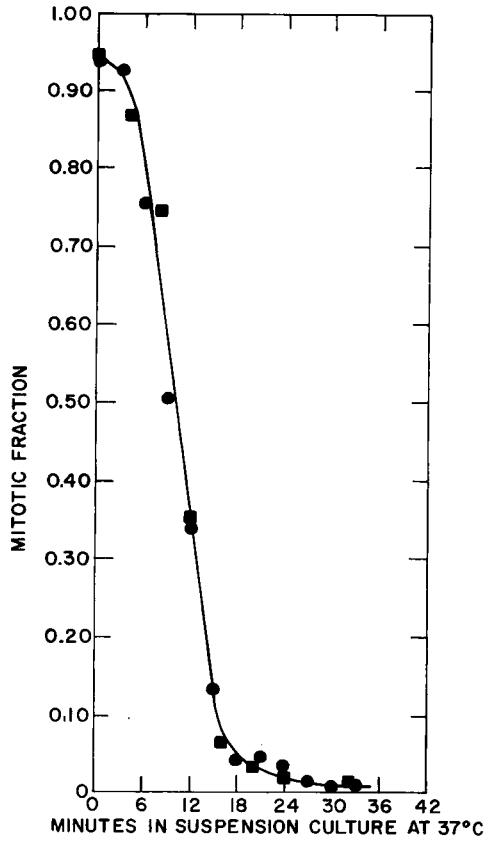


FIG. 9. Residual mitotic fraction as a function of time in suspension culture after selection from a monolayer. One collection (closed circles) was immediately placed in suspension culture and allowed to complete mitosis without delay. The other (squares) was immediately chilled in an ice bath, held 4 hours at 0°C, and then rapidly rewarmed to 37°C, at which time progress through mitosis resumed. The age width of the selected population (13 minutes) is the initial degree of synchrony achieved by the method.

Although this technique has proved useful in our hands, completion of mitosis in the cold has been reported (Buck *et al.*, 1967).

IV. Summary

Of the numerous methods available for inducing synchrony in mammalian cells, the most elegant in its simplicity is the Tolmach method of selective detachment of mitotic cells from a monolayer. Tightly phased populations of reasonable size can be produced at the beginning of the life cycle with virtually no delay and no apparent biochemical perturbation. The selection process follows the predictions of a simple model, the quality of the separation can be continuously monitored, and adjustments in several variable parameters can be made readily on the basis of volume spectrometry using a modification of the Coulter counter described in detail.

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