PHYSICAL STUDIES ON DNA FROM "PRIMITIVE" EUCARYOTES

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INTRODUCTION

Studies carried out over the years on bacteria and higher eucaryotes show several major differences between the two types of organisms in the nature of the DNA and chromosomes. Some of the differences are

1. The existence of a large percentage of interspersed, repetitive sequences in higher eucaryotes but not in bacteria.
2. The existence of chromatin containing large amounts of basic (histone) and nonhistone chromosomal proteins in higher eucaryotes but not in bacteria.
3. Bidirectional DNA replication from many initiation sites found in higher eucaryotes as compared to the single initiation site found in bacteria.

In light of these and other differences, eucaryotic microorganisms make interesting subject matter. Ultimately, studies on the nature of the DNA and chromosomes in these "primitive" eucaryotes may shed light on how these differences between bacteria and higher eucaryotes arose and how these differences may relate to the form and function of organisms (e.g., are repetitive sequences and histones necessary for differentiation). In much of the literature reviewed here, physical studies (especially DNA renaturation kinetics) play an important role. To understand this DNA literature, an understanding of the techniques and the pitfalls involved in using them is necessary. Therefore, we shall first discuss the major techniques used in physical studies of DNA. Techniques for the determination of molecular weights of very large chromosomal DNA molecules are discussed in greatest detail. While the number of molecular weight experiments on chromosomal DNAs is small, we believe that the determination of DNA size and form is an important and neglected area of research, one which we believe we are in an expert position to discuss. The remaining techniques are discussed only to the extent we deem sufficient for critical review.

MOLECULAR WEIGHTS OF CHROMOSOMAL DNA MOLECULES

Introduction

The size (molecular weight) and form (e.g., circular, linear, or branched) of chromosomal DNA molecules are important aspects of chromo-

some structure. Determining size and form of DNA in primitive eucaryotes permits us to answer the following questions: Are there one or many pieces of DNA in a single chromosome? What is the form of this DNA? Is it circular as in bacteria[1,2] or linear as is usually assumed for higher eucaryotes? Could some primitive eucaryotes have all their nuclear DNA in one circular piece as in bacteria, in contrast to the one or a few pieces of DNA per chromosome found in the higher eucaryote, *Drosophila*?[3] In what follows, the word DNA means linear, double-stranded DNA unless otherwise noted.

Experimental Problems Unique to Large DNA

Determining the molecular weight of chromosomal DNA molecules is a very difficult problem mainly because of the unusually large molecular weight and large root mean square radius of these DNAs. In Table 1 the molecular weights and contour lengths for DNA from several sources are listed and compared to typical synthetic polymers. It is seen that DNA molecules in general are much larger than synthetic polymers; those from primitive and higher eucaryotes can attain molecular weights in the billions and contour lengths of macroscopic dimensions if the DNA exists in almost chromosome-size pieces or larger.

Another factor making DNA unusual is its stiffness. To bend a DNA molecule 90° without strain requires a piece of DNA between 500 and 1,000 Å long. A typical synthetic polymer can bend 90° in 10 to 50 Å of its length. This high degree of stiffness makes small DNA molecules rodlike, but the large DNA molecules that we are concerned with here (10^7 to 10^{12} daltons) are long enough to be considered random coils. The "size" of a random coil is conveniently described by its root mean square (rms) radius, the radius which describes the volume that the random coil takes up in solution. This is illustrated schematically in Figure 1. It is seen that both the unusually large molecular weight and stiffness contribute to give DNA an extremely large hydrodynamic radius.

Most techniques for measuring molecular weights of polymer molecules were developed over the last 40 years or so, and most were developed for use on the smaller synthetic polymers and cannot be used easily or at all to obtain molecular weights of large chromosomal DNA. Among those which cannot be used at all and probably will remain unusable are end-group analysis, osmotic pressure, sedimentation-diffusion, equilibrium sedimentation, and light scattering. We will now briefly discuss why these techniques are inapplicable and then will discuss in detail the usable or potentially usable techniques of viscosity, viscoelasticity, sucrose sedimentation, autoradiography, and electron microscopy.

End-group analysis measures the number of DNA molecules. By knowing the number of DNA molecules and their concentration in grams per milliliter, one can then calculate molecular weight.

TABLE 1

Size of DNA from Various Sources

Source	Approximate molecular weight range (daltons)	Contour length[a] (cm)
Typical synthetic polymer	$10^4 - 10^5$	$5 \times 10^{-6} - 5 \times 10^{-5}$
Viral and bacteriophage chromosomal DNA	$10^6 - 10^8$	$5 \times 10^{-5} - 5 \times 10^{-3}$
Bacterial chromosomal DNA	$10^9 - 5 \times 10^9$	$5 \times 10^{-2} - 0.25$
Primitive eucaryote chromosomal DNA	$10^9 - 10^{11}$[b]	$5 \times 10^{-3} - 5.0$[a]
Higher eucaryote	$10^9 - 10^{12}$[b]	$5 \times 10^{-2} - 50.0$[b]

[a]Contour length is the actual length of the DNA molecule as traced along its helical axis; that is, the length of the molecule if it were a rigid rod. DNA values calculated assuming 1 μm = 2×10^6 daltons.
[b]Values calculated assume one piece of DNA per chromosome.

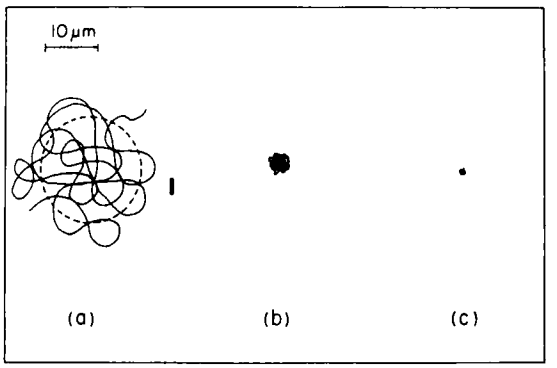

FIGURE 1. Schematic representation of random coils. (a) A *B. subtilis* chromosomal DNA random coil (M = 2 × 10^9 daltons) compared to the *B. subtilis* organism (black rectangle). The dashed line circle represents the sphere with radius equal to the rms radius, R_G. (b) A random coil of the same molecular weight as the *B. subtilis* DNA but with less stiffness, giving rise to a denser coil with smaller R_G. (c) A synthetic polymer random coil. It has such a small R_G that it appears here almost as a dot.

A simple calculation shows why end-group analysis is too insensitive. Suppose we have a solution of DNA molecules of molecular weight 5 × 10^9 daltons and at the high concentration of 1 mg/ml. The concentration of DNA molecule ends would be 4 × 10^{-10} mol/l, which is a very small concentration to detect chemically or with radioactive labels of typical specific activity.

Osmotic pressure is a colligative property and thus is proportional to the number of molecules in solution. Again, because solutions of very large DNA contain so few molecules, osmotic pressure is too insensitive to determine molecular weights. In fact, the maximum molecular weights measurable by osmotic pressure are about 10^6 daltons (Flory, p. 273).[4] For both end-group analysis and osmotic pressure, it is the high molecular weight of large DNA which leads to small numbers of molecules in solution.

The method of sedimentation-diffusion requires, among other things, the measurement of the diffusion coefficient, D. The rms distance a molecule diffuses in a given direction, $\langle X^2 \rangle^{1/2}$, in a time t, is given by the relationship:

$$\langle X^2 \rangle^{1/2} = \sqrt{2Dt} \qquad (1)$$

The diffusion coefficient is related to molecular size for spherical molecules by the Stokes-Einstein relationship:

$$D = \frac{kT}{6\pi\eta_0 R} \qquad (2)$$

where

- k = the Boltzmann constant (= 1.38 × 10^{-16} erg/deg);
- T = absolute temperature;
- η_0 = the solvent viscosity in poises;
- R = the radius of the sphere.

For the spherically symmetric, random coil molecules, the appropriate radius to use in this equation is the hydrodynamic radius, R_e, which may be approximately equated to the rms radius, R_G:

$$R_G \simeq R_e \qquad (3)$$

For DNA assuming a persistence length of 750 Å (the distance along the backbone of a DNA molecule necessary to bend the molecule 90° with no strain), R_G in centimeters is related approximately to molecular weight M (in daltons) by:

$$R_G = 1 \times 10^{-8} M^{1/2} \qquad (4)$$

For example, using Equations 1 through 4, we can calculate the time for a DNA molecule of molecular weight 5 × 10^9 daltons to diffuse 1 mm, a short, measurable distance. This time is 5,000 hr, a clearly impractical time. It is the unusually larger rms radius of DNA which causes such slow diffusion.

The equilibrium-sedimentation technique involves sedimenting polymer molecules slowly in a centrifugal field until the equilibrium distribution of polymer concentration at various positions in the centrifuge cell is reached. Equilibrium is reached when the flow due to the sedimenting polymer molecules is balanced exactly by the flow due to back diffusion as the concentration of polymer molecules builds up in the bottom of the cell. Analysis of the concentration vs. position-in-the-cell curve at equilibrium can yield molecular weight (higher molecular weight polymers give a steeper curve as they diffuse more slowly and sediment more). Details of this method may be found in Flory[4] (p. 307), Tanford[5] (p. 384), or Van Holde[6] (p. 110). The slow rate of attainment of equilibrium has always

been a major drawback with this technique. The time to reach equilibrium is inversely proportional to the diffusion coefficient,[7] and since the diffusion coefficient is roughly inversely proportional to the rms radius (Equation 2), the time to reach equilibrium is roughly proportional to the rms radius. For large DNA, which has a large rms radius, the time to reach equilibrium is impractically large. A crude calculation for DNA of 5×10^9 daltons using Equation 11 in Reference 7 yields time to reach equilibrium of 10^4 to 10^5 hr, a clearly impractical time.

Another technique, used widely by synthetic polymer chemists, is light scattering. In order to determine molecular weights, the intensity of light scattered at various angles must be extrapolated to zero angle. The larger the dimensions of the macromolecule (i.e., the rms radius in the case of DNA), the steeper the extrapolation to zero angle (see Tanford,[5] p. 275, or Van Holde,[6] p. 180, for details). In order to insure an accurate extrapolation, the light scattered must be measured at such small angles that the transmitted light coming from the incident beam (zero angle) interferes with the scattered beam. Thus, the problem for molecules of large rms radius is purely the technical one of how to measure the intensity of a scattered beam in the presence of a transmitted beam. Despite the problems, DNA molecular weights of 25×10^6 daltons have been measured successfully by light scattering.[8] The extension to very much larger DNA molecules seems unlikely.

It is unfortunate that none of the techniques described above can be used to determine molecular weights of DNA molecules in the 10^8 to 10^{11} dalton range, since all of those techniques yield *absolute* molecular weights. That is, they yield molecular weights without any assumptions regarding shape of the DNA molecules and without recourse to empirical formulas obtained by measuring a physical property (such as sedimentation coefficient) for DNAs of several already known molecular weights. The hydrodynamic techniques (intrinsic viscosity, viscoelasticity, and sucrose sedimentation) all rely on extrapolations of empirical formulas, where the long extrapolations might affect their accuracy, as will be seen.

The Problems of Mechanical Breakage and Aggregation

Before discussing in detail the usable and potentially usable techniques, it is appropriate here to discuss the important general problems of mechanical breakage and aggregation of DNA. Chromosomal DNA molecules, because of their stiffness, unusually large contour lengths (in the centimeters in some cases), and widths of molecular dimensions (the helix is only 20 Å diameter), are extremely fragile. The slightest shear stresses can break them. For example, the usual laboratory operations (pipetting, mixing, and pouring) used in isolating and handling DNA break it into pieces of molecular weights between 5 and 15×10^6 daltons; thus, a chromosomal DNA of molecular weight 5×10^9 daltons will be broken into several hundred pieces of roughly equal size by normal laboratory operations.

In order to measure molecular weights of chromosomal DNA molecules one must, of course, make sure that enough of the molecules are intact to make the measurement meaningful. To obtain pure DNA, the DNA is usually isolated from other cellular materials using biphasic solvent extractions (see DNA isolation section of this review). Using a phenol-water extraction procedure (with extreme care) on DNA from *E. coli* cells, Massie and Zimm[9] were able to isolate pure DNA of molecular weight 2.5×10^8 daltons. To avoid breakage they were always careful not to shake or pour solutions containing DNA. When solutions were to be transferred from one vessel to another, they were transferred using an inverted 10-cc pipette (wide mouth to reduce shear stresses) and were pipetted at rates of 1 ml/min or less, making sure that no liquid flowed down the sides of the vessels. Even with such care, there may have been breakage of most of the molecules since the actual molecular weight of the *E. coli* chromosome is about 2.5×10^9 daltons.[1,10] Thus, isolation of pure DNA with molecular weight greater than 10^9 daltons is a difficult, or nearly impossible, task.

Cell Lysis Procedures

All recent molecular weight measurements of chromosomal DNA, therefore, have been made on gently prepared whole-cell lysates. Since these lysates contain all of the other cellular components besides the DNA, care must be taken that these components do not influence the molecular weight measurements. For example, a highly positively charged protein molecule may interact with negatively charged DNA molecules, causing aggregation and giving anomalously high molecular weights. Also, one must worry about DNA degradation by nucleases in the cell lysates giving anomalously low molecular weights.

Examples of several lysis procedures are

presented in Table 2. The chemicals and enzymes are included in these procedures to prevent both DNA aggregation and degradation, and the mechanics of the procedures are designed to prevent (hopefully) mechanical breakage of DNA. For example, the high salt concentrations ($[Na^+] > 1\ M$), high temperatures ($T \geq 50°C$), high EDTA concentrations (about 0.25 M), and the use of proteases, detergents, urea, and guanidinium chloride are designed to deactivate, denature, and degrade nucleases and other enzymes and proteins. The high temperatures, high salt concentrations, and use of negatively charged detergents should help prevent electrostatic DNA-protein interactions and aggregations. Such aggregations are expected to occur more readily in higher eucaryote cell lysates where the amount of histone roughly equals the amount of DNA. Bacterial cells can be lysed and the DNA released, presumably unaggregated, in low salt concentrations using a nonionic detergent, Brij® 58 (see Table 2).

While it is not possible to completely rule out enzymatic or hydrolytic degradation, mechanical breakage, and aggregation, one can at least determine in a given set of experiments whether or not these possibilities are likely. Since one can reasonably expect that the amount of DNA aggregation might depend on DNA concentration, salt concentration, temperature, etc., these can be varied and molecular weight measurements made under the different conditions. If the same molecular weight is obtained, aggregation is then unlikely. One type of aggregation which might not be affected by changing any of these variables is entanglement of DNA molecules in the nucleus before the nucleus is lysed. Such physical entanglement may take days to disappear by diffusion of the coils (imagine two entangled bundles of string which can be disentangled only in a few ways). As a specific example of these kinds of checks, yeast DNA molecular weights appear independent of DNA concentration in both sedimentation[11] and viscoelasticity[13] and independent of salt concentration in viscoelasticity.[13]

At higher DNA concentrations (see Bloomfield[14] for example), all hydrodynamic measurements of molecular weight show concentration dependence because of interactions among DNA molecules. It is sometimes difficult to separate this "normal" concentration dependence from the effects of aggregation.

Enzymatic DNA degradation will most likely occur while the cells are being prepared for lysis or during a short time after lysis. It is assumed that at long times after lysis nucleases are inactivated by the detergents, proteases, etc. in the lysis mixture. By changing the procedure for preparing the cells for lysis, especially by lengthening the time of various steps in the procedure, smaller DNA should result if degradation is taking place in these steps. As a specific example, the molecular weight of yeast chromosomal DNA as determined by viscoelasticity does not change as the spheroplasting and incubation times in thioglycolate are varied.[13]

Hydrolytic degradation might occur when larger DNA is incubated for long times at high temperatures. To check for this, molecular weight measurements should be repeated at time intervals of over a few days. Most kinds of lysates do not show appreciable hydrolytic degradation.[3,10,13]

Mechanical breakage is probably the most difficult to check for and guard against. The working assumption is that chromosomal DNA is protected from mechanical breakage as long as it is kept in its condensed state in the cell or cell nucleus; therefore, intact cells (dead or alive) may be shaken, poured, pipetted, etc. without breaking the DNA. More or less harsh mechanical treatment of intact cells does not seem to affect DNA size, at least in nonfragile cells (for example, cells with walls as opposed to fragile spheroplasts). In any case, mechanical lysis from handling of cells should be checked in the microscope and DNA molecular weight measurements made under different handling conditions if possible.

The point at which mechanical breakage is most worrisome is at the exact moment of lysis. Chromosomal DNA molecules exist in the cell nucleus in a highly compacted state (a small rms radius) as compared to random coil DNA in solution. This compacted state may be likened to a large piece of spring wire stuffed into a small box. As the cell walls and nuclear membranes break during lysis and as the structures holding the DNA in its compact state (e.g., histones) lose their interactions with DNA, the DNA expands into its random coil form where the rms radius is often very much larger than the cell from which it came (see, for example, Figure 1a). This expansion may be very rapid and likened to the spring wire jumping out of the opened box. The shear stresses generated during this expansion may be enough to break the chromosomal DNA molecules. One can

TABLE 2

Some Typical Lysis Procedures[a] for Preparing Cell Lysates Containing Intact Chromosomal DNA

Bacteria[10]	*Drosophila*[3]	Yeast[11]	Algae[12]
Cells resuspended in BA buffer (contains boric acid, 0.195 *M* Na^+, 0.01 *M* EDTA, pH 8.3) and heated to 70°C for 15 min. Cells cooled to 0°C and lysed as follows: 1.0 ml cells in BA buffer in 2 mg/ml lysozome 2.0 ml of 2 mg/ml pronase in 0.195 *M* NaCl 2.0 ml of 10% Brij® 58 detergent in BA buffer The lysate is then incubated at 50° for 12 hr.	1.0 ml of freshly prepared chilled cell suspension is added to 1.8 ml of 1% sodium decyl sulfate detergent. After 20 min, the mixture is cooled to 50°C and 1.0 ml of freshly prepared HET-pronase added. HET-pronase contains 0.45 *M* EDTA, 1.8 *M* Na^+, 2 mg/ml pronase, pH 9.5. The lysate is then incubated at 50°C for at least 4 hr.	After an extensive spheroplasting procedure to destroy the cell wall, a small volume of cells is layered on a sucrose gradient containing 1% Sarcosyl® detergent, 0.01 *M* EDTA, 1 *M* NaCl, pH 8 at room temperature and incubated for 15 min.	Cells suspended in 1 ml HET kept on ice. Prepare solution of 0.5 ml 16 2/3% (w/w) Sarcosyl®, 3.0 ml 6 *M* guanidinium chloride, and 2.25 g solid urea and cool on ice. Pipette cells (1 ml) into urea, detergent, guanidinium chloride solution; cover with parafilm; and invert quickly. Then pour total lysate into incubation chamber. Incubate 20 hr at 50°C.

[a]Exact details concerning these procedures may be found in the original references.

attempt to control for this type of breakage by slowing the actual lysis, but it is difficult to quantitate what one means by "slowing" the lysis; furthermore, slower lysis may result in nuclease degradation of the DNA. How much, if any, DNA is mechanically broken during lysis is not known; however, it appears that not all the DNA is broken, at least in bacteria, as indicated by molecular weight measurements.[10,15,16] Thus, the possibility of aggregation and mechanical, hydrolytic, and enzymatic breakage must be controlled and kept in mind when making measurements on cell lysates.

Viscometry

The measurement of viscosity of DNA solutions is perhaps the quickest and cheapest way (in terms of required instrumentation) to obtain molecular weights. The quantity which may be related to molecular weight is the intrinsic viscosity, denoted by the symbol $[\eta]$. The intrinsic viscosity may be related to the solvent and solution (solvent + DNA) viscosities as follows. We first define the relative viscosity, η_{rel}, and the specific viscosity, η_{sp} as:

$$\eta_{rel} = \frac{\eta}{\eta_0} \tag{5}$$

and

$$\eta_{sp} = \frac{\eta}{\eta_0} - 1 \tag{6}$$

where

η_0 = the solvent viscosity;
η = the solution viscosity.

The intrinsic viscosity $[\eta]$ is defined as:

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} \tag{7}$$

where

c = the concentration of DNA.

An equivalent definition of intrinsic viscosity is

$$[\eta] = \lim_{c \to 0} \frac{\ln \eta_{rel}}{c} \tag{8}$$

Thus, the procedure for determining intrinsic viscosity is to measure the solution viscosity (η) at several different DNA concentrations, plot $\eta_{sp/c}$ or $\ln\eta_{rel/c}$ on the ordinate axis vs. DNA concentration (c) on the abscissa, then extrapolate the data (which should fall on the straight line at low DNA concentrations) to zero DNA concentration. Then, the intercept on the ordinate axis is the intrinsic viscosity. Such plots are shown in Figure 2 for T2 bacteriophage DNA, a DNA of about 1.3 $\times$ 10^8 daltons molecular weight. Since DNA concentrations are usually expressed in grams per milliliter or grams per deciliter (dl), intrinsic viscosity has the units milliliters per gram or deciliters per gram. In Figure 2 the intrinsic viscosity is about 320 dl/g.

Theory (see Flory,[4] p. 310; Van Holde,[5] p. 141; or Tanford,[6] p. 390) and experiment show that intrinsic viscosity may be related to molecular weight for random coil polymers by a formula of the form:

$$[\eta] = K M^a \tag{9}$$

where

M = molecular weight;
K = a constant;
a = a constant.

For DNA, experiments show that the formula takes on a slightly different form (because short DNA becomes rodlike):

$$[\eta] + b = K M^a \tag{10}$$

where

b = a constant.

Measuring intrinsic viscosities on several DNAs of known molecular weight, Crothers and Zimm[17] have found the empirical formula:

$$[\eta] + 5 = 1.371 \times 10^{-3} M^{0.665} \tag{11}$$

Other investigators obtain slightly different formulas. For example, Leighton and Rubenstein[18] obtain:

$$[\eta] + 5 = 1.683 \times 10^{-3} M^{0.653} \tag{12}$$

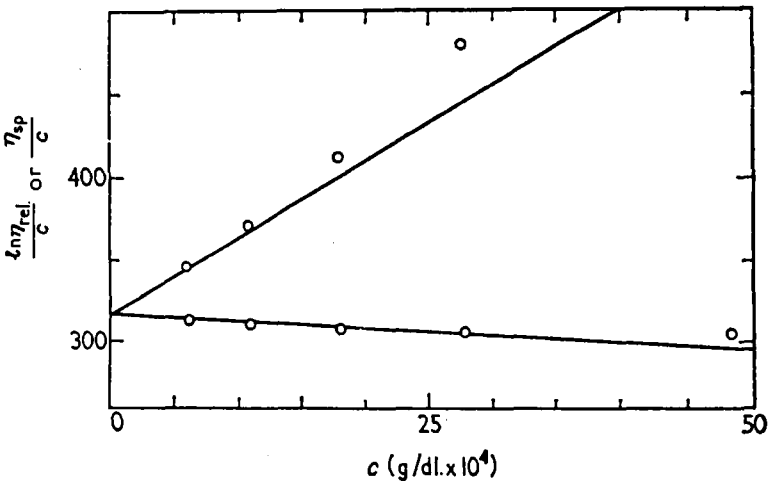

FIGURE 2. Ratio of specific viscosity, η_{sp} (upper plot), or logarithm of relative viscosity, η_{rel} (lower plot), of T2 DNA to concentration, c, plotted against concentration. The viscosities were measured at a shear stress of 0.0011 dyn/cm², a low enough shear stress so that non-Newtonian effects are not seen. (From Crothers, D. M. and Zimm, B. H., *J. Mol. Biol.*, 12, 525, 1965. With permission.)

Both formulas require that intrinsic viscosities be expressed in deciliters per gram. These formulas were determined using DNA of molecular weights between 2×10^5 to 1.2×10^8 daltons, and both are for DNA under the conditions of 0.2 *M* Na^+, 20 to 25°C, and pH 7.

The intrinsic viscosity of a DNA solution depends only slightly on cation concentration and does not depend on temperature, at least in the range where the double-stranded helix is stable.[19] Both these empirical formulas can be expected to give similar, accurate molecular weights in the 10^5 to 10^8 dalton range. For example, Equations 11 and 12 give $M = 6.0 \times 10^7$ daltons and $M = 6.1 \times 10^7$ daltons, respectively, for a DNA of $[\eta] = 200$ dl/g, about 2% difference. However, for a very large DNA with $[\eta] = 5{,}000$ dl/g, the molecular weights calculated from Equations 11 and 12 are $M = 7.4 \times 10^9$ daltons and $M = 8.2 \times 10^9$ daltons, respectively, for a difference of 11%, actually a better than expected agreement. This increase in percent difference is the result of "extrapolating" these empirical formulas to a molecular weight range far removed from that in which they were determined. This error, coupled with possible large errors in determining $[\eta]$ for very large DNA, can lead to large errors in estimates of molecular weights. For example, a 25% error in $[\eta]$ produces a 38% error in M due to the small power dependence ($a = 0.665$) of $[\eta]$ on M. For a discussion of the general problem of determining absolute DNA molecular weights in the 10^5 to 10^8 dalton range for use in developing empirical formulas from these data, see Freifelder.[20]

Perhaps the major problem in determining DNA molecular weights by viscometry is that solutions of DNA exhibit non-Newtonian behavior; that is, the viscosities of these solutions vary with the applied shear stress or shear rates (velocity gradient).* Specifically, the viscosity (and hence the specific viscosity and intrinsic viscosity) of DNA solutions decreases as the applied shear stress in increased. This is illustrated in Figure 3 for T2 DNA. The inset of the figure shows that at low enough shear stresses the viscosity remains constant with shear stress.

The physical reason for this non-Newtonian behavior is illustrated schematically in Figure 4. In part a of the figure, a naturally spherically symmetric random coil DNA is shown. In the presence of small velocity gradients (shear rates), illustrated in part b, the random coil retains its

Shear stress, S, is defined as the shearing force per unit area of solution. This shearing force (being unequal on different parts of the solution by definition) causes different parts of the solution to move at different rates. The shear rate, g, or velocity gradient is a measure of the difference in the rates of movement of different parts of the solution. For Newtonian liquids shear stress is always proportional to shear rate, the coefficient of proportionality being the viscosity, η; that is, $S = \eta g$. for a more detailed discussion, see Van Holde (p. 141).

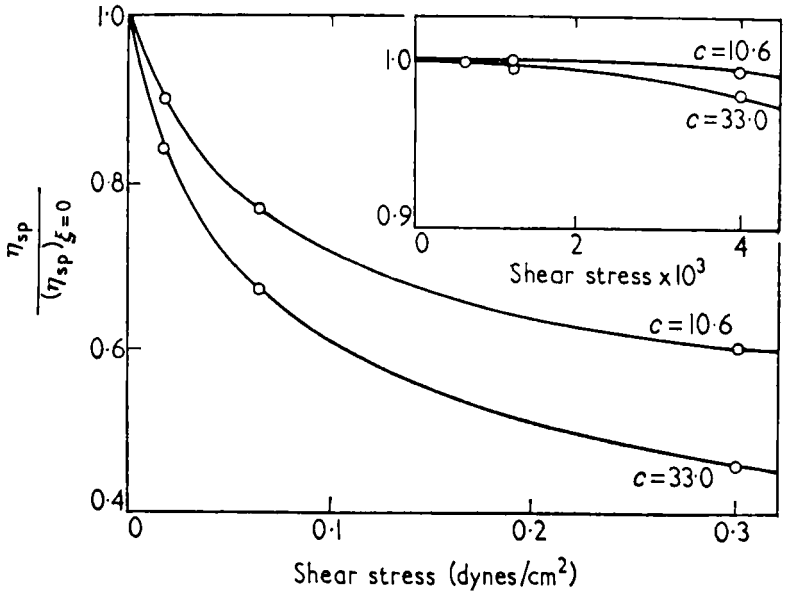

FIGURE 3. Dependence on shear of the specific viscosity, η_{sp}, of T2 DNA, expressed as the percentage of the value at zero shear. Curves for two DNA concentrations, c, are shown. Concentrations are expressed in milligrams per milliliter. (From Crothers, D. M. and Zimm, B. H., *J. Mol. Biol.*, 12, 525, 1965. With permission.)

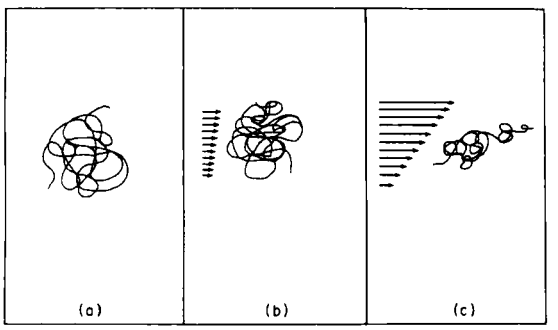

FIGURE 4. Schematic representation of the effect of high-velocity gradients on the configuration of random coil DNA molecules. The length and direction of the arrows represent the magnitude and direction of the solvent velocity.

natural, spherical symmetry. Higher velocity gradients distort the random coil, as illustrated in part c. To relate this phenomenon to viscosity in crude terms, the increase in viscosity of DNA solutions over the viscosity of the solvent alone is due to the increased frictional resistance on the solvent molecules flowing by the DNA. The distorted DNA molecules present less "surface area" to the oncoming solvent molecules; thus, the frictional resistance and viscosity are reduced. Because larger DNAs experience a greater difference in solvent velocities, distorting them more, and because they distort more easily, one might expect that solutions of large DNA molecules will show more pronounced non-Newtonian behavior. This is indeed the case, as is illustrated in Figure 5 where experimental results for T7 DNA ($M = 25 \times 10^6$ daltons) and T2 DNA ($M = 1.2 \times 10^8$ daltons) are shown. Also shown in the figure is a hypothetical curve for a DNA of $M = 5 \times 10^9$ daltons.

In order to determine molecular weights, the intrinsic viscosities of undistorted molecules (zero shear rate values) must be found in order to use empirical formulas. For technical reasons, it is difficult to measure viscosities at shear rates below 1 sec^{-1} in Couette, Cartesian-diver viscometers.[10,21] At this lowest shear rate the intrinsic viscosity of DNA ($M = 5 \times 10^9$ daltons) would be $[\eta]_g \sim 2{,}320$ dl/g, compared to a zero shear rate value of $[\eta]_o \simeq 3{,}860$ dl/g from Equation 11, yielding the anomalously low $M = 2.3 \times 10^9$ daltons, using Equation 11.

It may be possible, however, to correct for non-Newtonian effects. Several theoretical results (see Bloomfield[14] for references) predict that intrinsic viscosity should vary with shear rate as:

$$[\eta]_g = [\eta]_0 (1 - \alpha\tau_1^2 g^2 + \ldots) \quad (13)$$

where

$[\eta]_g$ = the intrinsic viscosity at some shear rate g;
$[\eta]_0$ = the intrinsic viscosity at zero shear rate;
α = a constant;
τ_1 = the longest relaxation time of the DNA of molecular weight, M.

The exact meaning of τ_1 is deferred to the next section. For the present all we need to know is that τ_1 is a strong function of molecular weight (for example, $\tau_1 \alpha M^{1.665}$ in 0.195 *M* Na^+) and can be calculated if M is known. Examination of Equation 13 reveals that when $[\eta]_g/[\eta]_0$ is plotted against $g\tau_1$ for DNAs of different molecular weight, instead of g alone (as in Figure 5) all the data should fall on the same "master" curve. Such a plot is shown in Figure 6 for T2 DNA (M = 1.2×10^8 daltons and τ_1 = 0.57 sec) and T7 DNA (M = 2.5×10^7 daltons and τ_1 = 0.046 sec). While the amount of data available for making this figure is small, the two DNAs do fall almost on the same curve compared to Figure 5. If this master curve idea is correct, there is a way to obtain molecular weights by obtaining $[\eta]_g$ at several shear rates, then trying several τ_1 values until a best fit with the master curve is obtained. From this best fit τ_1 value, molecular weight can be calculated from the known relationship between τ_1 and M. This method assumes that the DNA of unknown molecular weight is homogeneous in size.

To determine whether this method is practical or sensitive enough requires many experiments on DNAs of known molecular weight to obtain a good master curve and experiments on very large DNA (M > 5×10^8 daltons) of known molecular weight to see how well the method works. This work remains to be done. At present, viscoelasticity appears to be the method of choice for determining molecular weights of DNA with M > 5×10^8 daltons. Since viscoelasticity is practically the only game in town, it is necessary that results from viscoelastic experiments be corroborated by other techniques. Measurement of intrinsic viscosity is possibly one such technique.

Measurement of viscosities at several low shear rates requires special instrumentation. Usually viscosities are measured in capillary viscometers in which the time required for the solution to fall through a capillary tube is measured. Under the appropriate conditions this time is directly proportional to the viscosity of the solution (see Van Holde,[6] p. 174, for details). For large DNA, these viscometers are not practical because shear rates cannot easily be varied, except by using

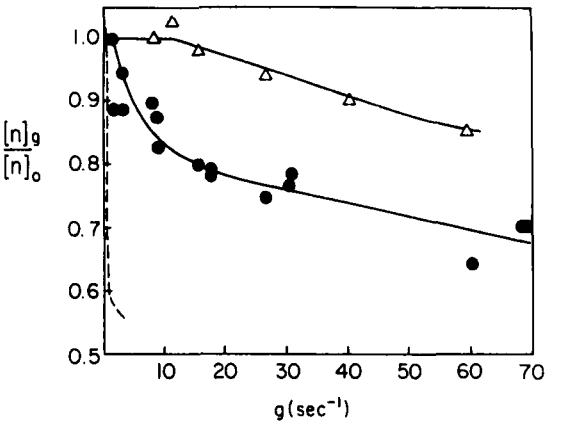

FIGURE 5. Intrinsic viscosity of DNA at various shear rates. The ordinate is intrinsic viscosity, $[\eta]_g$, at shear rate, g, normalized by dividing it by its zero shear rate value, $[\eta]_0$. The shear rates, g, have all been arbitrarily normalized to water at 25°C. (Δ) One experiment on T7 DNA (M = 25×10^6 daltons). (•) Three experiments on T2 DNA (M = 1.2×10^8 daltons). (– – –) Expected plot for a DNA of M = 5×10^9 daltons. (From Klotz, unpublished results.)

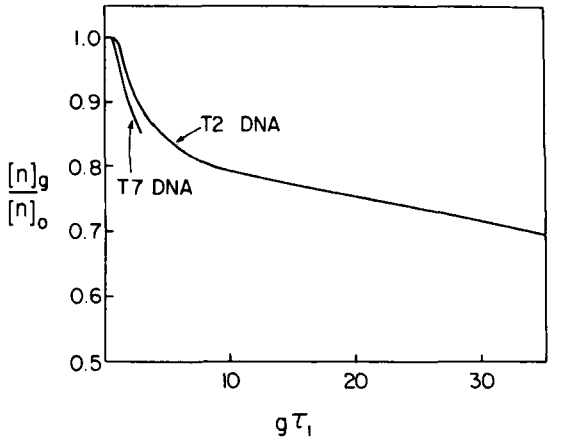

FIGURE 6. Intrinsic viscosities of T7 and T2 DNA plotted against shear rate times relaxation time. The data used in constructing this plot were taken from the solid lines, not the points, in Figure 5. (From Klotz, unpublished results.)

different viscometers with different size capillaries, etc. Typical shear rates in a capillary viscometer are in the neighborhood of 10^2 to 10^3 sec^{-1}. For large DNA, one would like to work in the shear rate range of 0.1 to 100 sec^{-1}. Couette or concentric cylinder viscometers can achieve these low shear rates. In such viscometers the rate of turning of a cylinder or rotor is used to measure viscosity. One such viscometer, which is capable of precise viscosity measurements (0.5% precision) is the Zimm and Crothers[22] viscometer in which the inner rotating cylinder is centered using the surface tension of the solution. This viscometer is quite acceptable for pure DNA solutions, but will not work on cell lysates because in lysates a protein film develops at the surface of the solution, which tends to jam the inner rotor so it will not turn.

For work on cell lysates, a Cartesian-diver, Couette viscometer, in which the inner cylinder (rotor) is completely submerged in the solution was developed by Gill and Thompson.[21] A schematic diagram of an improved version of that viscometer is shown in Figure 7. The rotor is an inverted Kel-F plastic tube, open at the bottom and closed at the top. An air bubble will be trapped in the top of such a rotor when it is placed in solution. By applying pressure to the top of the chamber which holds the solution and rotor, the size of the air bubble is reduced, thus making the overall density of the rotor greater, so it will begin to sink. By adjusting the pressure, the rotor can be made to stay suspended in the solution.

In order to keep the rotor suspended at the same height throughout an experiment, a servo mechanism is used. This servo consists of a light beam directed at the bottom of the rotor by a light pipe. With the rotor at the correct height, half the light beam is stopped by the rotor and the other half passes under the rotor to the other side of the sample chamber, where it is incident on the photocell (or photoresistor). As the rotor drifts up or down, more or less light is incident on the photocell. The photocell is connected to the input

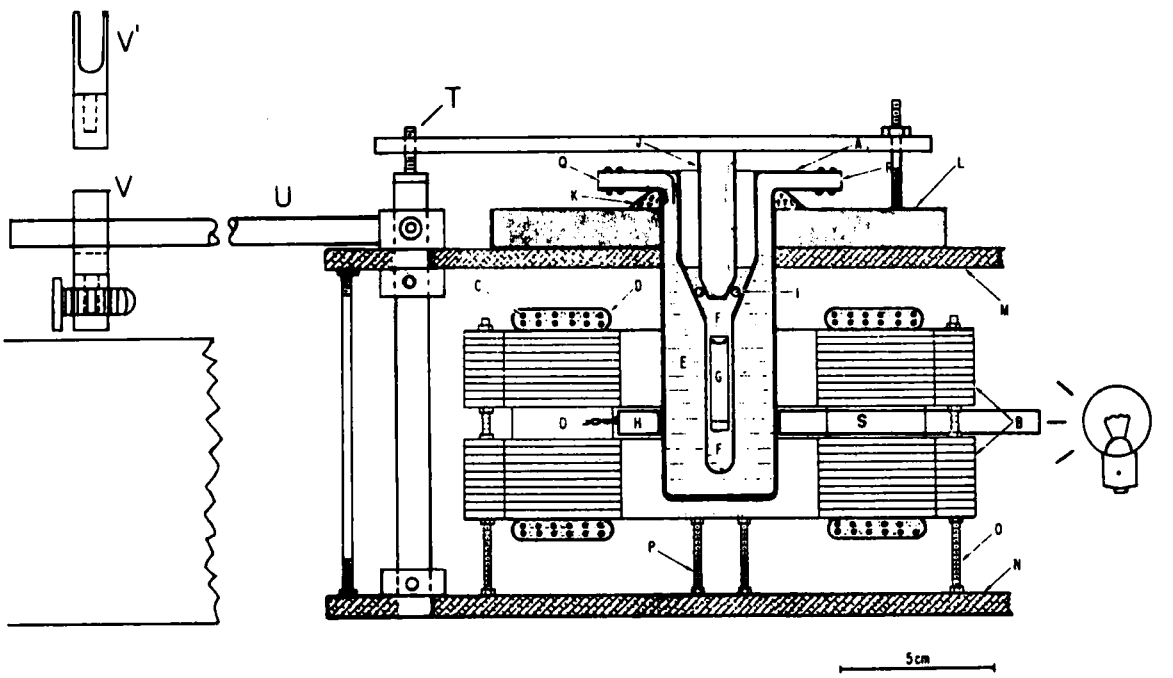

FIGURE 7. Schematic diagram of a Cartesian-diver viscometer. The viscometer consists of a glass sample chamber (F) surrounded by thermostatted water jacket (E) and magnet coils (B). It contains a rotor (G). The rotor is a Cartesian diver kept vertically centered in the chamber by a system which varies the pressure on vertical bar J and its O-ring gasket (I). During use the rotor intercepts the upper half of a light beam emerging from light pipe S and striking photoresistor H, which is biased to control the input voltage to a Heathkit® recorder. Movement of the recorder arm in response to rotor height variations is transmitted to threaded shaft T via arm U, which rides in yoke V (front view V') designed to fit the recorder's penholder. Two opposing sets of magnet coils (D) driven by 60-Hz sine waves 120° out of phase are used to turn the rotor.

of an inexpensive strip chart recorder. The recorder pen arm will move as the electrical signal from the photocell varies with the light intensity. The pen arm is connected directly to the pressure mechanism, so that its movement changes the pressure exerted on the top of the chamber and hence keeps the rotor height constant.

On the inside bottom of the rotor is a metal ring (preferably silver or gold). This ring serves to stabilize the rotor by lowering its center of gravity and acts as a conductor for the eddy current drive which turns the rotor. The eddy current drive consists of two sets of alternating current electromagnets. One set generates an eddy current in the metal ring in the rotor; the field of the other set, which is 120° out of phase from that of the first set, interacts with the eddy current to produce a torque which turns the rotor. A description of such a rotor drive is given by Chapman et al.[23] Further details of construction of such an instrument may be found in References 10 and 21.

Using an instrument with the magnetic rotor drive described above, relative viscosities are found simply, from the time for one rotation of the rotor. Specifically,

$$\eta_{rel} = \frac{t_r}{t_{r_o}} \tag{14}$$

where

t_r = the time of rotation for the solvent;
t_{r_o} = the time of rotation of the solution.

There is one other phenomenon, dilatancy or rheopexy, which can occur when measuring viscosities of solutions of large DNA. The dilatant effect is opposite to the usual non-Newtonian effect; that is, the viscosity of the solution increases as the shear rate increases. This effect was first observed for DNA on cell lysates of *E. coli* by Thompson et al.[24] As the shear rate is jumped from a low value or zero to a higher value, the viscosity remains constant for a short while, then rises rapidly, and finally levels off at a higher constant value. This is illustrated in Figure 8. The dilatant effect becomes more pronounced as the DNA size, DNA concentration, and the shear stress are increased. Viscosity changes of more than tenfold have been observed on some bacterial cell lysates.[25]

The physical reason for dilatancy is unknown. It is presumed that it is shear-dependent aggregation of DNA due to molecules moving at different speeds in the viscometer (because their centers of mass are at different distances from the rotor and hence are in regions of different solvent velocity) bumping into each other and aggregating either due to entanglement or some protein-mediated binding of the DNAs.

To the best of our knowledge, dilatancy has been observed only in cell lysates containing DNA of $M > 10^9$ daltons. Thus far, it has been possible to avoid dilatancy by reducing DNA concentration and using small shear rates, at least for DNA in the 10^9 dalton molecular weight range. Dilatancy seems to be present in some lysis mixtures and not others, indicating that proper choice of lysis procedures may avoid the problem. More experimental study of dilatant effects appears necessary. It would also be desirable to have a theoretical explanation of this phenomenon.

The discussion thus far has dealt only with linear, double-stranded DNA. Some chromosomal DNA molecules of primitive eucaryotes may indeed be circular, as in bacteria.[1,2] At the same molecular weight, a solution of circular DNA molecules will have considerably less intrinsic viscosity than linear DNA molecules. Theory[26] predicts that $[\eta]_{linear}/[\eta]_{circular} = 1.55$ for the same molecular weight. Experiments on the linear and circular forms of λ DNA[27] yield $[\eta]_{linear}/[\eta]_{circular} = 1.6$, in good agreement with theory.

The problem here is that *a priori* one usually does not know if the chromosomal DNA is circular or linear, and if the wrong form is assumed, a large error in calculated molecular weight will result. For example, an intrinsic viscosity of 2,490 dl/g will yield a molecular weight of 2.6×10^9 daltons, using Equation 11, if linear DNA is assumed. However, if the DNA is really circular, its true molecular weight is 5×10^9 daltons. Conversely, if the molecular weight is known, intrinsic viscosity might be used to establish form.

The size of the single-stranded DNA in chromosomes is also of interest. Intrinsic viscosities of single-stranded DNA as a function of cation concentration have been studied by Rosenberg and Studier.[28] At high cation concentrations, intrinsic viscosities are very low, since the flexible single strand can collapse into small hydrodynamic volumes because the cations reduce

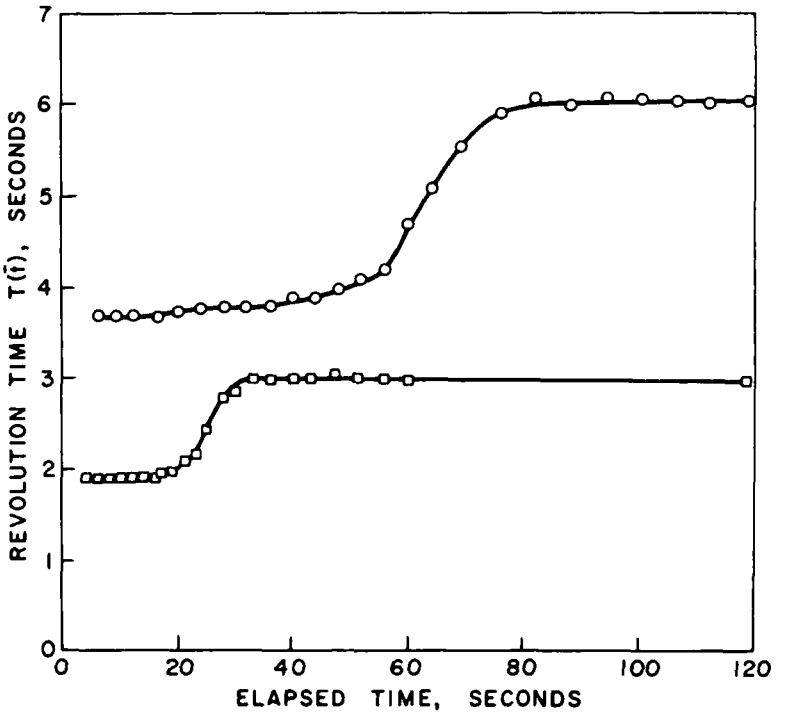

FIGURE 8. Rotor revolution time T(t) (proportional to viscosity) vs. elapsed time after shear rate jump for an *E. coli* cell lysate. (•) 25-fold shear rate jump. (□) 50-fold shear rate jump. (From Thompson, D. S., Hays, J. B., and Gill, S. J., *Biopolymers*, 7, 571, 1969. With permission.)

electrostatic repulsions among phosphate groups; therefore, it should also be possible to eliminate non-Newtonian effects under these conditions. In low cation concentrations, the intrinsic viscosities of single strands can even be greater than those for double strands. Thus, it should be possible to pick cation concentrations to make measurements convenient and possibly free from non-Newtonian and dilatant effects.

Lysates of single-stranded DNA may be prepared using NaOH to denature the DNA. Details of lysate preparation may be found in Uhlenhopp and Zimm.[29] A major problem in working with such lysates is the possible existence of breaks in the single strands due to nuclease activity in the preparation of lysates. Thus, it may be impossible to identify "naturally occurring" single-strand breaks from those caused by nucleases. It takes many random, single-strand breaks to cause a double-strand break, so this may not be a severe problem when studying double-stranded DNA.

In conclusion, while viscometry has not yet been used successfully to obtain DNA molecular weights much greater than 10^8 daltons, with careful study of non-Newtonian and dilatant effects it may be possible to extend the useful range of viscometry into the 10^9 dalton range. Since the onset of non-Newtonian effects appears to depend on relaxation time (and hence strongly on molecular weight), studying the shear rate dependence of intrinsic viscosity may provide a fairly sensitive way of obtaining molecular weight. The chief advantages of viscometry are that the instrumentation is inexpensive and measurements can generally be made quickly. The chief disadvantage is that for a solution of DNA heterogeneous in size only an average molecular weight is obtained, not a distribution of molecular weights. In cell lysates, where some of the molecules may be mechanically or enzymatically broken, this low average molecular weight will not at all reflect the true chromosomal DNA size if breakage is widespread.

Viscoelasticity

Measurement of DNA viscoelasticity has already been used to obtain chromosomal DNA

molecular weights greater than 10^{10} daltons.[3] Because of the relative ease with which the viscoelastic technique can be applied, especially to DNA in the 10^9 dalton range, and because viscoelasticity does not seem to show anomalous behavior as sedimentation and viscosity do, it appears at present to be the technique of choice for determining chromosomal DNA molecular weights. First, a qualitative description of the technique is given, and then a more quantitative discussion of the technique and results of experiments are discussed.

The viscoelastic technique measures the time of relaxation of DNA molecules back to a random coil configuration, after the molecules have been previously distorted by a solvent velocity gradient. The heart of the instrument is a concentric cylinder, Cartesian-diver viscometer (similar to that pictured in Figure 7). In such a viscometer there is a practically linear solvent velocity gradient, with the highest solvent velocity (equal to the tangential velocity of the rotor) at the rotor and zero solvent velocity at the outer cylinder (shown schematically in Figure 9a). In such a velocity gradient, large DNA molecules are distorted (Figure 9c) from their normal spherically symmetric (Figure 9b) random coil configuration.

In a typical viscoelastic experiment the rotor is made to turn for a period of time, and then the power driving the rotor (external magnetic fields) is shut off. The rotor then drifts in the same direction for a short time due to its inertia and the inertia of the surrounding solution. Then it begins to move in the *reverse direction*, where the rotor angle as a function of time, $\theta(t)$, decays as a single exponential at long times for a homogeneous DNA solution. This process is illustrated in Figure 10.

The reason the rotor motion decays exponentially in the reverse direction is that the distorted DNA molecules (Figure 9c) relax back to their statistically most probable, spherical, random coil configuration when the velocity gradient is removed. As they do this, they push the solvent in the opposite direction from the applied velocity gradient, and the solvent in turn pushes the rotor in the reverse direction. This process is illustrated in Figure 9d.

Rotor angle vs. time data obtained from these experiments may be used to obtain DNA molecular weight as follows. A single exponential decay can be represented by an expression of the form:

$$\theta(t) \propto e^{-t/\tau}$$

where

- t = the time after the magnet power has been turned off;
- τ = a measure of the time for exponential decay.

Larger τ values mean slower exponential decay. In the language of rheologists, such an experiment is

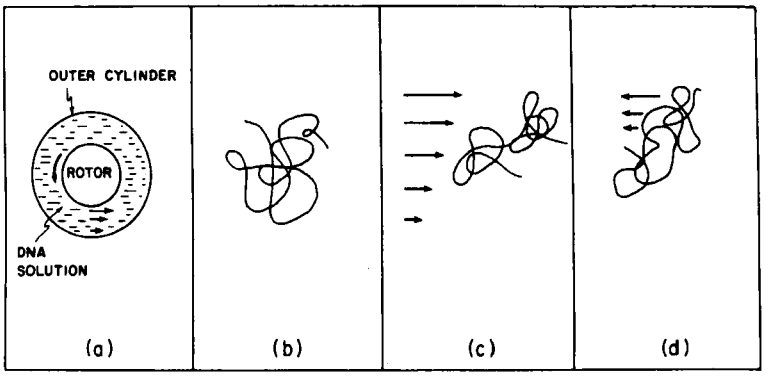

FIGURE 9. Schematic representation of viscoelasticity. (a) Top view of the Couette viscometer. The straight-shafted arrows represent the velocity gradient. The curved arrow represents the direction of motion of the rotor. (b) Schematic drawing of an undistorted, spherically symmetric random coil DNA molecule. (c) The same DNA molecule in a linear velocity gradient. (d) The DNA molecule, after the velocity gradient has been removed, relaxing back to its undistorted configuration.

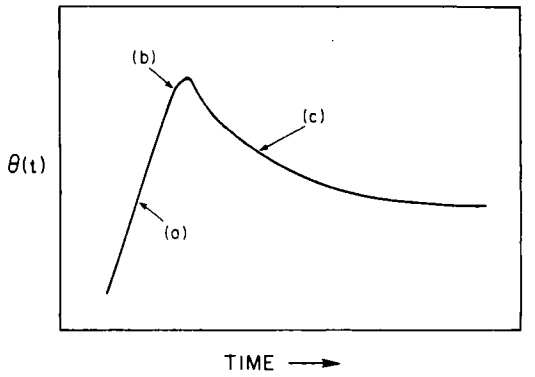

FIGURE 10. A schematic representation of a relaxation curve. (a) Rotor turning at a constant velocity, so that rotor angle, $\theta(t)$, varies linearly with time. (b) Power driving the rotor turned off at this point. (c) Exponential decay of rotor angle in the reverse direction due to relaxation of the largest DNA molecules.

called a creep-recovery experiment, and its characteristic exponential decay time, τ, is called a retardation time. When extrapolated to zero concentration, retardation times are equal to relaxation times, where the relaxation time is an intrinsic property of a random coil macromolecule which can be related to molecular size.

Larger DNA molecules cause slower exponential decays (large τ). Very qualitatively, larger DNA molecules are distorted more easily and to a greater extent. Thus, it will take a larger molecule more time to relax back to its random coil configuration. Theory[30,31] and experiment[17] show that τ may be related to molecular weight, M, by a formula of the form:

$$\tau = KM^{1.67} \qquad (15)$$

where

$K = 1.17 \times 10^{-14}$ for linear DNA at 50°C and in a buffer containing 0.195 *M* Na^+;
$K = 3.29 \times 10^{-15}$ for circular DNA at 50°C and in a buffer containing 0.195 *M* Na^+.

Thus, τ is a sensitive measure of molecular weight due to the 1.67 power dependence.

For solutions of DNA heterogeneous with respect to molecular weight, the viscoelastic technique measures mainly the higher molecular weights. The following illustrates this point. For a solution of DNA molecules where N_i is the number of molecules of molecular weight M_i, the rotor angle as a function of time is given by a formula of the form:

$$\theta(t) \propto \sum_i N_i \tau_i^2 e^{-t/\tau_i}$$

where

τ_i = the retardation time of the species of molecular weights M_i.

TABLE 3

Approximate Retardation Time and Intensity of Relaxation for Molecules of Molecular Weight M and M/2

Molecular weight	M	M/2
Retardation time	τ	$\tau/3$
Intensity	τ^2	$\tau^2/9$

Take the simple example where there are only two molecular weight species in solution, one of molecular weight M and the other with molecular weight M/2. Assume they are present in equal number so the N_i are equal and we can neglect them. Let τ be the retardation time of molecules of molecular weight M. Table 3 illustrates the "intensity" contribution of the two species to the relaxation curve, $\theta(t)$. Thus, not only do the half-sized molecules relax about three times as fast, they also contribute only 1/9 the total intensity of the large molecules, so that almost all of the relaxation curve will be due to the larger DNA.

To measure retardation time all one needs is a method for measuring the angle turned by the Cartesian-diver rotor as a function of time. How this is accomplished, in one type of instrument, is shown (schematically) in Figure 11. Details concerning construction of the instrument may be found in References 23 and 31. An example of the recorder output is given in Figure 12a.

The above description of viscoelasticity is oversimplified, mainly because a random-coil molecule actually has many relaxation times.[30] These many relaxation times, τ_k, are given by a formula of the form:

$$\tau_k = \alpha_k M \eta |\eta| / RT \qquad (16)$$

where

M = the molecular weight of the molecule;

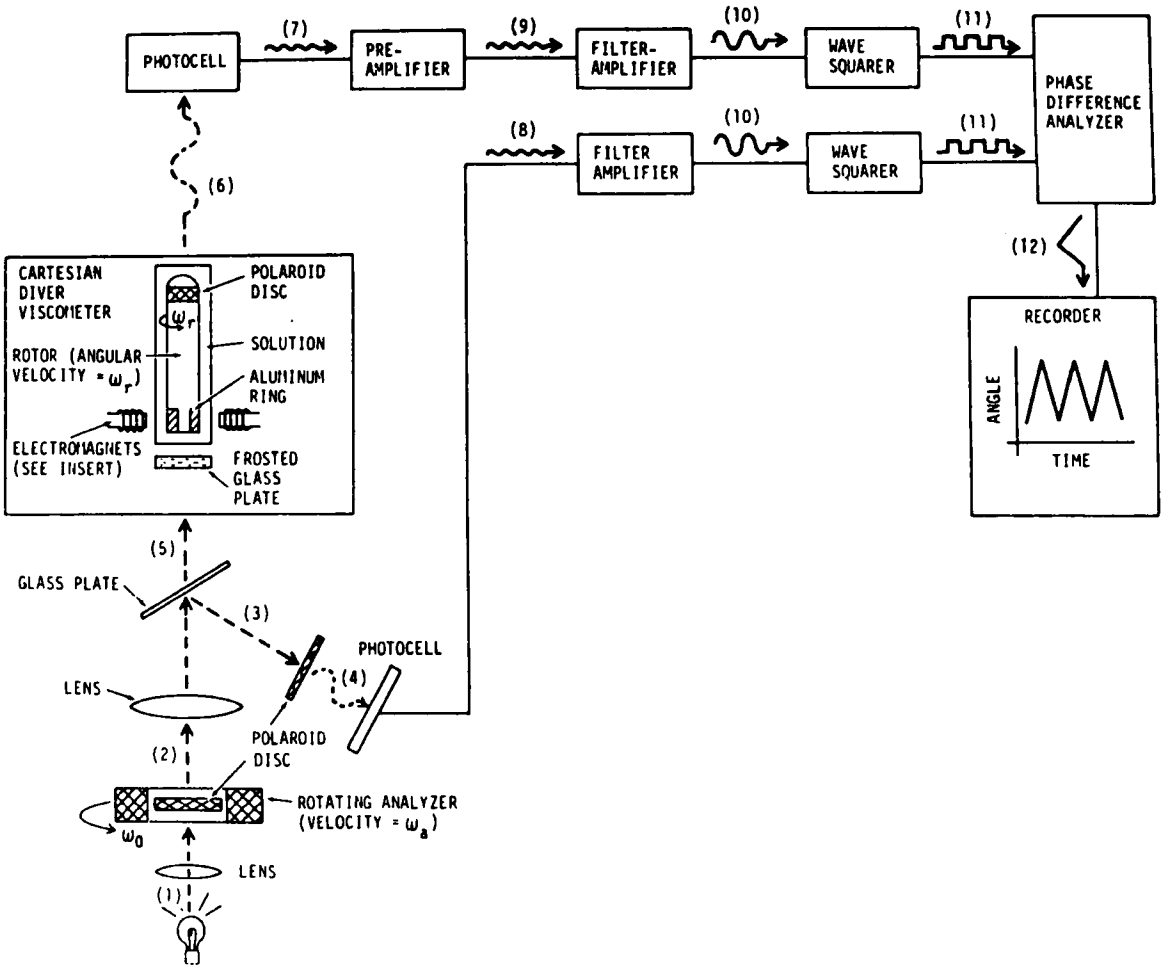

FIGURE 11. Schematic diagram of apparatus for measuring DNA retardation times. Dashed arrows represent light; solid arrows represent electrical voltage. The shapes of the arrow shafts show either intensity or voltage variation with time. All signal shapes refer to a rotor moving with constant angular velocity. The numbers in parentheses refer to the description in the text. Unpolarized white light (1) passes through a rotating polarizer (angular velocity ω_a), and the now polarized light (2) is focused on a glass plate where 15% of the light is reflected (3) through a stationary Polaroid® disk, so that the light which reaches the photocell (4) has an intensity varying sinusoidally in time with angular frequency, ω_a. The normal to the glass plate is at an angle of less than 10° from the incident light beam in order to minimize polarization during reflection. The rest of the light (about 85%) passes through the glass plate (5) and then through a frosted glass to diffuse the light so that the light impinging on the Cartesian-diver rotor is always even in intensity. For purposes of this description, assume the rotor to be moving with constant angular velocity, ω_r. (The rotor is made to turn through interaction of eddy currents in the aluminum ring with a pair of alternating current electromagnets.) The light leaving the Polaroid disk in the rotor (6) has intensity varying sinusoidally in time with frequency $\omega_a - \omega_r$, where $\omega_a > \omega_r$. Both light signals are converted into sinusoidally varying voltages (7 and 8) using photocells which are, in turn, amplified and filtered (9 and 10) to eliminate high- and low-frequency noise, squared off (11), and fed into a phase difference analyzer which converts the difference in phase:

$$\theta = \omega_a t - (\omega_a - \omega_r)t = \omega_r t$$

into a DC voltage (12). This voltage is fed into a strip-chart recorder to give rotor angle θ vs. time. (During a retardation-time experiment, of course, the output on the recorder will not be the triangular pattern shown in Figure 1, but will be an exponential-like decay curve.) Peak to peak on the sawtooth pattern corresponds to 90° of rotation of the rotor. More details concerning design of the instrument may be found in Reference 31. (From Klotz, L. C. and Zimm, B. H., *Macromolecules*, 5, 471, 1972. Copyright by the American Chemical Society.)

η = the solvent viscosity;
$[\eta]$ = the intrinsic viscosity of the macromolecule;
α_k = a constant that depends on the index k and also to some extent on the structure of the random coil.

For linear DNA, $\alpha_1 = 0.449$, $\alpha_2 = 0.139$, $\alpha_3 = 0.072, \ldots$ so that $\tau_2 = 0.310\ \tau_1$, $\tau_3 = 0.160\ \tau_1, \ldots$. In the limit of zero DNA concentrations, the angle turned by the rotor, $\theta(t)$, due to the relaxation of a homogeneous solution of DNA possessing all the relaxation times is given by:

$$\theta(t) = \frac{w_r \eta_{sp}}{\sum_n \tau_\eta} \left[\sum_{k=1}^{N} \tau_k^2 \exp\{-t/\tau_k\} \right] \quad (17)$$

where

η_{sp} = the specific viscosity;
w_r = the rotor speed *before* the magnetic drive is turned off.

Thus, the "intensity" of the relaxation of the kth mode is proportional to τ_k^2. Since $\tau_1^2 >> \tau_2^2 >> \tau_3^2$, Equation 17 says that most of the relaxation intensity (rotor recoil) should be in the longest normal mode. That this is indeed the case is evidenced by the practical linearity of the semilog plot of a typical DNA relaxation at long times, presented in Figure 12b. Only at very short times can the very small component associated with the faster retardation times be detected. Therefore, one "sees" only the longest retardation time. In what follows, when we talk of relaxation time or retardation time we mean the longest relaxation time or longest retardation time.

In a typical experiment, retardation times are measured on solutions of either purified DNA or cell lysates at several DNA concentrations. The values are then extrapolated to zero concentration to obtain the longest relaxation time (see Figure 13). Retardation times are obtained from the slopes of semilog plots as in Figure 12b or by integrating the area under the original, exponential relaxation curve.[32]

We will use the following symbols here: τ is the symbol for the raw retardation time values measured from the lysates, uncorrected for buffer viscosities and temperature. τ^0 is τ extrapolated to zero DNA concentration and is obtained from a τ vs. concentration plot. $\tau^0_{w,50}$ is τ^0 corrected to the standard conditions of 50°C and H_2O as the solvent by the formula:[3]

$$\tau^0_{w,50} = \frac{0.00549T}{323\eta_0}\ \tau^0 \quad (18)$$

In this formula, T is the absolute temperature, η_0 is the solvent viscosity, and 0.00549 is the viscosity of water in poises at 323° K.

From $\tau^0_{w,50}$ molecular weight can be obtained in the following way. Insertion of Equation 11 for intrinsic viscosity into Equation 16 for τ_1 yields:

$$\tau^0_{w,50} = 1.29 \times 10^{-14} M^{1.665} \quad (19)$$

at 50°C and 0.195 *M* Na^+ in H_2O for linear DNA.

Under circumstances where one can also measure intrinsic viscosity in the viscoelastometer (for example, with bacteriophage DNAs[10]), there are three ways of calculating molecular weights from the relaxation time and intrinsic viscosity data: (1) from the intrinsic viscosity alone using Equations 11 or 12, (2) from the relaxation time alone using Equation 19, and (3) from *both* the relaxation time and intrinsic viscosity using Equation 16. As shown in Table 4, all three methods give accurate molecular weights for T2 DNA (molecular weight about 1.2×10^8 daltons) and T7 DNA (molecular weight about 2.5×10^7 daltons). The data in this table indicate that relaxation time may be used successfully to obtain molecular weights. Also, Thompson and Gill[33] measured the longest relaxation time, τ_1, for T2 DNA using optical birefringence to follow the relaxation. The numerical value of molecular weight computed from the measured relaxation time agreed very well with that obtained in other ways. More recently, Callis and Davidson[34] using optical dicroism have measured the longest relaxation time of T4 DNA, T4 DNA half-molecules, λ_{b2b5c} DNA, and λ_{b2b5c} half-molecules at different temperatures and in solvents of varying viscosity. They have essentially verified Equation 16 for the dependence of relaxation time on molecular weight, solvent viscosity, and temperature.

The real interest in viscoelasticity, however, is for measuring DNA molecular weights greater than 10^9 daltons. As an example, results for DNA in *B.*

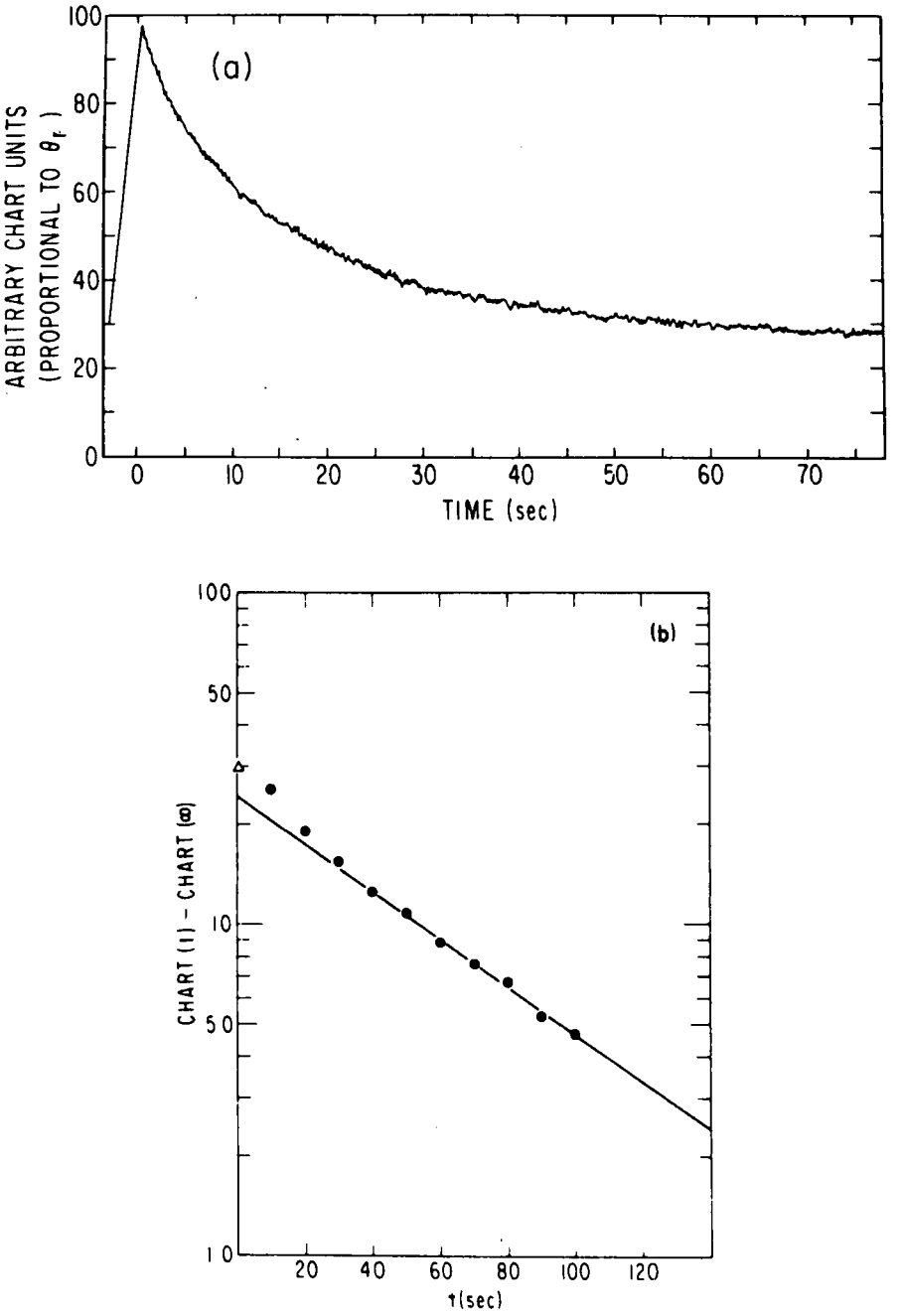

FIGURE 12. (a) Photograph of a typical recorder tracing for T2 DNA in a solvent consisting of 75% glycerol-25% BBES buffer. The DNA concentration is 14.9 μg/ml, T = 25° C. The shear stress at steady state rotation is 0.007 dyn/cm², and the steady state angular velocity of the rotor is 7.32 × 10^{-3} rev/sec. The figure shows both the steady state motion of the rotor when $t < 0$ and the DNA relaxation when $t < 0$. (b) Semilog plot of typical *B. subtilis* W23 cree-recovery experiment. DNA concentration 2.3 μg/ml, shear stress 0.155 × 10^{-2} dyn/cm². The retardation time, τ, calculated from the slope of the straight line in the plot is 61 sec.

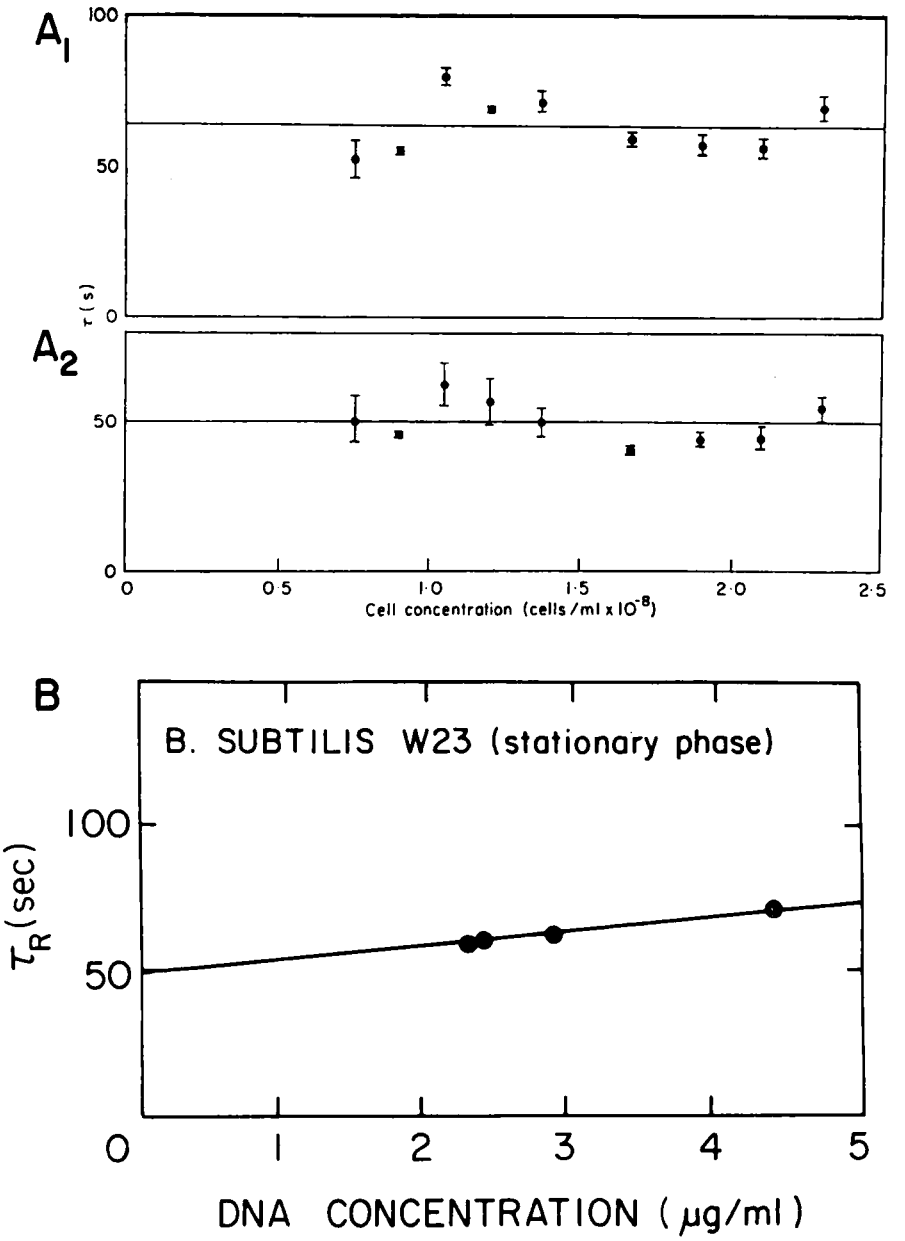

FIGURE 13. Retardation-time data plotted against DNA concentration for *B. subtilis* W23 stationary-phase cell lysates and yeast log-phase cell lysates. (a) Retardation times at 50°C in 2 *M* Na^+ vs. concentration for lysates of log-phase *S. cerevisiae*. In A_1 τ values were calculated by the semilog method; in A_2 τ values were calculated by the integral method. Each point is the average of between four and eight retardation-time measurements made on a given lysate. The error bars show the average deviation of the measurements made on a lysate from their average. (From Lauer, G. D. and Klotz, L. C., *J. Mol. Biol.*, 95, 309, 1975. With permission.) (b) *B. subtilis*. The average retardation-time extrapolated to zero concentration is $\tau^0_{W,50} = 47$ sec, T = 50°C, $[Na^+] = 0.195$ *M*. (From Klotz, L. C. and Zimm, B. H., *J. Mol. Biol.*, 72, 779, 1972. With permission.)

TABLE 4[a]

Molecular Weights for Various Samples of T2 and T7 DNA Calculated by the Three Methods Described in the Text

Sample[b]	$[\eta]$ (cm^3/g)	$\tau^0_{w,50}$ (sec)	Molecular weights[c] from: $M = 19.8([\eta]+5)^{1.5}$ ($\times 10^{-6}$)	$M = 6.2 \times 10^{12} \frac{\tau^0_{R.W.}}{[\eta]}$ ($\times 10^{-6}$)	$M = 1.56 \times 10 (\tau_{R.W.})^{0.60}$ ($\times 10^{-6}$)
T2 DNA	30,500	0.57	111	115	112
T2 DNA	32,000	0.57	120	110	112
T2 DNA	29,400	0.48	106	101	101
T2 DNA[d]	29,000	0.50[d]	104	107	103
T7 DNA	10,400	0.046	23	27	25

[a]Revised from Klotz, L. C. and Zimm, B. H., *J. Mol. Biol.*, 72, 779, 1972. With permission.
[b]All experiments run at 25°C.
[c]The formulae here are for 25°C. Consequently, they differ numerically from those in other parts of the text.
[d]From Thompson and Gill,[33] relaxation time measured by flow birefringence.

subtilis[10] are shown in Table 5. The expected value for *B. subtilis* chromosomal DNA molecular weight is about 2.0×10^9 daltons.[2,35] Assuming linear DNA, it is seen that the molecular weight calculated from intrinsic viscosity alone (1) is low, that calculated from the ratio or relaxation time and intrinsic viscosity (2) is high, and that calculated from relaxation time alone (3) is just about right. The fact that methods 1 and 2 give low and high results, respectively, probably indicates that some of the chromosomal DNA is broken. Broken DNA can reduce $[\eta]_0$ significantly but not change $\tau^0_{w,50}$, since viscoelasticity "sees" mainly the biggest molecules. For the same reason, method 3 is expected to give the most reliable results for the largest DNA in the presence of some broken DNA. The $[\eta]_0$ values may also be low because the correction for non-Newtonian effects may be inaccurate (see previous section).

As is seen from Table 5, roughly a twofold difference in molecular weight is obtained if it is assumed that the largest DNA is circular. However, even 10 to 20% full-sized linear DNA in the presence of 80 to 90% full-sized circles will give a relaxation time characteristic of linear DNA, since linear DNA with the same molecular weight as a circle has a relaxation time 3.5 times that of the circle. Thus, only a small percentage of circles broken once into full-size linears will give the linear result. Also, if we are seeing any circles, they should degrade in time first to full-size linears (with a larger retardation time) and then to small linears. Upon degradation, the retardation time of *B. subtilis* and *E. coli* cell lysates drops continuously without an initial rise, indicating that the largest DNA is probably linear. It is not possible, however, to completely rule out circles with these data. In summary, the molecular weight using method 3 and assuming linears is probably the most accurate. Similar results have been obtained for *E. coli* chromosomal DNA.[10]

The largest chromosomal DNAs studied to date by viscoelasticity are those of several species of *Drosophila* studied by Kavenoff and Zimm.[3] They obtained molecular weights from several *Drosophila* species and several strains of those species, with molecular weights ranging from 2 to 6 $\times 10^{10}$ daltons. A summary of their results is presented in Table 6. Their results approximate that the whole DNA content of the largest chromosomes exists as one linear piece of DNA, at least under the lysis conditions of their experiments. However, due to possible errors in extrapolating the relaxation time, molecular weight formula, Equation 19, to the 10^{10} dalton range and a possible error in the correction from 2 *M* Na^+ (the conditions of their experiments) to 0.195 *M* Na^+ (the condition for which Equation 19 is valid), the molecular weights may only be accurate within a factor of two. The relative molecular weights of the various species and strains tested fall in the expected order, lending further support to the validity of their results.

Recently, lower values of molecular weight for *Drosophila melanogaster* have been obtained by Roberts and Klotz[12] using a much different lysis solution (see Table 2) which contains high concentrations of the strong protein denaturants urea and

TABLE 5

Calculations of Molecular Weight of the DNA in *B. subtilis* W23 Stationary-phase Cell Lysates Using Viscosity-retardation Time Measurements

Assuming linear DNA		Assuming circular DNA	
Formula	Molecular weight $\times 10^{-9}$	Formula	Molecular weight $\times 10^{-9}$
(1) $M = 19.8 \times [\eta]_0^{1.5}$	$1.0^{+0.9}_{-0.5}$	(1) $M = 39.0 \times [\eta]_0^{1.5}$	$2.0^{+1.8}_{-1.0}$
(2) $M = 1.1 \times 10^{13} \times \frac{\tau^0_{R.W}}{[\eta]_0}$	3.2 ± 0.6	(2) $M = 2.4 \times 10^{13} \times \frac{\tau^0_{R.W}}{[\eta]_0}$	6.9 ± 1.3
(3) $M = 2.2 \times 10^8 \times (\tau^0_{R.W})^{0.60}$	$2.0^{+0.7}_{-0.4}$	(3) $M = 4.7 \times 10^8 \times (\tau^0_{R.W})^{0.60}$	$4.3^{+1.5}_{-1.5}$

Note: The cells were grown 16 to 18 hr into stationary phase, $[\eta]_0 = 1390 \times 280$ dl/g and $\tau^0_{R.W} = 40 \pm 9$ sec, 50°C. The formulae require that the viscosity be expressed in cm^3/g units: $[\eta]_0$ in dl/g $\times 10^2 = [\eta]_0$ in cm^3/g. In the limit of zero concentration $\tau^0_{R.W} = \tau_1$, where τ_1 *is the longest relaxation time in the theory* (see Equation 1). The exceptionally large error estimates on line 1 reflect the large possible error in extrapolating the Crothers and Zimm[17] empirical formula up to the 10^9 dalton range.

From Klotz, L. C. and Zimm, B. H., *J. Mol. Biol.*, 72, 779, 1972. With permission.

TABLE 6

Molecular Weights of *Drosophila* Chromosomal DNA from Several Species

Species and strain	DNA content of largest chromosome	Molecular weight from viscoelasticity
Melanogaster		
Cultural cells	43×10^9	41×10^9
Wild type	43	41
Inversion	43	42
Translation	59	58
Hydei		
Wild type	?	40
Deletion	>40	24
Americana	~2x(*Virilis*)	79
Virilis	?	47

guaniadinium chloride. With this lysis solution the molecular weights found are 6 to 9×10^9 daltons. (However, Roberts obtains approximately the Kavenoff result when her procedure is used.) The reasons for the difference between the two results are not known. Four possibilities are (1) more mechanical breakage in the Roberts procedure leading to smaller DNA, (2) aggregation of DNA in the Kavenoff procedure leading to larger DNA, (3) naturally occurring protein-DNA linkers which are disrupted by the strong denaturants in the Roberts procedure, or (4) dilatant effects in the Kavenoff lysates not present in the Roberts lysates. Clearly, the differences in these molecular weights must be accounted for.

There are several advantages and some disadvantages in using the viscoelastic technique as compared to the other hydrodynamic techniques (sedimentation and viscosity). Among the advantages, measurements are quick to make. In order to measure a retardation time, τ, the rotor is allowed to relax for a period of time equal to about 4τ so that a good base line is obtained from which τ may be calculated. *B. subtilis* DNA, for example, has a retardation time of about 1 to 2 min, so a measurement takes about 4 to 8 min. *Drosophila melanogaster* DNA has retardation times of about 1 hr, so a measurement takes about 4 hr. In comparison, sedimentation experiments (to be discussed later) may take a few days or

more, a time in which DNA degradation might take place.

Another advantage is that relaxation time is a very sensitive function of molecular weight, for example, $\tau^0_{w,50} \alpha M^{1.67}$ in 0.195 *M* Na^+ compared to $[\eta]_0 \alpha M^{0.67}$ on 0.195 *M* Na^+. Thus, a twofold increase in molecular weight means about a 300% increase in $\tau^0_{w,50}$ but only an 80% increase in $[\eta]$. Sedimentation coefficient is even a less sensitive function of molecular weight than intrinsic viscosity. Another advantage of this strong dependence of relaxation time on molecular weight is that extrapolation error is minimized. Recall that extrapolation error results from the fact that the empirical formulas relating relaxation time to molecular weight (Equation 19, for example) are derived from molecular weights obtained in the 10^6 to 10^8 dalton range, yet these empirical formulas are frequently used in the 10^9 to 10^{11} dalton range; any imprecision in the formulas in the 10^6 to 10^8 dalton range is magnified in the extrapolated 10^9 to 10^{11} dalton range. As a specific example, two different empirical formulas have been generated for DNA in 2 *M* Na^+. One was obtained by directly measuring relaxation time for three linear DNAs of known molecular weight in the 10^8 to 3×10^9 dalton range.[13] These direct measurements yielded:

$$\tau^0_{w,50} = 3.8 \times 10^{-14} M^{1.600} \quad (20)$$

The second method of generating an empirical formula was to insert Ross and Scrugg's[19] intrinsic viscosity data for DNAs up to about 10^8 daltons into Equation 16, finally obtaining:

$$\tau^0_{w,50} = 2.75 \times 10^{-14} M^{1.606} \quad (21)$$

For $\tau^0_{w,50}$ = 125 sec, Equation 2 yields M = 5.0×10^9 daltons and Equation 21 yields M = 5.6×10^9 daltons, similar values. Thus, the extrapolation error for these two different empirical formulas is small for relaxation time measurements. Also, imprecision in measurement of $\tau^0_{w,50}$ leads to smaller errors in molecular weight than does intrinsic viscosity. For example, a 25% error in $\tau^0_{w,50}$ yields only a 15% error in M.

One worry not yet discussed about extrapolations is that we have assumed that $\tau^0_{w,50}$ can always be related to M by a formula of the form, $\tau^0_{w,50} = KM^a$. Any imprecision in the empirical formulas is assumed to be only in the K and a values. If, however, the relationship between $\tau^0_{w,50}$ and M is more complicated in the 10^9 to 10^{11} dalton range (as might be evidenced by curvature in a log $\tau^0_{w,50}$ vs. log M plot), the extrapolated formulas could yield molecular weights grossly in error. There is no evidence that such complicated behavior exists and no reason to suspect that it does, but these formulas should be checked by molecular weight measurements on several DNAs by other techniques to make certain. This again illustrates the need for other precise, easily usable molecular weight techniques in the 10^9 to 10^{11} dalton range.

A third advantage of viscoelasticity is that relaxation times remain constant over a wide range of shear rates (shear stresses). In contrast, intrinsic viscosities of very large DNA show pronounced non-Newtonian behavior, and sedimentation coefficients of very large DNA are strongly dependent on rotor speed, an effect which is basically a shear stress effect. In Figure 14 relaxation time and intrinsic viscosity vs. shear stress are plotted for T2 DNA. It is seen that relaxation time remains constant over a 25-fold range of shear stress while intrinsic viscosity changes significantly. Constancy of relaxation time with shear stress has been observed for DNA in *B. subtilis, E. coli, Saccharomyces cerevisiae* and *Drosophila* cell lysates.[3,10,13] However, in these studies the shear stresses were varied only over a two- to fivefold range.

At very high shear stresses, dilatant effects set in (see viscometry section). While dilatancy can increase the intrinsic viscosity severalfold, the retardation time is affected only by a factor of two or less, an estimate based on only a few unpublished observations. There is a need to study the effects of dilatancy on retardation time. Dilatancy can usually be avoided, however, by making measurements at low shear stresses or by making measurements after initially turning the viscoelastometer rotor only a few degrees. Massa[36] has shown that relaxation time is not affected much by such short rotor windups.

As a standard practice, hydrodynamic measurements (sedimentation, viscosity, and viscoelasticity) are extrapolated to zero polymer concentration in order to eliminate interactions and excluded volume effects among molecules, which usually show up as concentration dependence of

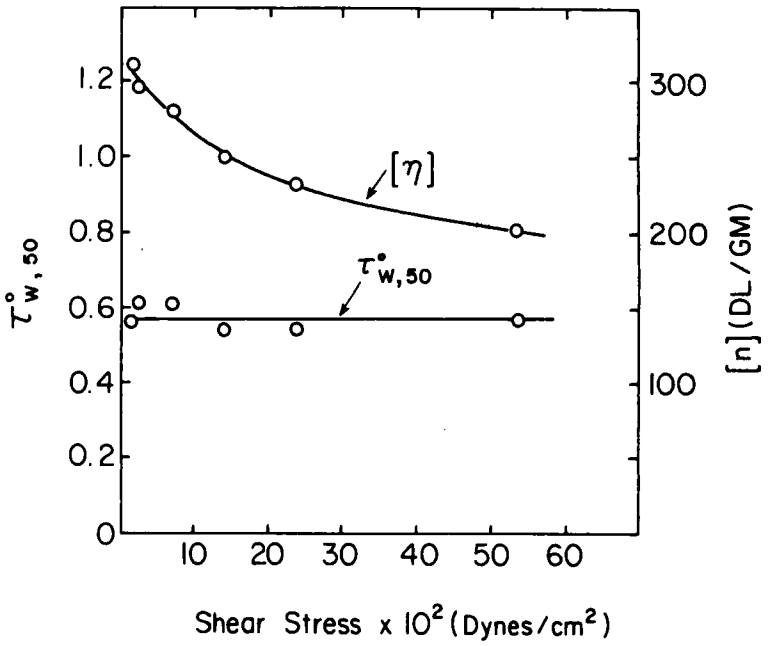

FIGURE 14. Shear stress dependence of relaxation time and intrinsic viscosity for T2 DNA. (From Chapman, R. E., Jr., Klotz, L. C., Thompson, D. S., and Zimm, B. H., *Macromolecules*, 2, 6, 1969. Copyright by the American Chemical Society.)

retardation times. For some types of cell lysates in some lysis mixtures, retardation time does not seem to depend on concentration, for example, *S. cerevisiae* cell lysates in 2 *M* Na^+ (see Figure 13a). Under these circumstances, retardation times obtained at several concentrations may be averaged, thereby eliminating the need for extrapolation to zero DNA concentration and eliminating errors associated with such extrapolations. In any event, where it exists the concentration dependence of retardation time is usually mild and linear (see Figure 13b) so that extrapolations to zero DNA concentration should be relatively accurate. The reasons for concentration dependence or lack of it are not yet understood.

Probably the most important advantage of viscoelasticity for the study of chromosomal DNA is the fact that the technique sees only the largest DNA molecules. This means that even in the presence of large percentages of mechanically broken or degraded DNA, as might be found in cell lysates, the molecular weight of the intact DNA may be obtained if it represents about 15% or more of the total DNA. In contrast, intrinsic viscosity yields an average molecular weight which heavily reflects broken or degraded DNA. Sedimentation patterns, on the other hand, show the whole distribution of DNA molecular weights which is, of course, the best situation.

This unique property of viscoelasticity may lead to one minor disadvantage and to what may be a major disadvantage in some cases. The minor disadvantage is that molecular weight distributions are difficult to obtain because smaller chromosomal DNA and broken DNA are not easily detected by the technique. The possible major disadvantage is that retardation time is expected to be extremely sensitive to DNA aggregation. To take a simple case, two linear DNA molecules aggregated end to end will act like a single molecule twice as big and therefore possess about three times the retardation time of two unaggregated DNA molecules. Clearly, aggregation of several DNA molecules can lead to very large retardation times and anomalously high molecular weights.

Possible types of aggregation, how they might be prevented, and how they might be detected have been discussed in detail earlier in this review. We just wish to reemphasize here that the absence of aggregated DNA in bacteria[10] and yeast[11, 13, 37] has not been definitely shown, but control experiments indicate that aggregated DNA is probably not present.

More direct methods of detecting aggregation are sorely needed. There may be a way to detect aggregation using only the data from viscoelastic measurements alone by using rotor recoil data along with retardation time. The most convenient recoil datum is the angle which the rotor has relaxed due to the largest retardation time only. This quantity is easily found by extrapolating semilog plots of the longest exponential decay back to zero time. This is illustrated in Figure 12b where the extrapolated value is 68 arbitrary chart units. This chart unit data can then be converted to degrees recoil which is given by the symbol Γ_1. This recoil can then be related to other known quantities through the formula for dilute solutions:

$$\Gamma_1 = \frac{kT\omega_r \tau^2 L_1}{\eta_0 \eta_{rel}} \quad (22)$$

where

- ω_r = the rotor speed (before the magnets are turned off);
- τ = the longest retardation time;
- η_0 = the solvent viscosity;
- η_{rel} = the relative viscosity;
- kT = the usual meaning;
- L_1 = the number of largest DNA molecules per cubic centimeter in the lysates.

The procedure is to calculate Γ_1 from the measured τ, η_{rel}, etc. and see if it agrees with that expected, which is calculated from the known number of cells used to prepare the lysate. An example of use of recoil data along with relaxation time to both calculate molecular weight and estimate heterogeneity is found in Reference 38.

DNA aggregation may be detected by comparing observed and calculated recoil. For example, in a gel (which is the extreme limit of aggregation) the relaxation time may remain small but recoil is total; that is, the recoil equals the number of degrees the rotor was initially turned before relaxation.[39] Thus, lesser degrees of aggregation may be expected to be reflected in some manner in the rotor recoil. A careful study of the viscoelasticity of aggregated model systems would be useful.

At the present time, the largest DNA molecular weights which can be determined by viscoelasticity are probably in the mid to high 10^{10} range. One reason for this limit is rotor stability in the instrument. Since the rotor is totally suspended in the DNA solution (see Figure 7), slight convection currents can make it drift to the side of the sample chamber over a period of hours. Also, small vibrations in the instrument room can cause significant turning of the rotor over a period of hours. Since retardation times are on the order of a few hours for DNA in the 5×10^{10} dalton range, these effects become significant and make the completion of experiments difficult. Another effect, becoming significant in the 10^{10} dalton molecular weight range, is that DNA migrates toward the rotor as the rotor turns and eventually "wraps around" the rotor. The rate of migration is approximately proportional to the $M^{5/2}$ power. Thus, excessive turning of the rotor will deplete the solution of big DNA and cause a mucouslike aggregation of DNA on the rotor, making the acquisition of meaningful data difficult. Also, dilatant effects seem to be more pronounced with DNA in the 10^{10} dalton range. It may, of course, be possible to eliminate or circumvent all of these difficulties and extend the useful molecular weight range of viscoelasticity to over 10^{11} daltons. Such an extension, however, will require much cautious effort.

Viscoelasticity may also be used to determine molecular weights of single-stranded DNA. Since single-stranded DNA has a very small hydrodynamic radius in a given Na^+ concentration compared to double-stranded DNA of the same molecular weight, and since relaxation time and relaxation intensity are a strong function of hydrodynamic radius ($\tau\alpha [\eta] \alpha R_e^3$), relaxation time and intensity depend strongly on salt concentration. Single-stranded DNA random coils can be expanded tremendously in low Na^+ because low Na^+ does not effectively shield electrostatic repulsions among the nucleotides.[28] Uhlenhopp and Zimm[29] have determined molecular weight of alkali-denatured (pH 12.5), single-stranded *E. coli* chromosomal DNA in 0.1 *M* Na^+, finding the largest single-stranded DNA to be 1.3 to 2×10^9 daltons. Whether the lower or higher value is correct depends on interpretation of the results. Since *E. coli* double-stranded DNA has a molecular weight of approximately 2.7×10^9 daltons, the single-stranded DNA they have measured may be whole chromosome-sized single strands. This very

large single-stranded DNA is present in both log-phase cells (where DNA is replicating) and stationary-phase cells (where DNA is not replicating). This large DNA is very sensitive to degradation by both heat and high pH. The conditions for obtaining large amounts of very large DNA are discussed by the authors.[29] Measurements on alkaline lysates of mouse 3T3 cells indicate single-stranded DNA, with molecular weight in excess of 10^{10} daltons.[41]

In summary, viscoelasticity appears to be the technique of choice for obtaining molecular weights of DNA molecules in the 5×10^8 to 5×10^{10} dalton range. It is a relatively quick and sensitive technique to use and appears to be insensitive to factors, such as shear stress, which cause anomalous behavior in other techniques such as viscosity and sedimentation. Despite the apparent successes of viscoelasticity and the fact that no major discrepancies have been found in the results of viscoelastic measurements, there is a pressing need for the development or extension of other techniques to corroborate the results of viscoelastic experiments.

Sedimentation

The rate of sedimentation in centrifugal fields has long been used to determine molecular weights of macromolecules. (For further details concerning the following theory see Tanford,[4] p. 364; Flory,[5] p. 304; or Van Holde,[6] p. 98). Theoretically, the rate of sedimentation is related to molecular weight by:

$$S = \frac{M(1 - \bar{v}\rho)}{N_a f} \tag{23}$$

where

- S = the rate of sedimentation per unit centrifugal field;
- M = the molecular weight;
- $\bar{v}$ = the volume per gram of the macromolecule;
- ρ = the density of the solvent;
- N_a = Avogadro's number;
- f = the frictional coefficient.

That is, f is a measure of the frictional resistance to sedimentation for spherically symmetric random coils and is given by:

$$f = 6\pi\eta_0 R_e \tag{24}$$

where

- η_0 = the solvent viscosity;
- R_e = the hydrodynamic radius.

The sedimentation coefficient, S, is defined mathematically as:

$$S = \frac{dx/dt}{\omega^2 x} \tag{25}$$

where

- dx/dt = the rate of sedimentation (distance sedimented per unit time);
- ω = the centrifuge rotor speed.

Thus, $\omega^2 x$ is the radial acceleration experienced by a sedimenting macromolecule. It is this quantity calculated from the experimental data which may be related to molecular weight. As seen in Equation 23, S depends on solvent viscosity, density, and temperature besides molecular weight. Usually, S values reported in the literature are corrected in the obvious way to water, w, at 20°C. These corrected S values are denoted by $S^0_{20,w}$ where the superscript zero means the data have been extrapolated to zero macromolecule concentration also.

Theoretically, $S^0_{20,w}$ may be related to molecular weight for random coil molecules through the result of chain statistics, $R_e \propto M^{0.5\text{-}0.6}$ (see, for example, Tanford,[5] p. 150). Substituting this proportion into Equation 24 and then into Equation 23 yields:

$$S^0_{20,w} = K M^a \tag{26}$$

where

- a = 0.5 to 0.6;
- K = a constant.

The exact values of K and a must be determined empirically, since the exact relationship between R_e and M is incalculable theoretically. For DNA, Crothers and Zimm[17] have found the empirical formula:

$$S^0_{20,w} = 2.7 + 0.0152\ M^{0.445} \quad (27)$$

Another relationship:

$$S^0_{20,w} = 2.8 + 0.00834\ M^{0.479} \quad (28)$$

has been obtained by Freifelder.[20] These equations and others for the analytical centrifuge[42] give accurate molecular weights in the 10^5 to 10^8 dalton range where they were developed.

Measurement of DNA molecular weights by sedimentation in the analytical centrifuge is, unfortunately, not applicable to DNA larger than about 5×10^8 daltons for at least two reasons. The analytic ultracentrifuge requires purified DNA solutions because of the optical system (UV absorption) used to detect sedimenting DNA. It is very difficult to purify DNA of molecular weight over 5×10^8 daltons without mechanical breakage, as discussed earlier. Also, in order to detect DNA optically, sufficiently high DNA concentrations must be used (greater than 25 μg/ml). At such high DNA concentrations and particularly with very large DNA, part of the DNA pellets to the bottom of the centrifuge cell due to a rotor speed-dependent "aggregation" phenomenon.[43] The cause of this phenomenon is now thought to be the downward pull on nearby DNA molecules from the moving solvent surrounding other DNA molecules, thus making large numbers of DNA molecules sediment anomalously fast to the bottom of the cell.[44]

There is, however, another sedimentation velocity method developed over the last 15 years which avoids these difficulties and is currently applicable to DNA of molecular weight slightly over 10^9 daltons. The method is sucrose gradient sedimentation velocity or simply sucrose sedimentation.

A typical procedure for doing sucrose sedimentation experiments on DNA is to first prepare a linear 5 to 25% sucrose gradient in a plastic cylindrical centrifuge tube with the denser 25% sucrose on the bottom of the tube. The purpose of such a gradient is to stabilize the solution against mixing (conversely, a gradient with the dense 25% sucrose on the top would mix spontaneously as the denser sucrose sinks through the less dense sucrose). A cell lysate containing ^{3}H-radiolabeled DNA is either prepared on top of or gently placed on top of the gradient. (Care must be taken to make sure that the density of the lysate is less than 5% sucrose or the lysate will sink.) The gradient plus lysate plus a ^{32}P-radiolabeled "marker" DNA of known molecular weight is then spun in a preparative centrifuge until the DNA has sedimented a measurable distance (as established, usually, by trial and error). After sedimentation, the bottom of the centrifuge tube is punctured, and the solution dripping from the puncture hole is collected in a number of fractions of convenient volume. The radioactive counts in each fraction are then determined to locate and quantitate the DNA, and then the distance sedimented is calculated from the fraction number and volume of the fractions and known dimensions of the centrifuge tube. Molecular weights are obtained by comparing the distances sedimented by the marker DNA of known molecular weight and the DNA of unknown molecular weight using an empirical formula of the form:

$$\frac{D_1}{D_2} = \left(\frac{M_1}{M_2}\right)^k \quad (29)$$

where

D_1 and D_2 = the distances sedimented by the marker and the unknown DNA;
M_1 and M_2 = their molecular weights.

The types of sedimentation patterns one obtains are illustrated in Figure 15. How such sedimentation patterns are quantitatively related to molecular weights will be discussed later. Specific procedures for running sucrose sedimentation experiments on large DNA may be found in References 11, 15, 16, and 37. The first application of sucrose sedimentation to quantitate DNA molecular weights may be found in Burgi and Hershey.[45]

Sucrose sedimentation has many advantages over conventional sedimentation. By using radiolabeled DNA, very low DNA concentrations may be used (0.1 μg/ml or less), thereby eliminating both sedimentation coefficient concentration dependence and sedimentation anomalies brought on by high DNA concentration such as the "aggregation" anomaly observed by Rosenbloom and Schumaker.[43]

A second advantage is that the procedure may

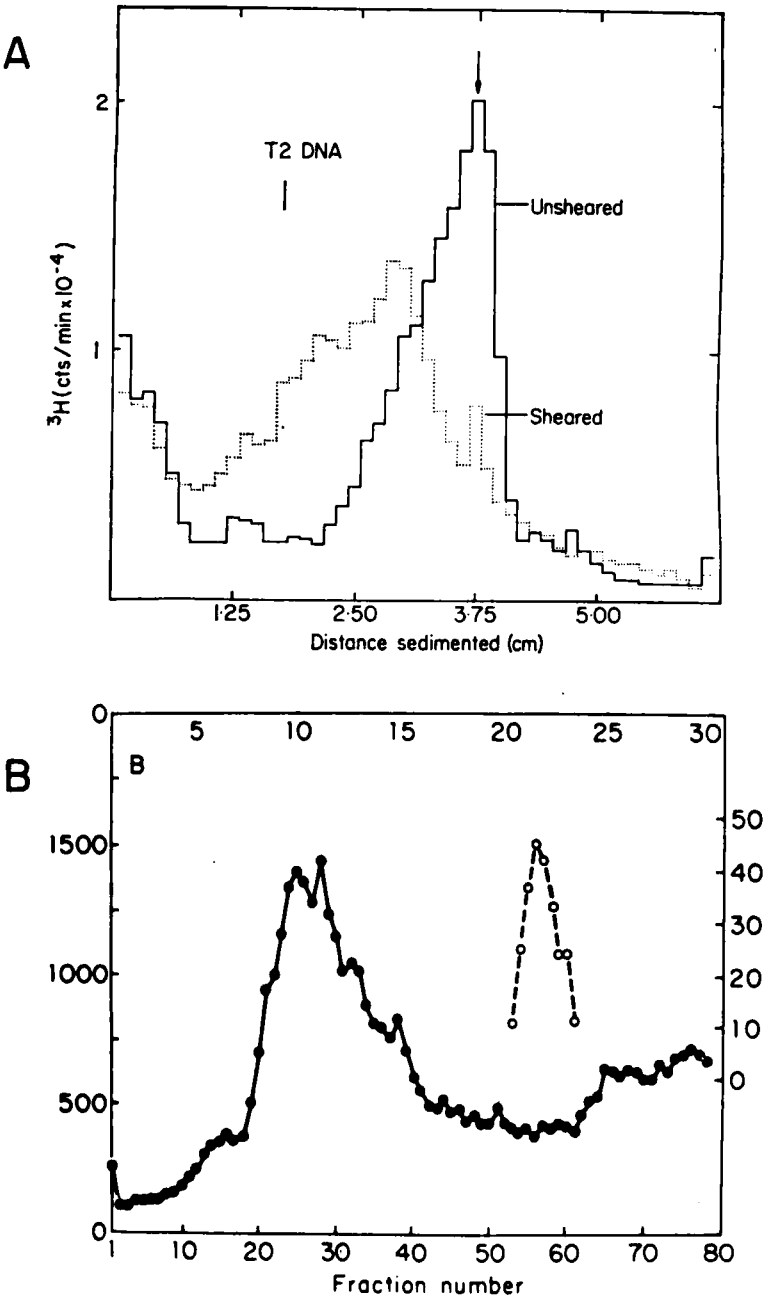

FIGURE 15. Sucrose sedimentation patterns of *B. subtilis* and *S. cerevisiae* chromosomal DNA. (a) *B. subtilis* DNA. Note that mild shearing significantly reduces the size of the DNA and increases the width of the size distribution. Centrifugation was for 30 hr at 6,000 rpm. (From Kavenoff, R., *J. Mol. Biol.*, 72, 801, 1972. With permission.) (b) *S. cerevisiae* DNA. (•) Yeast DNA. (O) T4 bacteriophage marker DNA (M 1.1 × 10.8 daltons). Centrifugation was for 26 hr at 10,000 rpm. (From Petes, T. D. and Fangman, W. L., *Proc. Natl. Acad. Sci. U.S.A.*, 69, 1188, 1972.)

be scaled up or down over a sizable range of DNA concentrations using different sizes of centrifuge tubes; thus, the technique may be used preparatively to separate DNA of different sizes. The fact that different size classes end up in different fractions also means that the technique yields the whole molecular weight distribution instead of a molecular weight average, as intrinsic viscosity does, or only an estimate of the biggest molecules in the population, as viscoelasticity does. In sucrose sedimentation, picking out larger intact chromosomal DNA molecules in the presence of many broken molecules is difficult, however.

The instrumentation for sucrose sedimentation is inexpensive, since only a preparative centrifuge is required in place of the complicated and expensive analytical centrifuge.

Perhaps the biggest advantage is that cell lysates can be used since the DNA in the cell may be specifically labeled with ^{3}H-thymidine, making it the only labeled substance in the cell. Thus, the breakage problem in preparing purified DNA is eliminated. The cell lysates may be prepared on top of the gradient, eliminating mechanical breakage that can occur from attempting to transfer lysed cells.

The first empirical formula relating distance sedimented to molecular weight of DNA was developed by Burgi and Hershey.[45] They determined that:

$$D_1/D_2 = (M_1/M_2)^{0.35} \quad (30)$$

using T2, T5, and λ bacteriophage DNAs as standards. Freifelder[20] has reevaluated this relationship and obtains:

$$D_1/D_2 = (M_1/M_2)^{0.38} \quad (31)$$

Because the distance sedimented varies only as molecular weight to the 0.38 power, sucrose sedimentation is relatively insensitive to DNA size as compared to viscoelasticity, where the relaxation time varies as molecular weight to the 1.67 power (in 0.195 *M* Na^+). Also, this low power dependence in sucrose sedimentation can lead to large extrapolation errors. Suppose, for example, T4 DNA ($M = 110 \times 10^6$ daltons) is used as the standard and suppose the ratio of distances sedimented for the DNA of unknown molecular weight and T4 DNA is 4.26. From the Freifelder relationship (Equation 31) the unknown molecular weight is calculated to be $M_2 = 5.0 \times 10^9$ daltons, and from the Burgi and Hershey relationship the unknown molecular weight is calculated to be $M_2 = 6.9 \times 10^9$ daltons, the two formulas giving a rather large difference. Similarly, a 25% error in ratio of distances sedimented gives a 66% error in molecular weight.

Of course, to reduce extrapolation error, a standard DNA of molecular weight closer to that of the unknown molecular weight might be used. Two possible standards are *B. subtilis* and *E. coli* chromosomal DNA. The main problem with using these as standards is that neither DNA has yet been sedimented completely intact.[15,16] In Figure 15, for example, the sedimentation pattern indicates DNA ranging in molecular weight from about 2.5×10^9 daltons to slightly bigger than T2 DNA, the largest value being a reasonable value for the *B. subtilis* chromosome. Even though their sedimentation pattern is not simple, these bacterial chromosomes can still make useful standards.

Considering the potentially large extrapolation errors, the Kavenoff[15] result for *B. subtilis* chromosomal DNA ($M = 2.5 \times 10^9$ daltons) and the Appleby, Rall, and Hearst[16] values for *E. coli* DNA ($M = 1.0$ to 3.0×10^9 daltons for their largest DNA) agree well with the accepted range for these two chromosomal DNAs, which is 2 to 3 $\times 10^9$ daltons.[1,2,11] Also, the Petes and Fangman[11] sucrose sedimentation result for the largest chromosomal DNA in yeast ($M = 1.4 \times 10^9$ daltons) agrees well with that obtained by viscoelasticity ($M = 2.0 \times 10^9$ daltons).

There is an anomaly associated with the sedimentation of very large DNA similar to the non-Newtonian effects seen in viscosity. They are similar in that both anomalies are induced by solvent velocities acting on the DNA molecules. Qualitatively, the physical reason for the sedimentation anomaly is as follows. The ends of a linear, nonsedimenting, random coil DNA molecule are on the average tucked inside the molecule. The solvent inside a sedimenting molecule moves at about the same rate as the molecule, so the ends experience little frictional resistance to motion. The solvent velocity gradient around sedimenting molecules will distort them, large molecules being distorted more. One effect of this distortion is that the ends of the random coil tend to drag behind the rest of the molecule. These exposed ends experience a frictional resistance to sedimentation

from the nearly stationary solvent through which they are passing. This increased frictional resistance increases the frictional coefficient, f, and hence decreases the sedimentation coefficient, leading to anomalously low calculated molecular weights (see Equations 23 through 26). A schematic diagram of this anomaly is presented in Figure 16. This anomaly has been observed for DNA as small as T5 bacteriophage DNA with M = 68×10^6 daltons.[46]

Zimm[47] has worked out a theory for the anomaly and has found that sedimentation coefficient can be expressed as a power series in DNA molecular weight, rotor speed, ω, and other parameters. He finds that:

$$S = S^0 (1 - D_2 y^2 + D_4 y^4 - \ldots) \quad (32)$$

where

- S = the expected sedimentation coefficient for a DNA of molecular weight M at some rotor speed ω;
- S^0 = the sedimentation coefficient in the limit of zero rotor speed;
- D_2 = a constant ($+2.887 \times 10^{-2}$);
- D_4 = a constant (2.051×10^{-3}).

The variable y is given by:

$$y = \frac{8.374 \times 10^{-24} M^2 (1 - \upsilon\rho)^2 (\text{rpm})^2 x}{T \eta_0 S^0} \quad (33)$$

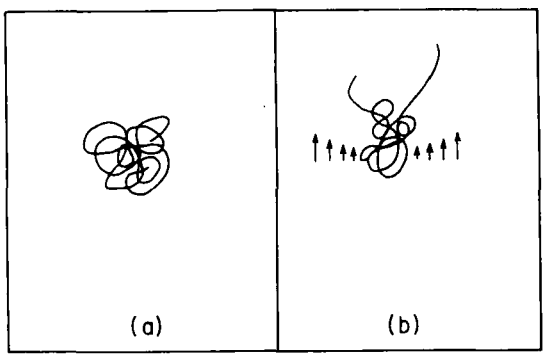

FIGURE 16. Schematic representation of the sedimentation anomaly. (a) A DNA random coil at rest (no centrifugal field). Note that the ends are tucked inside the coil. (b) A DNA random coil in a high centrifugal field. The direction of sedimentation is downward. The arrows denote the solvent velocity as seen by an observer moving with the center of mass of the DNA. The ends drag behind the coil exposed to moving solvent.

where

- x = the distance to the sedimenting DNA from the center of rotation;
- (rpm)= the rotor speed in revolutions per minute;
- T = absolute temperature;
- η_0 = solvent viscosity.

At low rotor speeds, only the $(1 - D_2 y^2)$ terms are significant, so Equation 32 becomes, after substituting for y in Equation 33,

$$S = S^0 - \frac{K M^4 (\text{rpm})^4}{S^0} \quad (34)$$

where

K = roughly a constant.

K is given by:

$$K = 2.02 \times 10^{-48} \frac{(1 - \upsilon\rho)^4 x^2}{T^2 \eta_0^2} \quad (35)$$

The exact value of K is calculated from the conditions of the experiment and the geometry of the centrifuge.

As seen from Equation 34, the deviation in sedimentation coefficient from its true value is a very strong function of both molecular weight and rotor speed. For example, a 2-fold increase in either molecular weight or rotor speed will cause a 16-fold increase in the deviation of sedimentation coefficient in the range where deviation occurs. Deviations of sedimentation coefficient (expressed as S/S^0, as a function of $y^{1/2}$, a quantity proportional to rotor speed) are plotted in Figure 17a for T7 DNA ($M = 25 \times 10^6$ daltons), T2 DNA ($M = 120 \times 10^6$ daltons), and *E. coli* DNA ($M = 2 \times 10^9$ daltons). *E. coli* DNA shows deviations at even the very low centrifuge speed of 3,500 rpm. Standard sedimentation experiments might be run at rotor speeds of 20,000 to 50,000 rpm; the deviations in sedimentation coefficient for chromosomal DNA the size of *E. coli* would be so great at these rotor speeds as to make calculation of a meaningful molecular weight impossible.

In the range of molecular weights and rotor speed where the deviation is slight (i.e., the $D_4 y^4$ term and higher terms in the power series are insignificant), a plot of measured S vs. $(\text{rpm})^4$

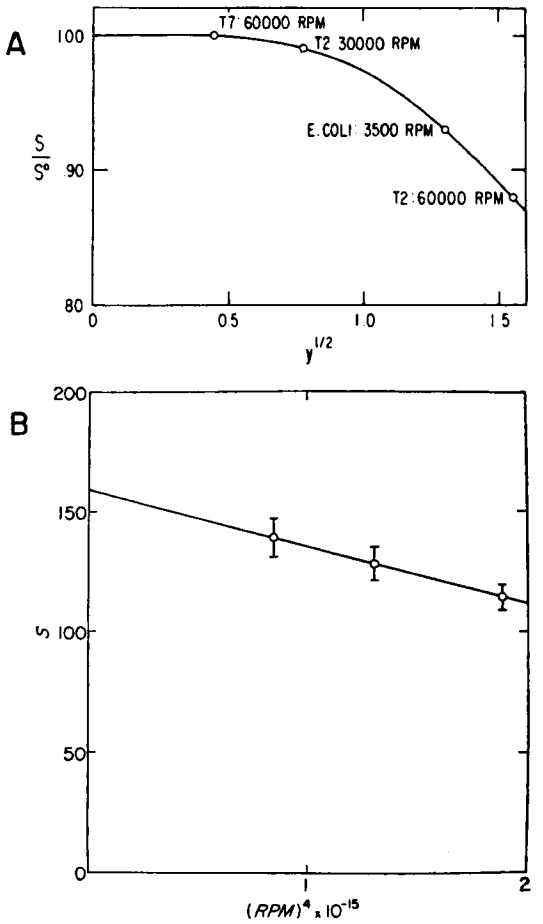

FIGURE 17. (a) Calculation of expected deviation in sedimentation coefficient for various DNAs at various rotor speeds. The abscissa, $y^{1/2}$, is proportional to the centrifuge speed. The points shown were calculated for T7 DNA ($M = 25 \times 10^6$ and $S^0_{20,\omega} = 32$), T2 DNA ($M = 120 \times 10^6$ and $S^0_{20,\omega} = 61.8$), and *E. coli* DNA ($M = 2.7 \times 10^9$ and $S^0_{20,\omega} = 150$, estimated), assuming the conditions: T = 293°, X = 5.2 cm, $1 - \bar{\nu}\rho = 0.436$ (for 10% sucrose), $1 - \bar{\nu}\rho = 0.457$ (for 0.2 *M* NaCl), $\eta(H_2O) = 0.01$. (From Zimm, B. H., *Biophys. Chem.*, 1, 279, 1974. With permission.) (b) Plot of S vs. $(RPM)^4$ for *B. subtilis* DNA. Values for the S of the *B. subtilis* W23 genome were measured relative to T2 DNA (57S). At each centrifuge speed indicated, S was measured with three different concentrations of *B. subtilis* DNA and extrapolated to zero DNA concentration. Here the values obtained for S at zero DNA concentration are plotted vs. $(RPM)^4$. (From Kavenoff, R., *J. Mol. Biol.*, 72, 801, 1972. With permission.)

should be linear, and the values of S extrapolated to zero rotor speed should yield S^0. Such a plot is shown in Figure 17b for *B. subtilis* DNA.[15] The linearity of the plot provides support for the theory.

This anomaly can reduce the sedimentation coefficient of a greater than 10^9 dalton molecular weight DNA so much as to make it sediment slower than a smaller molecule in the same centrifuge tube; that is, at a given rotor speed, a sedimentation coefficient (ordinate) vs. molecular weight (abscissa) plot has a maximum.[219,373]

One way to avoid this anomaly is to run experiments at very low rotor speeds along with extrapolations to zero rotor speed. Such experiments take a long time, however, since distance sedimented in sucrose varies only as $M^{0.38}$, whereas it varies as the square of the rotor speed (see Equations 25 and 31). Thus, for example, an experiment which takes 1 hr at 35,000 rpm takes 100 hr at 3,500 rpm to sediment the same distance. Even if the DNA in the lower rotor speed experiment is ten times bigger, it would take 40 to 50 hr to sediment the same distance.

Another way to reduce this anomaly and make experiments more practical has been studied by Appleby et al.[16] From Equations 34 and 35 it is seen that the deviation of sedimentation coefficient depends also on the quantity $(1 - \bar{\nu}\rho)^4$. For DNA in sucrose, $\bar{\nu} \simeq 0.6$ and $\rho \simeq 1.06$ g/cc, so that $(1 - \bar{\nu}\rho)^4 \simeq 0.018$. They use sucrose containing CsCl, so that $\rho \simeq 1.6$ g/cc and $(1 - \bar{\nu}\rho)^4$ are reduced to 2.6×10^{-6}. Using this sedimenting medium they have obtained $M = 1.0$ to 3.0×10^9 daltons, a reasonable value for the larger DNA in an *E. coli* cell lysate.

We can estimate that sucrose sedimentation, if carefully carried out at low rotor speeds, is usable for approximately sizing DNA up to 5×10^9 to 10^{10} daltons.

Circular DNA sediments only slightly faster than linear DNA. Both experiment and theory[48] give approximately $S_\ell/S_c = 0.9$, where S_ℓ and S_c are the sedimentation coefficients of linear and circular DNA of the same molecular weight, respectively. As an example of the difference in molecular weight obtained by assuming the two different shapes, for *B. subtilis* chromosomal DNA Kavenoff[15] calculates $M = 2.5 \times 10^9$ daltons assuming linears and $M = 1.8 \times 10^9$ daltons assuming circles.

However, using sucrose sedimentation it should be easy to distinguish circles from linears, since the sedimentation anomaly described above should not occur at all, or at least should not be nearly as severe for circular DNA. Thus, measuring sedimen-

tation coefficient at several rotor speeds should allow us to distinguish circles from linears.

Both intrinsic viscosity, $[\eta]$, and relaxation time, $\tau^0_{w,50}$, can be combined with sedimentation coefficient, $S^0_{w,20}$, to calculate molecular weight. It is assumed, of course, that the intrinsic viscosity is measured in a solution of DNA homogeneous in size. For very large DNA (where $[\eta] \gg 5$ and $S^0_{w,20} \gg 2.7$), Equations 11 and 27 may be rewritten as:

$$[\eta] = 1.371 \times 10^{-3} M^{0.5 + x} \quad (36a)$$

$$S^0_{w,20} = 0.0152 M^{0.5 - x} \quad (36b)$$

where

$$x \simeq 0.052.$$

The fact that the molecular weight exponents for sedimentation and intrinsic viscosity can be related through a common x value is a result of theory (see Tanford[5] for further details). Eliminating x between these last two equations yields:

$$M = 1.44 \times 10^4 (S^0_{20,w})^{3/2} [\eta]^{1/2} \quad (36c)$$

where $S^0_{20,w}$ is expressed in the normal Svedberg units and $[\eta]$ is in deciliters per gram. If, however, the DNA we are measuring is circular where $[\eta]_c = 0.643[\eta]_\ell$ and $S_c = 1.11\ S_\ell$, the measured intrinsic viscosity will be too low and the measured sedimentation coefficient too high.

The product:

$$S_c^{3/2} [\eta]_c^{1/2} = 0.94\ S_\ell^{3/2} [\eta]_\ell^{1/2} \quad (37)$$

so that the calculated molecular weight is almost the same whether the DNA is actually linear or circular. We can therefore calculate molecular weight independent of the form of the molecule.

The use of Equation 36 requires $S^0_{w,20}$ values, not sucrose sedimentation coefficients. $S^0_{w,20}$ can be calculated from sucrose sedimentation coefficients if one knows the viscosity and density of sucrose as a function of position in the centrifuge tube. Details of such calculations are given in Reference 49.

By eliminating intrinsic viscosity between Equations 16 and 36, we get an equation relating molecular weight to relaxation time and sedimentation coefficient for linear DNA:

$$M = 2.81 \times 10^6 (S^0_{20,w}) (\tau^0_{50,w})^{1/3} \quad (38)$$

where $\tau^0_{50,w}$ is in seconds and $S^0_{20,w}$ is in Svedbergs. If, however, the DNA we are measuring is circular where $\tau_c = 0.46\ \tau_\ell$ and $S_c = 1.11\ S_\ell$, the product:

$$S_c \tau_c^{1/3} = 0.86\ S_\ell \tau_\ell^{1/3} \quad (39)$$

so that the calculated molecular weight will be off by 14%, a rather small error. Thus, combination of relaxation time and sedimentation coefficient can be used to calculate molecular weight approximately regardless of form, provided one has accurate sedimentation coefficients, which are difficult to obtain.

Molecular weights of single-stranded DNA may be measured using sucrose sedimentation. The DNA is sedimented in an alkaline sucrose gradient,[50,51] and distance sedimented may be related to molecular weight using the empirical formula of Studier.[42] Anomalous sedimentation behavior at high rotor speeds similar to that for double-stranded DNA has been observed.[52] Since the hydrodynamic radius of single-stranded DNA can be reduced considerably using solvents with high Na^+ concentration, anomalous behavior might possibly be avoided.

In summary, it appears that with careful work, as a quantitative technique sucrose sedimentation might be extended to the low 10^{10} dalton range. Particularly interesting is the method of using dense CsCl-sucrose gradients to reduce the magnitude of the rotor speed anomaly.[16] The principal disadvantage of sucrose sedimentation seems to be the insensitivity of sedimentation distance to molecular weight; this insensitivity, coupled with the difficulty of precisely measuring distances sedimented, can lead to large errors in molecular weight compared to viscoelasticity and viscosity. The main advantage of sucrose sedimentation is that it gives the whole molecular weight distribution.

Hydrodynamic Studies of Replicating DNA

Using viscosity, sedimentation, or viscoelasticity, it might be possible to study replicating DNA and perhaps differentiate between modes of

replication such as linear branched Ys, figure-eight circles, or linear DNA with many replication bubbles as seen in eucaryotes.[53]

Very little experimental work or theoretical calculations on replicating DNA have yet been done. Bloomfield[48] has calculated the change in sedimentation coefficient as a function of degree of replication for linear Ys and has calculated the sedimentation coefficient for a figure eight with equal size loops (i.e., a circular DNA which has completely replicated but the two daughter molecules have not yet come apart). He finds that the sedimentation coefficient of the linear should increase 33% from start to finish of replication and that of the figure eight should increase 52%. With precise sedimentation data it might be possible to differentiate between these two forms. Theory has not yet been worked out for replication bubbles. Worcel and Burgi[54] have shown easily measurable differences in sedimentation distance between replicating and nonreplicating folded *E. coli* chromosomes, however.

A crude theory[32] shows that relaxation time is sensitive in detecting linear, branched replication, the relaxation time increasing by a factor of three for a fully replicated structure. Linear bubble structures will show very little change in relaxation time as replication proceeds, hence making them undetectable. No approximate theory exists for figure-eight structures. Log-phase *E. coli* cell lysates do give higher relaxation times than stationary-phase cell lysates, presumably due to replicating DNA.[32]

ELECTRON MICROSCOPY

Direct visualization of DNA molecules with the electron microscope is one of the most powerful tools presently available for the study of the physical nature of DNA. The technique was founded by the work of Kleinschmidt, Zahn and their co-workers who developed the basic methodology in use today.[55,56] In the Kleinschmidt technique, DNA and a basic globular protein (generally cytochrome c) are mixed in a rather small volume (50 μl is a typical size). The DNA and protein complex electrostatically, and the resulting complex is spread on an air-water interface, usually by allowing a drop of the solution containing the DNA-protein complex to slide down a ramp formed from a cleaned microscope slide onto the surface of a low molarity salt solution termed the hypophase. The salt concentration of the hypophase is such as to denature the basic protein, which floats on the surface spreading out to form a monolayer. The protein film which results contains the DNA molecules spread out in an easy-to-visualize form. This film can then be picked up on a plastic-coated microscope grid, stained with uranyl acetate, shadowed with a heavy metal, and viewed in the electron microscope (see Davis et al.[57] for a detailed description of the technique as currently practiced).

The literature on electron microscopy of DNA is far too vast to survey here and is not our field of expertise. (For a complete review of the literature through July 1973, see Younghusband and Inman.[58]) The following are simply a few points which came to our attention in examining the papers reviewed in this article: There is a difference between seeing a molecule once, a few times, or even a large number of times, in some cases, and proving that the form seen had biological importance. Certainly, molecules seen in low numbers should be considered with some degree of caution when the time comes to draw conclusions. Proper studies with the electron microscope include statistics on the various forms of molecules visualized, all molecules in a random survey being counted. Even when a given molecular form is found in reasonable abundance, some care must be exercised in judging its meaning. Particularly important is consideration of the steps used in purifying the DNA. What appears to be pure DNA in the electron microscope may be a complex of DNA and protein hidden by the overlayer of cytochrome or the given form may have resulted from a rearrangement of the DNA molecule which occurred in the isolation procedure. Finally, we may consider the limitations of the electron microscope in determination of the size of DNA molecules. When used to determine the molecular weight of uniform populations of molecules such as those found in the T-even phages of *E. coli*, the method is probably accurate to ±10% of the absolute value.[20] However, when measurements are made on larger DNAs, such as those of yeast chromosomal DNA, technical problems may occur. The chief difficulty is placing a molecule in the 5×10^8 dalton size range onto the viewing grid without breaking it. If the technique is used wherein a drop containing the DNA-cytochrome complex is allowed to slide down a

microscope slide, the molecules may be broken in placing the drop on the slide or as the drop slides down. Another technique is available[59] in which the DNA is allowed to diffuse into the already formed cytochrome film. This eliminates handling the DNA but raises another problem. Larger DNA molecules diffuse more slowly than small ones, and truly large DNAs may not diffuse up to the film at all. Hence, the molecules seen are weighted statistically toward smaller molecules, and large molecules may be missed entirely.

AUTORADIOGRAPHY

To a large degree, autoradiography is a complementary technique to electron microscopy for the study of DNA. Like electron microscopy, it is often used to study DNA replication and, to a lesser degree, to study the sizes of molecules. Although autoradiography gives none of the fine details seen in the electron microscope, it does allow the study of the kinetics of the replication process and is generally capable of disclosing larger DNA molecules than the electron microscope. Indeed, Kavenoff[60] has obtained pictures of molecules apparently as large as 2.5×10^{10} daltons. Once again the technique, if used for size determination, is subject to errors due to the presence of protein-DNA interactions. For papers detailing the technique of autoradiography, see Cairns[1] and Lark.[61]

DNA PER CELL MEASUREMENTS

Measurements of the DNA per cell of a given organism are perhaps the most tedious measurements which are routinely undertaken. Yet, such measurements are often of extreme importance. For instance, DNA per cell measurements when combined with renaturation kinetics (which give the haploid genome size) can be used to obtain the apparent ploidy of an organism. In the case of yeast, the exact DNA per cell is presently of great interest in the problem of trying to decide if one or more than one genetic linkage group is found on the largest piece of nuclear DNA.

In theory, DNA per cell can be estimated by making any of four fundamental measurements. Thus, one can quantitate the amount of (1) native DNA, (2) DNA-associated deoxyribose, (3) DNA-associated pyrimidine and purine bases, or (4) DNA-associated phosphate. Historically all four measurements have been made; however, at the present time most researchers use tests designed for either deoxyribose or for native DNA itself. The reasoning that leads to the choice of these two measurements is fairly obvious. Of the tests which measure DNA-associated properties, deoxyribose is the preferred measurement since only it is specific for DNA. Thus, in deoxyribose measurements it is only necessary to extract free (non-DNA) deoxyribose and any possible interfering substances, while sources of phosphate or nucleic acid bases need not be considered.

The two most widely used methods of deoxyribose estimation are the diphenylamine method of Burton[62] and the diaminobenzoic acid (DABA) method of Kissane and Robins.[63] The Burton method relies on a colored reaction product of deoxyribose and diphenylamine. The DABA method, on the other hand, makes use of the fluorescence of the reaction product of DABA and deoxyribose. Both methods are used extensively. Perhaps there is a slight trend in more recent literature in favor of the DABA method. This may be due to a somewhat decreased probability of encountering interfering substances with DABA. It is this chance of interfering substances which is the weakness of both deoxyribose measuring techniques. Cells can and sometimes do contain polysaccharides or other materials, which show up falsely as DNA in the measurements and which cannot be preextracted. This possibility has led to the development of methods which quantitate native DNA itself. These methods can be easily controlled for interfering substances by the use of a DNAse control in which the native DNA has been enzymatically degraded. The DNAse control sample should give the same reading as the blank. Hence, positively interfering cell constituents may be detected. The most widely used method which measures native DNA is the ethidium bromide method introduced by LePecq and Paoletti.[64] As the method is commonly practiced, cell lysates are treated with detergent, proteinase, and RNAse at elevated temperature. Ethidium bromide is then added and its fluorescence, enhanced by its intercalation into the DNA double helix, is measured relative to a DNAse-treated blank (e.g., see Klotz and Zimm).[10] This method, of course, relies on the ability of the researcher to obtain clear and, preferably, colorless lysates.

Of course, many other DNA estimation techniques exist, and the literature is full of articles

detailing slight modifications of the procedures named. Perhaps the only point we wish to make here is that there is a definite need to make DNA per cell measurements by more than one method. Often it is extremely desirable to use one method such as DABA, which should give an upper bound to the measurement (since positively interfering substances might exist) in conjunction with a second method, such as the ethidium bromide method, which should give a lower bound (since cell lysis may not be complete or some DNA may be denatured or degraded).

DNA ISOLATION

As has already been discussed, at times it is necessary to take great pains in the isolation of DNA to try to avoid breakage of very large DNA molecules. Usually in such work purity of the resultant product is of somewhat less concern than its size. Certainly, the largest molecules thus far isolated under conditions where the purity of the resulting DNA was judged to be good have molecular weights of no more than 5×10^8 daltons.[9] In instances where larger material was studied, very little, if any, purification had been performed on the crude lysates.

However, often it is necessary to isolate DNA of no more than 100×10^6 daltons. Indeed, for renaturation studies the DNA isolated seldom has a molecular weight of over 20×10^6 daltons. Under these circumstances a great deal more care can be taken to insure the purity of the isolated DNA. The literature on DNA isolation procedures is almost endless, but most methods seem to make use of one or more of three basic techniques.

1. The method of Marmur[65] is the traditional choice. Here the crude cell lysate (made in a buffer containing EDTA, SDS, and perchlorate) is deproteinized by extraction with phenol or a mixture of chloroform and isoamyl alcohol. Ethanol is then added to the deproteinized aqueous phase of the extraction to precipitate nucleic acids. RNA is later removed enzymatically.

2. When larger sized DNA is desired, a method using CsCl isopycnic centrifugation is used. In this procedure cell lysates are incubated in an EDTA, detergent, and proteinase mixture. After this incubation, solid CsCl is dissolved in the lysate to the appropriate density, and the lysate is centrifuged to equilibrium. The DNA band is then collected from the centrifuge tubes. (The exact origin of this procedure is uncertain but its popularization is probably due to Ruth Kavenoff.)

3. Finally there is the urea-phosphate method of Britten et al.[66] In this method crude cell lysates are made 8 *M* in urea, 0.12 *M* in phosphate, 0.1% in SDS, and 1 *M* in $NaClO_4$. The lysate is then gently sheared and applied to a hydroxyapatite (HAP) column. Proteins and RNA are washed off the column with a solution of 8 *M* urea-0.12 *M* phosphate, pH 6.8. Then urea is washed from the column with 0.12 *M* phosphate buffer. Finally, the DNA is eluted from the column with 0.4 *M* phosphate buffer. This method is extremely useful for isolating radioactively labeled DNA due to its simplicity and the small amount of equipment used.[66]

There are, of course, many variations on the above procedures. There are also many tricks used to remove troublesome contaminants such as polysaccharides. However, this is not intended to be a review of DNA isolation procedures. Instead, at this point we would like merely to make a comment on DNA isolation as commonly practiced. Specifically, it must be noted that a given isolation procedure seldom gives a 100% recovery of the DNA that was initially present. This criticism is perhaps most valid of the Marmur procedure as practiced at times, where recoveries may be at the 10 to 20% level. The other two methods give higher yields on the average, with yields of from 50 to 100% probability. However, for a given organism it is conceivable that any or all of the methods might give low yields. The problem with low-yield isolation is that a given portion of the DNA may be specifically lost. Thus, a satellite may be visible in DNA isolated by one method and not by another (e.g., Britten et al.[66]). Barring an isolation at 100% efficiency, the best precaution against specific loss of DNA would seem to be the use of at least two different methods of isolation.

DNA RENATURATION KINETICS

Introduction

Since the late 1960's, DNA renaturation kinetics has been a major technique for studying the nature of DNA sequences in all types of organisms. Specifically, DNA renaturation kinetic experiments can provide information on the amount of

genetic information an organism potentially contains (complexity); the amount of repetitive sequences and the number of times they are repeated; and the interspersion pattern of these repeats, i.e., whether they are located together (tandem repeats) or interspersed between non-repeated (unique) DNA.

Of all the experiments on DNA of primitive eucaryotes discussed in this review, renaturation kinetics experiments are probably discussed most frequently. To review the theory, experimental techniques and factors influencing DNA renaturation kinetic experiments in any detail would require a review article at least as long as this one. Furthermore, excellent recent reviews by experts in the field already exist.[67,68]

There is one aspect of renaturation kinetics, however, about which we disagree with many authors.[69,70] We believe that even under "ideal" conditions, nucleation rate-limited kinetics followed by optical density or S1 nuclease assay should not be pure second order with respect to the time course of the reaction. It has been previously predicted that such kinetics should be pure second order.[71] We wish to discuss this point in some detail here. After this we will mention briefly other major aspects of renaturation kinetics.

Alternative Theory of Nucleation Rate-limiting Kinetics

To make our point, let us take the case where all the single strands have the same length L. The sequence of events which takes place when two partially complementary single strands renature is shown in Figure 18. Renaturation, then, involves a series of steps: first, a step which follows second-order kinetics, in which the two strands nucleate (that is, form a single or a few base pairs); then, several steps where the base pairs "zip" one pair at a time to obtain, finally, a helix, in which all the complementary base pairs have formed. In the case where all the zipping steps are very fast so that zipping to the fully zipped helix may be considered instantaneous, the kinetics is nucleation rate-limiting. It is this case we consider here.

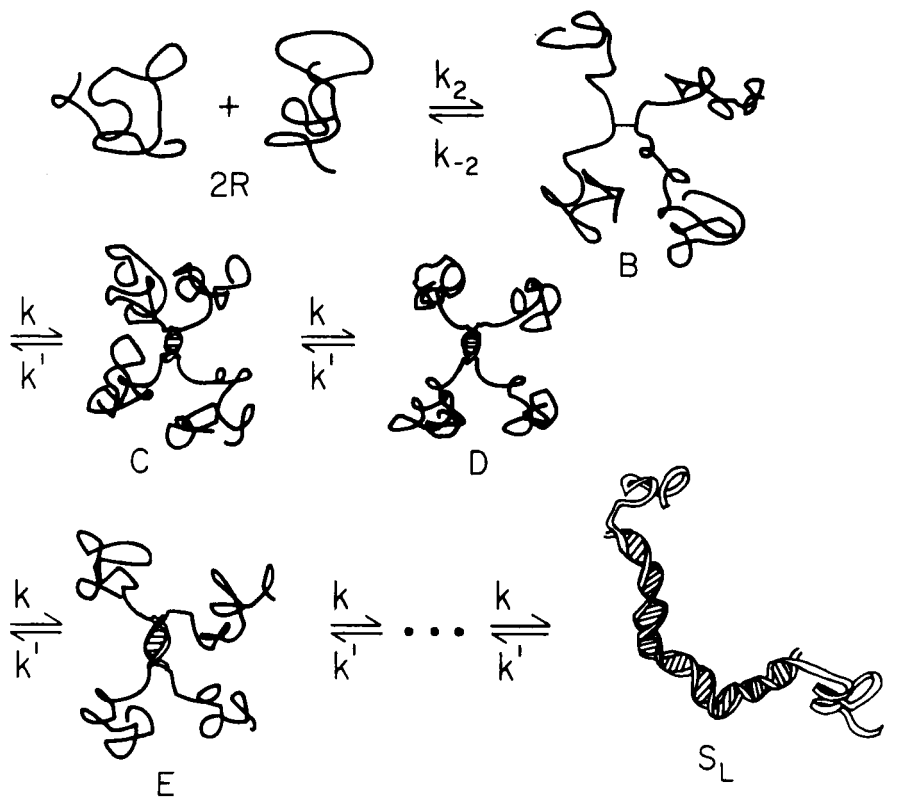

FIGURE 18. A schematic representation of the sequence of reactions involved in the renaturation of two partially complementary DNA single strands, L nucleotides long.

Under conditions where the single strands have been prepared by extensive random shearing of the DNA, the two fully zipped strands will only be partially complementary to each other. The number of zipped base pairs is a random function where, on the average, 2/3 L base pairs zip when all strands have length L.[72] Some possible products of the reaction are shown in Figure 19B. For the present we assume that three-stranded partially helical species (panel C) and concatemers (panel D) do not form. How these are taken into account will be discussed later. To summarize, our "ideal case" is

1. All single strands have length L.
2. Zipping is instantaneous, implying nucleation rate-limiting kinetics.
3. The partially helical duplex species (panel B of Figure 20) have, on the average, 2/3 L base pairs.
4. The single-strand ends on partially helical duplex species do not react.

In order to describe the time course of the renaturation reaction we define the following parameters.

- k_2 = The second-order rate constant for nucleation of free single strands of length L nucleotides.
- R_0 = The concentration of free Watson or Crick single strands at time t = 0.
- R(t) = The concentration of free Watson or Crick single strands at time t.
- S(t) = The concentration of nucleated Watson or Crick strands at time t. If zipping of the helix is instantaneous after nucleation, then S(t) is the concentration of partially helical duplexes at time t. Clearly, $R_0 = R(t) + S(t)$.
- A_0 = The concentration of nonhelical base pairs at time t = 0. In terms of strands, $A_0 = LR_0$.
- A(t) = The concentration of nonhelical base pairs at time t. If the zipping reaction of the pL base pairs of a nucleated duplex is instantaneous, then $A(t) = A_0 - pLS(t)$. For the ideal case, p = 2/3.

We assume that the nucleation of strands is a second-order reaction:

$$-\frac{dR(t)}{dt} = \frac{dS(t)}{dt} = k_2 (R(t))^2 \quad (40)$$

The rate of nucleation will follow this single, simple equation only if all single strands nucleate with the same rate constant, k_2. This will indeed be true if all the single strands have about the same GC content and the same length. That the single equation, Equation 40, is a good approximation for simple DNA, such as most bacterial and bacteriophage DNAs which have been mechanically sheared by the standard methods (sonication, French press, etc.), is confirmed by the fact that the kinetics when followed by HAP chromatography (which detects nucleation) is nearly pure second order.[73]

Both optical density and S1 assay follow the

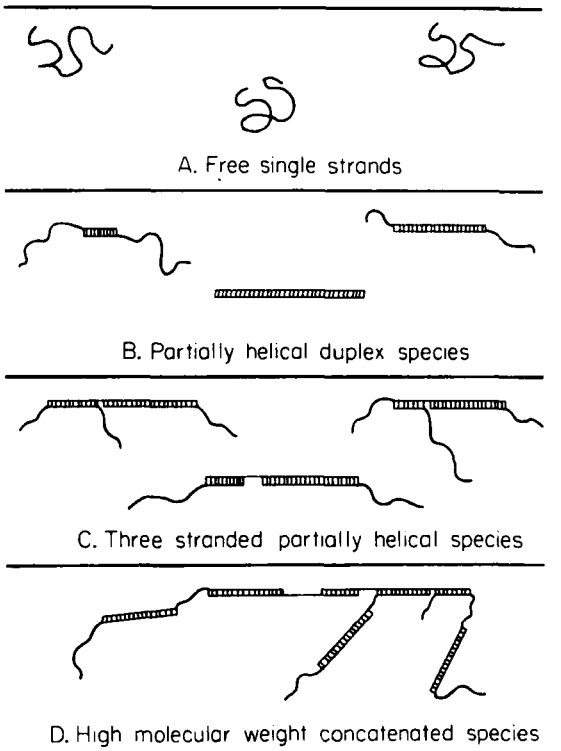

FIGURE 19. A schematic representation of the renaturation reaction of DNA single strands with a random distribution of partially overlapping sequences. Panel A represents the initial population of denatured single strands. The reaction of two single strands will yield a population of partially helical duplexes represented in panel B. The reaction of the tails of partially helical duplexes with free single strands or with each other will give such products as those depicted in panel C. Panel D shows some typical concatemers that will be formed after about 60% reaction.

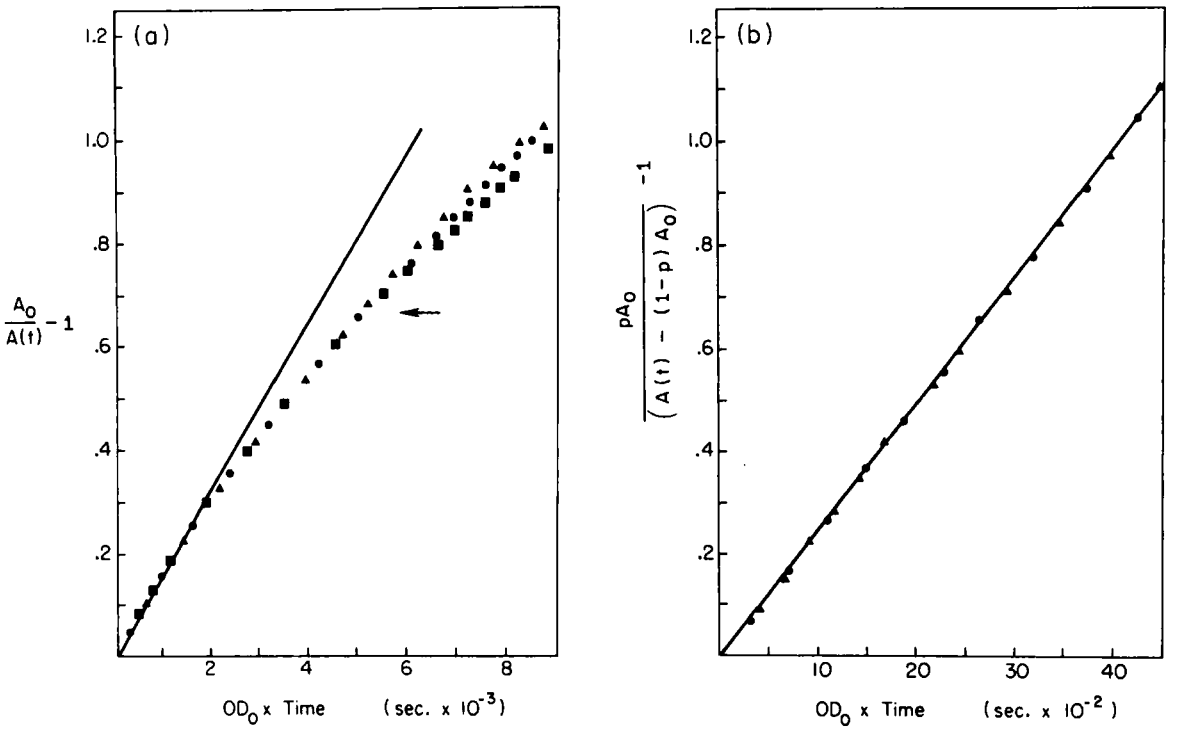

FIGURE 20. (a) Kinetic data for the renaturation of T2 DNA in 0.1 *M* Na^+ at 60° is graphed according to a standard RSO relationship with a concentration-normalized time axis. Data for *E. coli* DNA, renaturing in 0.2 *M* Na^+ at 70°C are included. The time axis for the *E. coli* DNA reaction is rate normalized, i.e., the time scale is $(k_2, E.\ coli/k_2, T2)\ OD_0\ t$. (•) T2 DNA $A_0 = 4.37 \times 10^{-5}$ M base pairs. (▲) T2 DNA $A_0 = 1.69 \times 10^{-4}$ M. (■) *E. coli* DNA $A_0 = 2.30 \times 10^{-4}$ M. The arrow indicates the 40% reaction point ($A_0/A(t) - 1 = 0.667$). (b) The kinetic data of part a replotted according to the modified RSO relationship (Equation 7), with p = 0.71. (•) T2 DNA, $A_0 = 4.37 \times 10^{-5}$ M. (▲) T2 DNA, $A_0 = 1.69 \times 10^{-4}$ M. In this figure, OD_0 stands for the initial denatured optical density which is proportional to DNA concentration.

concentration of nonhelical (or helical) base pairs as a function of time, A(t). In order to cast Equation 40 in terms of A(t) we proceed as follows.

From the relationship between R_0, S(t), and R(t), Equation 40 can be written as:

$$\frac{dS(t)}{dt} = k_2\ (R_0 - S(t))^2 \qquad (41)$$

Straightforward integration gives:

$$\frac{1}{(R_0 - S(t))} - \frac{1}{R_0} = k_2\ t \qquad (42)$$

If we now cast Equation 42 in terms of nonhelical base pair concentrations, A(t) and A_0, we have:

$$\frac{p}{(A(t) - (1-p)A_0)} - \frac{1}{A_0} = k_2'\ t \qquad (43)$$

where

$k_2/L = k_2'$, the nucleation rate constant per molar nucleotides.

Equation 43 can be algebraically manipulated to give the form:*

$$\frac{A_0}{A(t)} - 1 = \frac{p\ k_2' A_0\ t}{(1 + (1-p)k_2'\ A_0\ t)} \qquad (44)$$

Equation 44 predicts downward curving plots when $\frac{A_0}{A(t)} - 1$ is plotted against t – that is, the standard reciprocal second-order (RSO) plot.

Such downward curving plots are indeed observed for kinetics followed by S1 assay[73,74] and optical density.[69] An example of a curved RSO plot of optical density data is shown in Figure 20a. For kinetics followed by HAP chromatography, all nucleotides, whether they are in helix or single-

*At this point instead of using the average value, p, one can write a separate differential equation for each species with i = 1, . . . , L base pairs when $k_2 = ik_2$ and obtain the same results in a rigorous manner.

strand ends, are counted equally. This is equivalent to setting p = 1 in Equation 44, yielding:

$$\frac{A_0}{A(t)} - 1 = k_2' A_0 t \tag{45}$$

which is the equation for pure second-order kinetics. Data obtained by hydroxyapatite do indeed approximate second-order kinetics well.[73]

Real experiments, followed by S1 assay or optical density, however, are somewhat removed from the ideal case we have just considered. In the first case, the single-strand length varies from strand to strand. Theory[75] and experiment[74,75] show that the effect of length heterogeneity is to lower the average overlap of partially helical duplexes, so that in many cases for mechanically sheared DNA a fraction of p = 0.6 base pairs forms instead of the ideal p = 2/3 fraction.

Length heterogeneity can lower p in another way. As discussed before, longer single strands will react faster as they contain more nucleation sites (i.e., bases). These different reaction rates will also cause downward curvature in RSO plots, hence lowering the value of p because in a strictly empirical sense p can be used as a measure of downward curvature (lower p values more curvature). This effect, as we said before, must be slight because of the almost pure second-order nature of kinetic data obtained from HAP chromatography.

Another nonideal effect is that even in the first 40% of reaction some three-stranded partially helical species form so that more base pairs form than predicted from our ideal case. Rau[69] has worked out a theory which includes these three-stranded species and has shown that their existence can be taken into account approximately by simply increasing the parameter, p.

The Rau theory also shows that even if the bases in the single-strand ends of partially helical species have the same rate constant of nucleation per nucleotide as bases in free single strands, the kinetics will still not be pure second order; that is, RSO plots will still curve downward. The condition for pure second-order kinetics is that the rate constant times the average number of base pairs which zip up must be constant when any partially helical species reacts with any other partially helical species or free single strand. In order for that constancy condition to hold, clearly the rate constant of nucleation would have to be larger for partially helical species than for single strands, since fewer base pairs zip up when partially helical species nucleate. In fact, the rate constant of nucleation for partially helical species is about half that for free single strands.[69,73]

Thus, the parameter, p, which was originally defined as the average fraction of base pairs in partially helical species, now takes on a new meaning. It is now a parameter which includes the slight effect of length heterogeneity on reaction rate, the effect of formation of three-stranded species, and, of course, the major effect that helical duplexes are only partially complementary. It is now a parameter which is perhaps best determined experimentally, although a good theoretical estimate of it can be made.[69]

Analysis of curvature in RSO plots of renaturation kinetic data obtained by both optical density and S1 assay under nucleation rate-limiting conditions shows that the best-fit value of p is 0.70 for a fairly wide range of conditions.[69] Rearranging Equation 44 to yield:

$$\frac{pA_0}{(A(t) - (1-p)A_0)} - 1 = k_2' A_0 t \tag{46}$$

indicates that a plot of the left hand side vs. t should be linear using p = 0.7. Such a plot is shown in Figure 20b.

Another empirical equation derived by Morrow[74] which adequately describes downward curving RSO plots is

$$\frac{A(t)}{A_0} = \frac{1}{(1 + k_2 A_0 t)^n} \tag{47}$$

In summary, the main cause for downward curvature in RSO plots is the fact that, on the average, only a fraction (0.60) of L possible base pairs zip after nucleation. Other factors contribute to make p = 0.70, the best-fit value. Another way to look at downward curvature is that many researchers take the optical density of the native DNA as the infinite-time optical density, OD(∞), when analyzing renaturation kinetic data, so that the total extent of reaction is OD(0) - OD(∞) where OD(0) is the denatured optical density. Instead, they should be taking 2/3 [OD(0) - OD(∞)] for the ideal case. The above theory applies to about the first 40% reaction only, because formation of many-stranded species which will occur later in the reaction is not accounted for by this treatment.

Under conditions of high DNA concentration, high cation concentration, low complexity, or low temperature, we[69,70,76] believe that the kinetics followed optically continuously becomes even more complicated because the nucleation rate-limiting assumption no longer holds; that is, the rates of zipping of helices become comparable to nucleation rates. The reason for this slow zipping appears to be secondary structure in the single strands which must be broken as the double-stranded helix zips. The net effect of slow zipping is to artificially linearize RSO plots so that the kinetics appears to be second order. However, under these conditions the kinetics does show anomalous behavior such as dependence of the observed rate constant on DNA concentration and complexity in a way not predicted by simple theory. Details may be found in Reference 76.

In practice, it is best to work under nucleation rate-limiting conditions because data are easily interpreted using Equation 46. While it is not possible to list one convenient set of nucleation rate-limiting conditions for all experiments, in general such conditions are those where nucleation rate is slow (low DNA concentration, high complexity, and low cation concentration) and where secondary structure in the single strands is minimized (low cation concentration and high temperature). Two specific examples of nucleation rate-limiting conditions are

1. For *E. coli* DNA: 0.2 *M* Na^+ at 70°C at concentration 2.30×10^{-4} M base pairs and L ≃ 1,000 base pairs.
2. For T2 DNA: 0.1 *M* Na^+ at 60°C at concentration 1.69×10^{-4} M base pairs and L ≃ 1,000 base pairs.

Perhaps the best way to determine if conditions are nucleation rate-limiting is to determine if rate constants found either by the initial slope of an RSO plot or the slope of the modified plot (Equation 46) are concentration independent. For further details, see Rau.[69]

Very Brief Review of Renaturation Kinetics Literature

There are three commonly used ways to follow DNA renaturation kinetics: optical density, hydroxyapatite (HAP) chromatography, and S1 nuclease assay. Which method one chooses depends on several factors: the amount of DNA available, whether one wishes to separate repeated from unique DNA, the precision required, ease of data interpretation, etc.

In terms of versatility, HAP chromatography is perhaps the best. It may be used either analytically or preparatively. Both small amounts of radioactive DNA or large amounts of DNA may be used in many cases, making it possible to study both very fast and very slow renaturing DNA under the same experimental conditions. Data analysis of kinetics followed by HAP chromatography is simple, since such kinetics are pure second order to a good approximation. An excellent review of the experimental practice of DNA renaturation kinetics followed by HAP chromatography is that of Britten et al.[68]

Optical density is the most precise way to follow renaturation kinetics, since optical densities can be accurately measured. Most commonly, the kinetics are followed by continuously recording the optical density decrease at 260 nm as the DNA renatures. Since precise recording spectrophotometers exist, a large quantity of data can be collected conveniently. However, such experiments are limited to the range of optical densities acceptable to the spectrophotometer (0.2 to 20 OD, depending on cuvette optical path length). Higher OD (i.e., higher concentration) samples may be run if one employs an aliquot method such as that used in HAP chromatography or S1 assay experiments. Data analysis, as discussed before, can be complicated. An excellent current review of optical methods is that of Wetmur.[67] As discussed above, we disagree with the author as to one aspect of optical renaturations, i.e., we believe the kinetics are not pure second order under any circumstances of which we are aware, except renaturation of a single restriction fragment of a given DNA.

The most recent method developed for following renaturations is S1 nuclease assay. Details of this procedure are discussed by Morrow[74] and Britten et al.[73] The method, as do optical density experiments performed under nucleation rate-limiting conditions, follows the number of base pairs formed, so the kinetics will have the form described by Equation 46. Since radioactive DNA is generally used, low DNA concentration can be employed to study fast kinetics. Also, this method easily handles high DNA concentrations to study slow kinetics. Several articles illustrating the use of

DNA renaturation kinetics to study the DNA of organisms may be found in Reference 77.

A SUMMARY OF FINDINGS ON HIGHER EUCARYOTES

At this point we have outlined the basic techniques for the physical study of DNA and are ready to go on to the various specific findings about the DNA of primitive eucaryotes. First, however, it is necessary to pause for a brief summary of the findings made concerning the DNA of higher eucaryotes and, in light of this summary, to consider the reasons for studying the DNA of primitive eucaryotes.

By definition, all eucaryotes, high and low, are found to have some considerable portion of their DNA enclosed in a nuclear membrane. It is difficult to imagine an intermediate state in this regard (i.e., an organism with a partially formed nucleus), and certainly no extant organisms are known to show such an intermediacy. This nuclear DNA shows at least three properties which are shared, to a surprisingly universal degree, among all higher eucaryotes (plants and animals alike) and which stand out as jump discontinuities between procaryotic and higher eucaryotic DNA.

First, nuclear DNA is found associated with certain basic (histones) and nonbasic proteins (the rough ratios of DNA to histone to nonhistone proteins are 1g:1g:1g) to form chromatin. Though many interspecies differences exist in chromatin, particularly with regard to the nonhistone chromosomal proteins, the fundamental structure of the chromatin is emerging as a surprisingly constant form for both plant and animal species. Thus, it has been known for a considerable time that the amino acid sequence of some of the histones is amazingly constant in all organisms studied thus far. For instance, there is the famous example that histone IV has only two amino acid differences between cow and peas.[78-80] Similarly, histone IIb is highly conserved in evolution.[81] More recently, various workers (for a brief review, see Reference 82) have demonstrated that there is a very constant subunit structure in chromatin from rather diverse animal species. This structure, sometimes likened to beads on a string, consists of individual repeating units formed of roughly 200 base pairs of DNA interacting in a supposedly very specific manner with a group of histone molecules to form a basic monomeric structural unit of chromatin. This subunit organization was first suggested by Olins and Olins[83] from the appearance of whole-mounted chromatin from osmotically shocked nuclei as viewed in the electron microscope. Such chromatin quite literally looks like a series of beads on a string. Further evidence was gained from the work of Kornberg[84] on the association of histones which led him to suggest that histones exist in chromatin as stoichiometric assemblies of a small number of histone molecules with a length of DNA to form a monomeric unit. Also extremely suggestive of such a subunit composition was the finding by Hewish and Burgoyne[85] and others that limited nuclease digestion of chromatin chopped the chromatin DNA into a linear series of fragment sizes with a monomeric unit of roughly 200 base pairs. Taken as a whole, these findings strongly suggest a subunit structure for chromatin which is present in all organisms thus far studied. Although work on plants is at an early stage (McGhee and Engel[86] report similar nuclease digestion patterns for pea and mouse chromatin), the fact that histones seem to form the framework for the beads and that histones are conserved in plants and animals strongly suggests that plants and animals alike will be found to have the same basic chromatin structure – a structure which stands in stark contrast to the DNA of bacteria which has been found to have associated no histones. Chromatin is more properly a subject of a review of its own; e.g., see Elgin and Weintraub[81] for a recent review.

Second, in contrast to procaryotic DNA which has a small number of repeated-sequence elements (less than 1% of the genome), the DNA of higher eucaryotes has been found to have much higher amounts of repeated DNA, ranging from 10 to 90% of the genome, with the "typical" animal cell having roughly 30% repeated DNA arranged into two basic classes: (1) highly repeated DNA sequences generally found from 10^5 to 10^6 times per cell forming long blocks and associated with density satellites and centromeric heterochromatin and (2) so-called moderately repeated DNA present from 50 to 5,000 times per genome. The very elegant work of the Britten-Davidson group at the California Institute of Technology (for a summary, see Davidson et al.[87]) has revealed that much of the moderately repeated DNA is found as blocks of 300 bases of repeated-sequence DNA interspersed with longer blocks of unique-sequence

DNA some 1,200 or so bases long. (These numbers are average figures with considerable heterogeneity found in the actual distributions.) Moreover, the Davidson group has shown that the 5′ ends of most cellular messenger RNAs are transcribed from unique-sequence DNA adjacent to repeated DNA sequences. This, of course, is consistent with the idea that the repeated DNA sequences might function as control elements.[88] This structural organization of DNA in long blocks of highly repeated sequences, interspersed unique- and moderate-repeated DNA, and finally, long blocks of unique DNA has been found in animals as diverse as sea urchins and toads and appears to be the general, if not universal, model for animals. Of the animals studied, only *Drosophila* appears to be an exception to the rule in that its blocks of repeats are much longer than 300 bp, averaging 5,600 bp or more.[89]

Plants also have large amounts of repeated DNA, with the general finding being that the more DNA in the genome the larger the proportion which is repeated.[90] Though higher plants have the same basic sequence elements (highly repeated, moderately repeated, and unique) in their DNA, it has yet to be demonstrated that the moderately repeated elements are interspersed with unique DNA as in animal DNA, though the answer to this question should appear quite soon.

The final major distinction between the DNA of procaryotes and eucaryotes is the number of origins for DNA replication. Of the bacteria studied (*E. coli* and *B. subtilis*), it has been found that there is a single unique origin for DNA synthesis – synthesis which is at least initially bidirectional.[91] However, animals[92-94] show multiple origins for bidirectional DNA synthesis. These sites appear on the average at distances of 10 to 60 μm apart, though there is considerable divergence around the mean from organism to organism and even in a given organism at different times in development.[92-96]

Of course, there exist other notable differences between the DNA of procaryotes and higher eucaryotes. These include the presence in eucaryotes of organelles such as mitochondria and chloroplasts which contain DNA themselves, the presence of long inverted repeat sequences[97-99] in eucaryotic nuclear DNA which appear to be absent or present in much smaller amounts in procaryotic DNA, and the presence of a coiling cycle in eucaryotic chromosomal DNA varying the state of condensation of the DNA from interphase to metaphase. However, the three differences named above serve quite well to make a basic point: The fundamental design of higher eucaryotic DNA is strikingly different from that found in bacteria, and when only higher eucaryotes are considered, these differences stand out as discontinuities in evolution.

We can now turn to the original question – why study the DNA of primitive eucaryotes? Of course, from a purely scientific point of view there is no reason to favor the study of any given organism or group of organisms. However, as will soon become evident, higher eucaryotes have already been studied much more extensively than lower forms by almost every known technique. Given the immensity of the task of truly understanding any organism at the molecular level, the presently more advanced state of knowledge concerning higher eucaryotes has a sort of momentum of its own, to some extent demanding that an even larger proportion of the available expertise be applied to higher eucaryotic systems. Yet there remain persuasive arguments for studying primitive eucaryotes. In the first place, there are many inherent advantages to some of the more primitive systems. Thus, the yeasts, certain other fungi, and cellular and true slime molds present systems which grow rapidly and in a controllable manner and which are readily amenable to genetic analysis. In some cases the amount of DNA found in these organisms makes them easier to study than a mammalian system with its picogram quantities of DNA per cell. However, even when a primitive organism presents no obvious advantages over higher systems, its study may still be advisable to place some limits on the diversity of structure and function of eucaryotic DNA. Finally, there is at least a possibility that, in the process of exploring the diversity of DNA form in lower eucaryotes, insights will be gained into the manner of evolution of the DNA characteristics discussed above which serve to delineate higher eucaryotes from procaryotes. This is, of course, a risky proposition, for although we know that some modern phyla have fossil records dating back to the very beginning of eucaryotic evolution (i.e., well into the Precambrian), there is no guarantee that modern day representatives of ancient orders will retain primitive traits.

LOWER EUCARYOTIC DNA

Choice of Organisms

The exact choice of organisms to discuss in a review on primitive eucaryotes is, at best, difficult. For reasons of convenience, we have decided to deal with three major classifications of organisms: the algae, the fungi plus cellular and true slime molds, and the protozoans. Doubtless, we will offend some people for omissions and others for inclusions.

PRIMITIVE EUCARYOTIC ALGAE

Figure 21, taken from Loeblich, Jr.,[100] shows the currently known geological record of the various algal groups. It is apparent from the figure that certain algae, most notably the red and the green, present a continuous or nearly continuous geological record back to the times when eucaryotes supposedly first appeared. Other groups – the dinoflagellates and euglenoids – do not show continuous records back to the Precambrian, yet are believed, largely on a basis of microscopically observed attributes, to have diverged from the line of eucaryotic evolution almost as early as the red algae. (It should be stated at this point that it is problematic whether the various algal phyla should be placed as branches on a tree or as separate trees growing from a common protoeucaryotic pool.)

It is important to note that some modern divisions, though of ancient lineage, contain a rather wide spectrum of organizational complexity among the various members. Thus, the red algal group contains organisms ranging from apparently primitive single cells to giant seaweeds showing organ development, and modern day brown algae are all quite complex structurally. However, there exists the distinct possibility that living algae may retain DNA structural traits primitive enough to partially bridge the gap between the procaryotes and higher eucaryotes.

Unfortunately, the DNA (particularly the nuclear DNA) from the various algae mentioned above has been little studied. Thus, the discussion which follows is limited. However, there do exist enough data to merit discussion of both the evolution of nuclear DNA organization and the evolutionary origins of chloroplasts and mitochondria.

Phaeophyceae, Prasinophyceae, and Rhodophyceae

Almost nothing is known of the DNA from members of these three algal groups except perhaps a few DNA per cell determinations and chromosome counts. This is particularly sad in the case of the Rhodophyceae or red algae, for these algae not only have an extremely ancient fossil record but also show the closest relationship, with respect to their pigment complement and chloroplast structure, to the procaryotic blue-green algae (both groups contain chlorophyll a but not chlorophyll b or c, plus accessory pigments phycocyanin and phycoerythrin; both have unstacked photosynthetic lamellae; and both lack flagella at any stage), making them prime candidates for truly primitive forms. (For a review of the red algal biology, see Dixon.[101]) However, the only account we could find concerning the isolation of red algal DNA indicated that there were more problems than answers. Specifically, Nasatir and Brooks[102] found that the red alga *Griffithsia globulifera* contained large quantities of polysaccharides which tended to copurify with DNA through Marmur-style isolation procedures; moreover, they discovered a nuclease activity present in the alga. Brooks' answer to these problems was to isolate nuclei and then extract DNA from the isolated nuclei. However, this procedure is tedious and gives a marginally satisfactory product (hyperchromicity is 23%).

Recently, work has commenced in our laboratory on the DNA of the red alga *Porphyridium aerugineum.*[103] Although still at an early stage, study of this morphologically primitive species has shown that neither Marmur-style extraction procedures nor somewhat more sophisticated techniques involving banding of cell lysates in CsCl in the ultracentrifuge are of much use in DNA isolation. This is not surprising, since red algal polysaccharides contain negatively charged sulfate moieties and are present in such large amounts – up to 200 times the DNA content of the alga on a weight-to-weight basis.[103,104] Polysaccharides generally copurify with DNA to some extent even if they are uncharged; hence, the addition of negatively charged groups makes the chemical structure of the polysaccharide similar enough to DNA to make chemical distinction difficult at the preparatory level. The large quantities of polysaccharide further complicate matters. Thus, even though the buoyant density of the polysaccharide is not

identical to that of the DNA, the large quantity of polysaccharide simply overburdens the entire gradient in CsCl density gradient centrifugation. Finally, we have found the polysaccharides to be relatively insensitive to commercial enzymes such as amylase, β-galactosidase, or agarase.

However, the problem is not hopeless. The urea-phosphate-hydroxyapatite method of Britten[66] seems to be relatively efficient at separating the DNA from polysaccharides (as well as other cellular material). Moreover, since this method is so quick, nucleases, definitely a problem, have little time to work. The final product from HAP isolation seems to be contaminated at 260 nm (hyperchromicity still only 25 to 29%), but further purification by CsCl buoyant density centrifugation should improve the purification. DNA per cell measurements by the DABA method reveal a total DNA content of 7×10^{10} daltons.[103] This complexity is roughly comparable to that of the single-celled green algae (to be discussed later).

The next important questions to be answered about the DNA of red algae are as follows. (1) Are there any eucaryote-like repeated sequence elements in the nuclear genome? (2) What is the percentage of cellular DNA in chloroplast and nucleus in this cell? (The chloroplast physically occupied some 90% of the cell volume.[105])

While the DNA of red algae has been little studied by direct means, recently an indirect study has been made on the chloroplast DNA (chDNA). Specifically, Bonen and Doolittle[106] have examined the oligonucleotide sequences present in the 16S rRNA (believed to be the chloroplast rRNA) of a *Porphyridium* species and compared these sequences with those found in the blue-green alga *Anacystis nidulans*, the bacteria *B. subtilis* and *E. coli*, and in the 18S rRNA (believed to be the cytoplasmic rRNA) from *Porphyridium*. (As we will see later, it has been proven for other algal species that the chloroplast DNA does indeed code chloroplast rRNAs.)

The basic technique involves the isolation of P^{32}-labeled chloroplast 16S rRNA from the organism and its digestion with T_1 ribonuclease, which cuts at the 3′ side of guanidylate residues. The digested RNA fragments are fingerprinted by two-dimensional electrophoresis by the basic method of Sanger and co-workers,[107] after which the individual oligonucleotides can be sequenced.[108] The various oligonucleotides from a given 16S rRNA can be compared with those from other organisms, and the phylogenetic relationship of the organisms can at least be approximated. Thus, after allowances are made for chance sequence homologies it is found then that there is substantially no homology between the 18S cytoplasmic and 16s chloroplast rRNAs of *Porphyridium* – the two molecules sharing only 8 to 10 pentamers and 2 hexamers while two nonrelated RNAs of this size (i.e., 16S) could be expected to share 9 pentamers and 2 hexamers. On the other hand, the chloroplast 16s rRNA seems to have a strong sequence homology with each of the three procaryotic 16S rRNAs. For each oligonucleotide size class, the number of shared sequences exceeds the number expected for two unrelated RNAs by such a margin that the probability of the number of coincident species occurring by chance ranges from 1 in 100 to less than 1 in 100,000. The closest homology lies between the 16S chloroplast rRNA and those of *A. nidulans* and *B. subtilis*, where there are 11 to 12 shared oligonucleotides of size 7 through 12. The authors note that it is a bit surprising that the chloroplast rRNA shares more sequences with *A. nidulans* and *B. subtilis* than either of these two organisms shares with *E. coli*. Finally, the authors find that of the 31 sequences, pentamers or larger, shared by all three procaryotes, 25 are also found in the chloroplast rRNA while only 7 are found in *Porphyridium* 18S rRNA.

The basic point which the authors would like to address is the origin of the chloroplast. The two most widely favored theories on this subject are (1) the chloroplasts arose as an endosymbiotic blue-green alga and (2) the chloroplasts arose when a basic "protoeucaryote" (generally considered as a polyploid procaryote) compartmented part of its genome in a chloroplast membrane and other parts of its genome in nuclear and mitochondrial membranes. (For essays on this subject, see References 109 to 111.)

Now certainly the finding of a procaryote-like 16S rRNA in the chloroplast ribosome is consistent with the endosymbiotic hypothesis. However, it must be noted that the DNA which becomes compartmented in the chloroplast according to the second hypothesis would most likely have been procaryote-like as well. Hence, the proponents of compartmentalization need only argue for the presence of some evolutionary pressure for retention of the procaryote-like sequence of the chloro-

plast 16S rRNA or pressure against its retention in the nuclear genome. Such pressure might simply be tied in with the fact that the chloroplast genome is apparently present in multiple copies (see later discussion). Hence, the same mechanism which serves to keep the multiple copies relatively homogeneous in sequence, if indeed they are, might tend to preserve the primitive sequence of the 16S rRNA. Thus, it seems that the evidence gained from sequencing the 16S rRNA of the chloroplast strengthens only slightly the endosymbiotic hypothesis. (The authors themselves stated that the evidence seemed "most consistent with" the endosymbiotic hypothesis but did not rule out the second hypothesis.) Indeed, it can seriously be argued that attempts to distinguish between the two theories, while inherently valuable for the information provided concerning the nature of chloroplasts, are predestined to failure.

The Dinophyceae

When only clearly identifiable fossils are considered, the fossil record of the dinoflagellates extends no further than that of the higher plants (Figure 21). However, if dinoflagellate affinities are assumed for certain acritarchs (i.e., certain fossils known as acritarchs cannot be identified directly with any living order; however, some of these acritarchs bear striking resemblance to modern dinoflagellates), the dinoflagellate record can be extended into the Precambrian.[100] Thus, the fossil record is at least suggestive of an ancient origin of the group.

Microscopic examination of contemporary dinoflagellates at both the light and electron microscope levels reveals a fascinating picture for the chromosomes and nucleus. The organisms contain huge amounts of DNA (ranging from 3.8 pg in *Crypthecodinium cohnii*[112] to 200 pg in *Gonyaulax polyedra*[113]) arranged into numerous (an average value might be 80 with the range extending from 5 to 256), permanently condensed chromosomes.[114,115] Viewed in the electron microscope, the chromosomes reveal a banded pattern when cut longitudinally and a swirl or fingerprint-like pattern of chromatin fibrils when seen in transverse section (see Figure 22). Notably, these fibrils are 30 to 60 Å in diameter, smaller than eucaryotic chromatin fibrils and much closer to the fibrils seen in bacterial nucleoids. Moreover, the swirl pattern itself is like that seen in bacterial nucleoids.[116] Cytochemical stains fail to reveal any RNA or basic proteins in the chromosomes, in striking contrast to the situation in higher eucaryotes.[117] Recently, this old cytological observation has been largely confirmed by Rizzo and Noodén,[118,119] who isolated chromatin from several dinoflagellate species. They found the basic protein content to be 0.1 g or less per 1 g of DNA. One species of basic protein was found which migrated on an SDS polyacrylamide gel at approximately the same rate as corn histone IV. However, since this protein had an amino acid composition considerably different from known histones and was not present in stationary cells, they concluded that it was probably not a histone.

Mitotic division in dinoflagellates is apparently extremely primitive. No spindle is seen. Instead, at mitosis, cytoplasmic channels containing microtubules invaginate the nucleus, whose membrane remains intact, and the nucleus itself appears to ride along these cytoplasmic channels toward the two poles, finally pinching off to form the two daughter nuclei. The cigar-shaped chromosomes are seen to be attached at one of their ends to the nuclear membrane on the surface of the channels, and it is presumed that the movement of the membrane mediates the segregation of the chromosomes to the daughter nuclei.[120] In 1965, citing the appearance of the DNA fibrils, their lack of basic protein, and the primitive mitotic process, Dodge[121] suggested that the dinoflagellates might represent an intermediate condition between the procaryotic and eucaryotic states, which he called mesocaryotic.

The very unusual appearance of dinoflagellate chromosomes has led microscopists to construct models of the DNA fibril organization in the chromosomes. Four models in all have been suggested. In 1965 Giesbrecht proposed a model made up of one piece of DNA scaffolded on hypothetical non-DNA supporting structures.[122] Having studied the minor cycles of relative condensation and expansion found in both dinoflagellate chromosomes and bacterial nucleoids,[116] Giesbrecht was anxious to explain these movements in his model (Figure 23A). Also in 1965, Grassé et al.[123] proposed a model of the dinoflagellate chromosome which consisted of DNA-containing fibrils with no supporting structures. This model was polytenic (Figure 23B). In 1968 Bouligand et al. proposed a model likened to a stack of coins in which each "coin" was made up

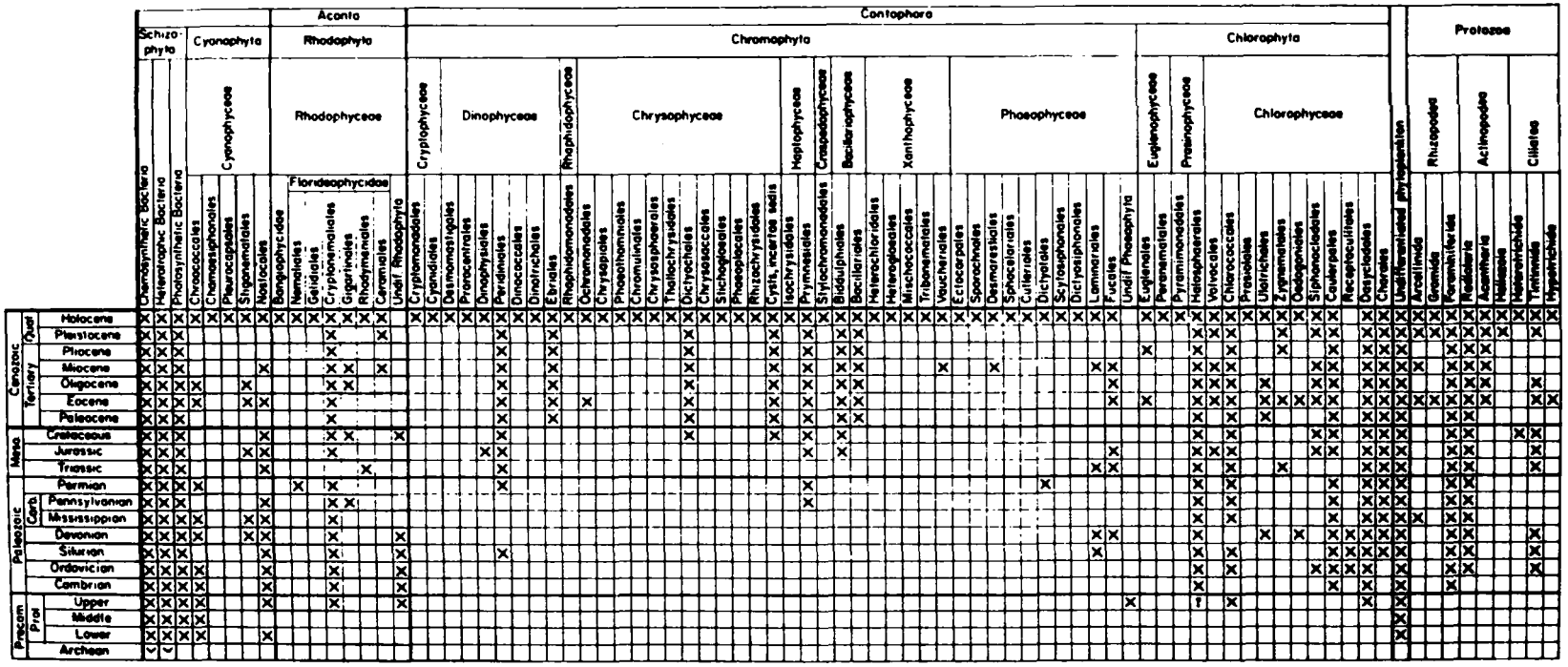

FIGURE 21. Geologic ranges, as presently known, for the major groups of Protista and Monera (bacteria and blue-green algae). (X) Known fossil occurrence. (After Loeblich, A. R., Jr., *Taxon*, 23, 277, 1974. With permission.)

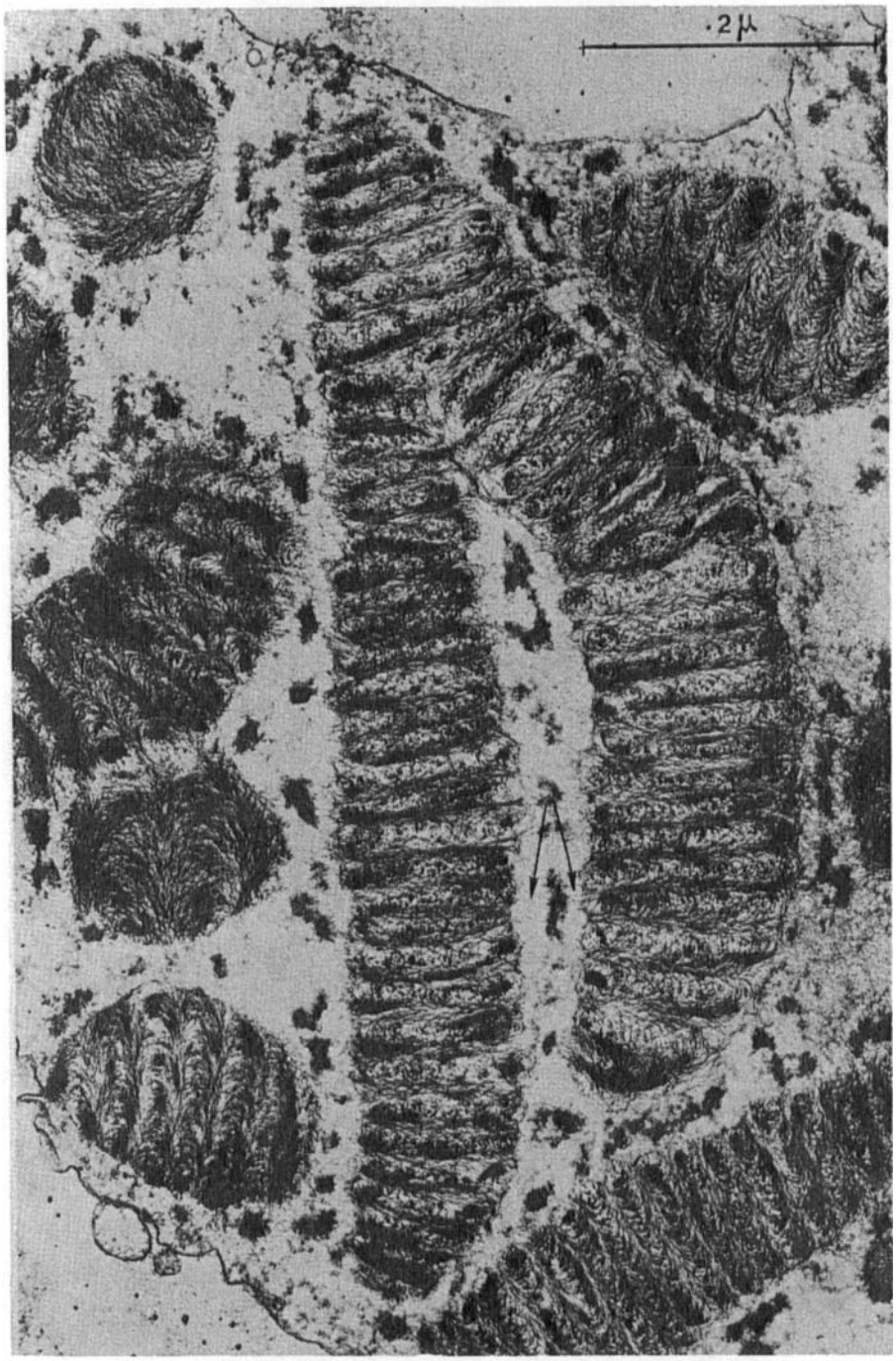

FIGURE 22. *P. micans* chromosomes shown in thin section. The V-shaped body is thought to be a dividing chromosome. The arrows point to fibrous attachments to granular border, located between the separated daughter chromosomes (X 21,900). (From Soyer, M. O. and Haapala, O. K., *J. Microsc.* (Paris), 19, 137, 1974. With permission.)

23a

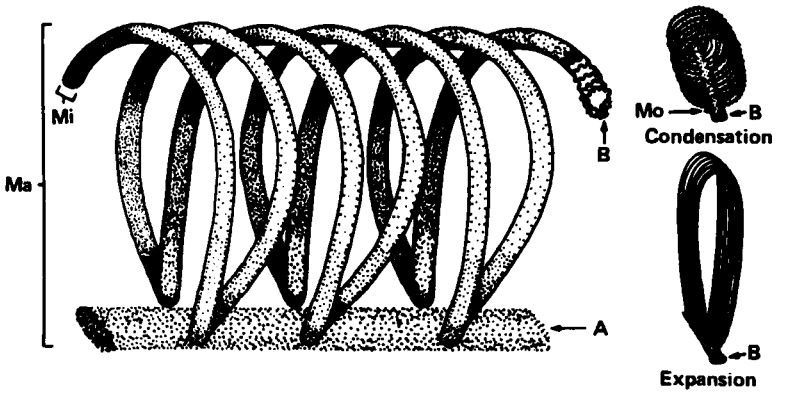

FIGURE 23. Dinoflagellate chromosome models. (a) Geisbrecht model. Schematic representation of the basic principles of the "serpentine coiling" system of the dinoflagellate chromosome. (Ma) Major system. (Mi) Minor system. (Mo) DNA containing filaments, forming a coiled molecular system. (A) Hypothetical supporting structure of the major system. (B) Supporting structure of the minor system. (Condensation) Magnified cross section of the minor system in a relatively condensed state. (Expansion) Magnified cross section of the minor system in a relatively expanded state. (After Geisbrecht, P., *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.*, 187, 452, 1962. With permission.) (b) Grassé et al. model. Diagram of a dinoflagellate chromosome in the stage of maximum disassociation of fibrils. (B) Band or disk of the chromosome. (f.a.) Arch of fibers. (f.t.) Cluster of transverse fibrils. (From Grassé, P. P. et al., *C. R. Acad. Sci.*, 260, 1743, 1965. With permission.) (c) Bouligand et al. model. Model possessing the essential geometric properties of the dinoflagellate chromosome; a cylindrical structure, similar to a stack of coins, is divided by equidistant planes containing parallel equidistant fibers of DNA perpendicular to the axis of the cylindrical structure. (From Bouligand, Y. et al., *Chromosoma*, 24, 251, 1968. With permission.) (d) Soyer and Haapala A model of the extrachromosomal loops of the dinoflagellate chromosomes (based on *Procentrum micans*). (a) The permanent packing of the chromatids is regulated by the supercoiling of the chromosomal DNA (inset). The regular spirality of chromatids is maintained by a locally operated mechanism marked with black rectangles in the junctions of periodic structures. There is one operating site (arrow) in the middle region of each whole turn of helical spiral of chromatid coil (thick spiral, opposite-handed spiral is not marked). The uncoiling regulator sites are thus situated in every junction of periodic structures seen in thin sections; all the chromatids pass a single site twice. The onset of gene activation is preceded by uncoiling of the chromatids in certain loci exposing structures comparable to the newt lampbrush chromosome loops. (b) The development of a large loop inplies simultaneous release of supercoiling in adjacent regulator sites (corresponding portions of the rectangles are marked white). During gene activity a matrix (dotted line) is accumulated around the extrachromosomal loops. The regions of uncoiling of the dinoflagellate chromosomes are considered as functional equivalents of chromomeres of higher eukaryotic organisms. (From Soyer, M.-O. and Haapala, O. K., *Chromosoma*, 47, 179, 1974. With permission.)

23b

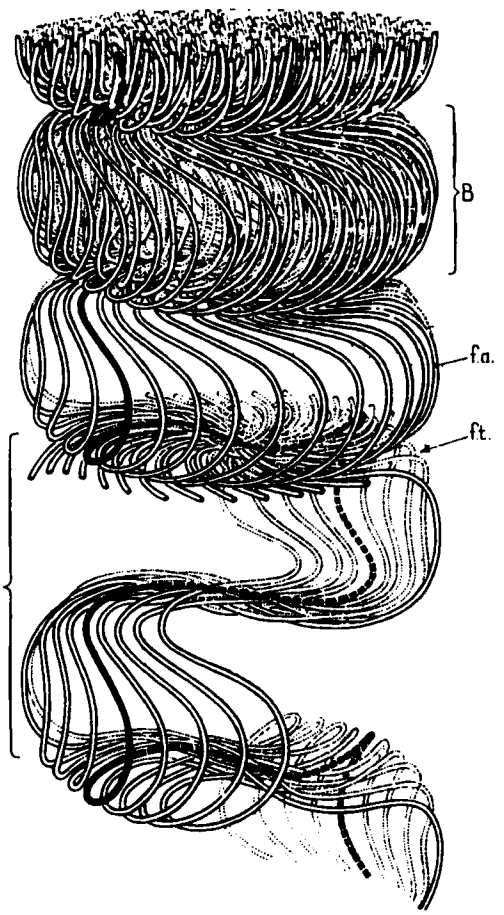

FIGURE 23B

of parallel rows of DNA helix arranged at right angles to the axis of the stack. As each new "coin" was added, the alignment of the parallel fibers was rotated through a small angle relative to the DNA in the preceding "coin" (Figure 23C).[124] In this model the exact number of DNA pieces in the chromosome was left undetermined, possibly as low as one molecule per chromosome, possibly as high as in the millions. The model bears a most striking similarity to ψ DNA of Lerman[125] and to the orientations of molecules of liquid crystals. Most recently, Haapala and Soyer[126-128] have proposed that the DNA in dinoflagellate chromosomes is procaryotic in nature, with each chromosome being polytene and composed of 700 to 1,500 supercoiled circular pieces of DNA scaffolded at least in part by RNA. Almost all of Haapala and Soyer's evidence for this model comes from examination of chromosomes in the electron microscope, either in thin section or as whole-mounted, spread chromosomes. As the abundance of models suggests, it is difficult to the point of impossibility to decipher the true structural arrangement of the DNA fibrils from thin sections or whole mounts. Haapala and Soyer did make one attempt to demonstrate these supposed supercoiled circular chromatids by spreading them on a cytochrome film by the Kleinschmitt technique.[129] Unfortunately, this paper may be criticized on several points. (1) They dealt with too few molecules – counting only 25 from *C. cohnii* and only 4 from *Prorocentrum* – making little mention of the number or description of the other structures they must have seen. (2) In the case of *C. cohnii* (the only organism where even a modicum of molecules was counted), the observed size (30 ± 5) is not out of the range expected for mitochondrial DNA or DNA from proplastids (if, indeed, proplastids contain DNA). (3) In no case were open circles seen. Open circles are readily identifiable as such. Instead, the supposed circles from *C. cohnii* were branched or straight linear structures. While such structures might be tightly coiled, closed circular molecules as the authors suggest, they might as easily be bits of chromatin which, though composed of highly twisted DNA held in this twisted configuration by protein, need not be formed from circular DNA. Indeed, apparently no effort was made to deproteinize the DNA as isolated; moreover, a blowup of a "broken supercoiled circle" from *Prorocentrum* definitely reveals just such a structure (i.e., a fragment where supercoiling still exists even after the molecule has been broken – such supercoiling must be the result of some interaction of DNA and protein). In final review the paper presents no molecules at all which are clearly identifiable, either as circles or as supercoiled circles.

We can now turn to the biochemical studies done on dinoflagellate DNA, which unfortunately are all too few. Franker has reported the isolation of DNA from the dinoflagellate which is endosymbiotic in the coelenterate *Anthopleura elegantissima*. This DNA consists of nuclear and cytoplasmic fractions separable by isopycnic ultracentrifugation. The nuclear DNA contains a small fraction of 5-methylcytosine as its only rare base.[130] Franker has also studied the process of DNA replication in *C. cohnii* by the technique of thermal elution chromatography on hydroxyapatite of radiolabeled DNA isolated from

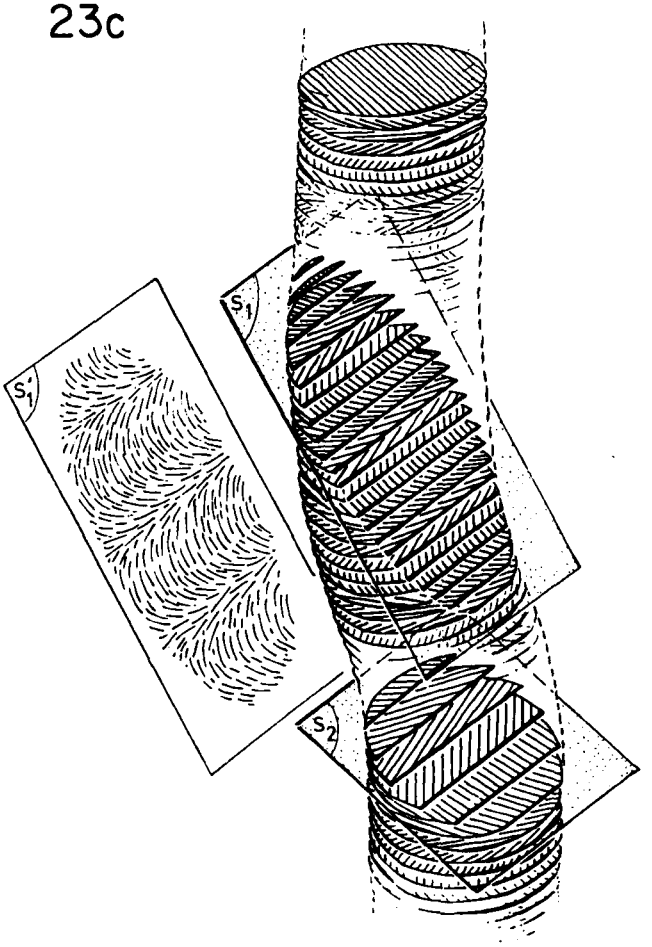

FIGURE 23C

synchronized cells at various points in the cell cycle. He found that S period occurred relatively late in the cell cycle (hours 5.5 to 8 in the 9-hr cycle) and that a component of the DNA which eluted from HAP at high temperatures was synthesized late in the S period.[131] In 1973 Rae[132] isolated DNA from *C. cohnii* and obtained the optical melting profile, the buoyant density distribution by CsCl isopycnic centrifugation, and a chromatographic analysis of the base composition. His findings were that *C. cohnii* had a T_m of 68.5°C in 0.0195 *M* Na^+, corresponding to a G + C percent of 36.5 and a buoyant density in CsCl of 1.715 g/cc (relative to *E. coli* at 1.710 g/cc), corresponding to a G + C percent of 56.1. The chromatographic analysis settled this discrepancy, showing that the true G + C percent was 41 with roughly 40% of the thymine substituted for by 5-hydroxymethyluracil. The presence of this rare base, which has otherwise been reported only in certain phages of *B. subtilis*, would be sufficient to account for the apparently high buoyant density and low T_m. It must be noted that all chromatographic systems do not resolve 5-hydroxymethyluracil; hence, its presence in the DNA of the endosymbiont studied by Franker is not ruled out.

More recently, this laboratory[112,133] has studied the renaturation kinetics of DNA isolated from *C. cohnii*. As can be seen in Figure 24, the DNA evidently contains sequences of different kinetic complexities. The actual numbers obtained reveal that roughly 56% of the DNA is repeated with various kinetic classes of repeats present and that about 44% of the DNA is composed of sequences found only once (or at most two to three times) in the haploid genome. These numbers were obtained by monitoring renaturation of DNA 500 to 550 nucleotides in length by HAP chromatography. When DNA of length 3,000

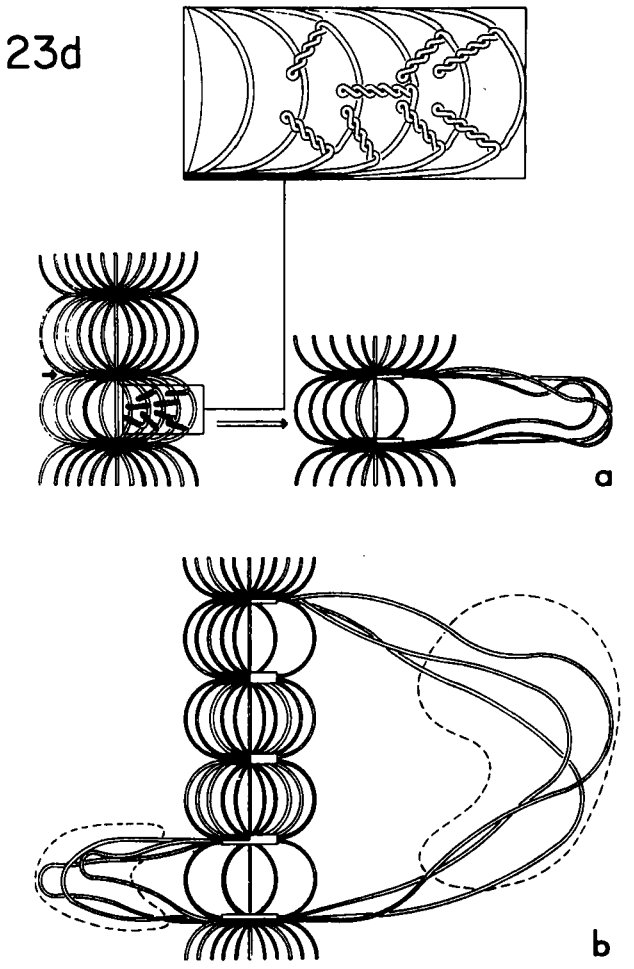

FIGURE 23D

nucleotides was used, the amount of DNA binding HAP at the low C_0t of 20 mol ℓ^{-1} sec increased from 56 to 76%. Similar behavior has been found in higher eucaryotic DNA where it has been proven to be the result of the interspersion of short (roughly 300 base pairs) repeated-sequence elements with longer unique DNA sequences. When the duplex repeated-sequence DNA binding HAP at low C_0t values (2.0 to 100 mol ℓ^{-1} sec) was optically melted, it displayed a T_m 6.5°C lower than sheared native DNA and a considerably decreased hyperchromicity compared to sheared native DNA. This behavior, universally characteristic of DNA from higher eucaryotes, is once again thought to arise due to the presence of imperfectly repeated sequence elements interspersed with unique elements. The imperfect nature of the repeats leads to the formation of mismatches in the renatured duplexes, which brings about a lowering of the T_m and some decrease in hyperchromicity (about 1% decrease for each 1% of mismatch). The remaining decrease in hyperchromicity arises due to the presence of single-stranded tails hanging off the duplex, occasioned by the still unrenatured unique-sequence elements. Thus, in two important criteria (melting behavior of renatured repeated-sequence elements and effect of length of the renaturing DNA on the apparent amount of repeated DNA), the dinoflagellate DNA behaves in a manner quite typical of the DNA of higher eucaryotes. Allen et al. also report preliminary results, using S_1 nuclease to digest away the single-stranded regions of renatured repeated-sequence duplex, which are consistent with the above interpretation. These data, obtained by renaturing DNA at long

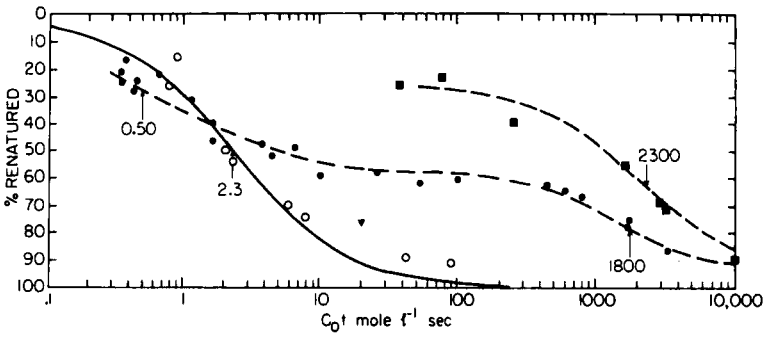

FIGURE 24. The kinetics of renaturation as measured by HAP chromatography of DNA. (O) *E. coli.* (•) *C. cohnii.* (■) Human nuclei. The points are corrected to those expected for fragment lengths of 450 base pairs in 0.12 *M* phosphate buffer (pH 6.8) at 60°C. The closed triangle (▲) represents the corrected percent of *C. cohnii* DNA bound at C_0t 20 at fragment length of roughly 3,000 base pairs. The line through the *E. coli* points is an ideal curve for second-order kinetics of renaturation with a $C_0t_{1/2}$ of 2.3. The $C_0t_{1/2}$ of the fast renaturing 55 to 60% of the *C. cohnii* DNA has been marked at 0.50, and the $C_0t_{1/2}$ of slow renaturing component has been marked at 1,800. The data have been corrected by the researchers. Note that the terminal value for the *E. coli* renaturation is 90% (quite generally, HAP renaturation reaches a terminal value of 92 ± 3%). However, the $C_0t_{1/2}$ of the reaction has been chosen at 50% (not 45%). We use this practice systematically, but it should be considered as contributing an error of up to roughly 15% to our calculations. (After Allen, J. R., Roberts, T., Loeblich, A. R., III, and Klotz, L. C., *Cell*, 6, 161, 1975. With permission.)

fragment lengths (roughly 3,000 base pairs) and then digesting the resulting material with S_1 nuclease which selectively removes single strands, suggest that the repeated-sequence elements are some 400 to 450 bases in length and compose some 30 to 40% of the genome.

The presence of unique DNA in *C. cohnii* is, of course, extremely important for all future builders of chromosome models. It indicates that the organism cannot be polytene (unless the various DNA strands in a given chromosome contain different information), nor can the chromosomes be polyploid. It can be noted at this point that Robert Tuttle and Alfred Loeblich have obtained nitrosoguanidine-induced pigment and nutritional mutants of *C. cohnii* at frequencies consistent with the haploid nature of the genes involved.[133,134]

Allen et al. found that some 5 to 10% of the DNA renatured faster than they could measure. They treated such DNA as "zero-time binding" DNA. Such DNA has been found in all higher eucaryotes studied. It has been identified as resulting from the concentration-independent formation of hairpin loops within single strands (i.e., it is not the result of a second-order kinetic process, but rather is the result of a concentration-independent first-order process). However, Allen et al. did not demonstrate that this DNA is indeed a zero-time binding fraction and not a highly repeated satellite. This is a rather important point, for, as we shall see, there is doubt whether any algae possess the equivalent of the rapidly renaturing DNA satellites found in higher eucaryotes.

Allen et al. also reported one other finding worthy of note. They measured DNA per cell for logarithmic and stationary cultures of three dinoflagellates. In each case, they found that the cells from log cultures contained roughly twice as much DNA per cell as did those from stationary cultures (7.3 vs. 4 pg for *C. cohnii*, 67 vs. 27 pg for *Gyrodinium resplendens*, and 15.4 vs. 7.3 pg for a *Gymnodinium* species). Now, this observation could be explained by assuming that dinoflagellates have no G_1 period in their cell cycle (i.e., that no time elapses between the end of cell division and the onset of DNA synthesis) and that DNA synthesis time is short relative to the length of the cell cycle. However, no difference is seen for the rapidly growing *C. cohnii* with 7-hr division times and the slowly growing *G. resplendens*, whose division time is 72 hr. Hence, one can at least imagine that there may be another cause for the phenomenon. It is worthwhile noting, in this regard, that *C. cohnii* is sometimes seen to give rise

to division cysts, in which one parent cell has given rise to eight progeny. The specific cultures used by Allen et al. were not monitored for this trait. Alternatively, the value in stationary cultures could be low due to some type of degradatory process in the cells. Dinoflagellate cultures are much more prone to die out after reaching stationary phase than are those of most algae. Hence, it is possible either that some stationary cells contain no DNA or that all stationary cells degrade a part of their DNA.

While the renaturation kinetics of dinoflagellate DNA clearly suggests that the organisms are haploid or at most diploid or triploid and that they are certainly not highly polytene or polyploid, the question still arises as to the number of DNA pieces present in dinoflagellate chromosomes. We have attempted to answer this question using the viscoelastic technique.[12] Using the high salt, high EDTA, detergent-proteinase system of Kavenoff and Zimm,[3] we obtained lysates which showed a decrease in the size and intensity of recoil with time (in the case of one organism, the *Gymnodinium* species), gave recoils typical of DNA aggregates (in the case of *G. resplendens*), or gave a combination of the two results (in the case of *C. cohnii* lysates). By switching to a highly protein-denaturing buffer system (6 *M* urea, 3 *M* guanidinium hydrochloride, 4% Sarcosyl® detergent, 0.08 *M* EDTA, pH 9.5 incubated at 50°C) we were able to remove the apparent DNA aggregation problem. However, the problem of decay of the intensity of recoil and retardation time in time remained. In the case of two of the organisms, *G. resplendens* and *C. cohnii*, we obtain relaxation curves characteristic of DNA in the $5 \pm 1 \times 10^9$ dalton range at 24 hr after lysis. After another 24 hr at 50°C, the values fall to those expected for DNA of $4 \pm 1 \times 10^9$ daltons, and the recoil intensity falls some 80% (rough estimate). In the case of *Gymnodinium* species #160, no recoil was detectable after the first 24 hr. This behavior is consistent with the presence in the lysates of large DNA pieces, ranging from 1/5 chromosome size in *C. cohnii* (which has 2.4×10^{12} daltons of DNA and 100 chromosomes, giving an estimate of 2.4×10^{10} daltons for a chromosome-sized DNA molecule) to 1/20 of chromosome size in *G. resplendens* (which has 1.6×10^{13} daltons of DNA and roughly 150 chromosomes, giving a size of 1.1×10^{11} daltons for a chromosome-size piece of DNA), degrading slowly in time. As the molecules decay, the size and intensity of the viscoelastic recoils decrease. That no recoil is observed in the lysates of the *Gymnodinium* species might mean that the degradation process is occurring much faster in the lysates. Such behavior could be caused by the presence of an extremely durable nuclease in the cell lysates or by regions of weakness, such as single-stranded regions, within the molecules themselves. Indeed, we see somewhat the same qualitative behavior in large-scale DNA isolation, where cell lysates (containing EDTA, perchlorate, and SDS) give larger yields of DNA in a Marmur-style[65] isolation procedure if all steps involving heating of the crude lysate are deleted. This effect is only very slight for *C. cohnii* but is extreme for *Gymnodinium* species #160 where incubation of the crude lysate at 60°C for 10 min reduces the DNA yield manyfold. Such behavior is indicative of nuclease activities.

However, it is also possible that the observed phenomenon in viscoelastic experiments is due to the slow coming apart in solution of chromosome structures composed of very many pieces of DNA. Thus, we feel that the viscoelastic results cannot be used to make definitive statements about the strandedness of the chromosomes.

In summary, the sequence composition of dinoflagellate nuclear DNA is apparently organized much like that of higher eucaryotes, even though its chromatin is entirely different, more closely resembling the bacterial condition. Unfortunately, dinoflagellate DNA replication has not yet been studied at the molecular level. For those building models, one should consider that dinoflagellates are haploid, being neither highly polyploid nor polytene. The similarity of the dinoflagellate chromosomes to bacterial nucleoids, when viewed in cross section by thin sectioning, is striking; hence, the desire to say that each dinoflagellate chromosome is made of one piece of DNA, like the bacterial nucleoid, is strong. However, there is no firm basis for such an argument. The idea that dinoflagellate DNA is scaffolded into its three-dimensional form by much the same process as that used in bacteria is much more easily defended. This explains the lack of histones[118, 119] and the bacteria-like appearance of the DNA fibrils in thin section.

Of the models mentioned earlier, the most likely candidates (i.e., most likely to the authors of this review) seem to be the penny stack model,[124] with the assumption that the entire

stack is made of one piece (or a few pieces) of DNA, or the model of Haapala and Soyer,[126-128] again modified so that there is one strand (or a small number of strands) of DNA looped around many times, instead of many strands looped once. However, this choice of models represents a far from uncontested position. As mentioned before, there is certainly no proof of the proposition that each chromosome is composed of one DNA molecule. More importantly, some people in the field doubt that the unique-sequence DNA disclosed by renaturation kinetics (and the DNA in which mutations were obtained at frequencies consistent with the haploid nature of the genes) is the same DNA found in the visible, condensed chromosomes. Rather, they suggest that the chromosomal DNA itself is polytene, while the unique-sequence DNA has some other location in the cell. To us such a view seems improbable because it demands that roughly half the cellular DNA, comprising 95% of the sequence information of the cell, must not be located in the chromosomes.

Chlorophyceae or Green Algae

In regard to their DNA, the green algae are probably the best studied algal group. Though extremely ancient in origin, this group is generally considered to have been the predecessors of the higher plants. Moreover, green algae can be and have been studied quite profitably with genetic techniques. Hence, the Chlorophyceae make a most felicitous subject for review. Since the organism *Chlamydomonas reinhardtii* is at once one of the most simple green algae and the best studied of the group, we will deal chiefly with it, bringing up findings concerning other green algae only to make specific points. Indeed, with *Chlamydomonas,* for the first time we are forced to pick and choose from an abundant literature to find those papers we feel to be most important.

C. reinhardtii contains, of course, at least three separable species of DNA: nuclear, chloroplast, and mitochondrial. Of the three, the chloroplast and nuclear DNAs have been most studied, with only small attention given recently to the mitochondrial species. Before going on to the first two types, we can quickly summarize data (which, except as noted, come from a single study, Ryan et al.)[135] concerning the mitochondrial DNA. Mitochondrial DNA shows up on CsCl buoyant density gradient centrifugation runs as a peak at ρ = 1.706 g/cc. In alkaline CsCl it appears as a single symmetrical peak of ρ = 1.720 g/cc, indicating that there is no nucleotide bias between the complementary strands. T_m measurements are consistent with buoyant density measurements of native DNA in CsCl, i.e., both indicate the same G + C content. The DNA is reported to renature at about the same rate as the phage T4 DNA. This is a kinetic complexity equivalent to some 100 X 10^6 daltons of DNA. (However, Gillham[136] cites a personal communication from the same authors which indicates a smaller kinetic complexity of 16 X 10^6 daltons.) This last point is of evolutionary significance: Animal mitochondrial DNAs have been found to have uniform kinetic complexities on the order of 10 X 10^6 daltons, while higher plants have been found to have much higher kinetic complexities, on the order of 70 X 10^6 daltons. Fungi, too, have been found to have rather large mitochondrial genomes, with yeast, for example, showing a kinetic complexity of some 50 X 10^6 daltons. (For a review, see Reference 137.) Only in the case of the algae has the information on mitochondrial DNA complexities remained cloudy. As we shall see, the situation in *Euglena,* where perhaps the most work has been done, is still unclear, with some workers suggesting a very small genome on the order of 2 X 10^6 daltons and others suggesting a much larger size of 40 X 10^6 daltons. A more complete discussion of the situation will follow in the section on *Euglena.* For the moment, two things must be pointed out. (1) The reference cited[135] is only an abstract of a presentation which we did not attend; hence, it is impossible for us to evaluate the techniques employed. (2) If the mitochondrial situation in algae bears any resemblance to the chloroplast renaturation work, renaturation kinetic measurements made early in the study may be off by a factor of two or more.

At least three groups have reported on the kinetic complexity of the chloroplast DNA of *C. reinhardtii.* Bastia et al.,[138] using optical methods, determined a complexity of 194 X 10^6 daltons. They reported that all the DNA was of a single kinetic class, though differential analysis of melting curves revealed that the base composition of the DNA was quite heterogeneous. Wells and Sager,[139] who also used optical methods, reported a kinetic complexity of roughly 200 X 10^6 daltons, with a minor repeated-sequence component making up some 10% of the DNA.

More recently, Howell and Walker[140] have performed the same experiment using HAP to monitor renaturation, and they also found a genome complexity of 200 X 10^6 daltons with no apparent repeated DNA sequences, but with some 10% of the sequences binding HAP at "zero time," i.e., faster than allowable by a second-order reaction. Similarly, the chloroplast DNA of *Chlorella*, another genus of green alga, has been found, both optically and by HAP, to have a kinetic complexity of 200 X 10^6 daltons.[141]

The following comments can be made about these findings. (1) The presence of a small amount of repeated DNA remains open to question. On the one hand, the measurements made by optical methods give mixed results, with one group finding repeated DNA and the other not. Moreover, the shape of Wells and Sager's curves at early times in the renaturation are reminiscent of the type of curving caused by first-order intrastrand base pairing,[142] a phenomenon which inevitably shows up in optical renaturation, but which can differ in degree from one type of DNA to the next. On the other hand, the HAP data presented by Howell and Walker are insufficient to rule out or rule for an amount of repeated DNA of 10% or so. (2) The exact size of the chloroplast genome in *Chlamydomonas* and *Chlorella* is probably still uncertain, even though the data on the subject very consistently place the answer at 200 X 10^6 daltons. The reason for this *caveat* is as follows: The chloroplast DNAs of higher plants have been found to be circular molecules in the size range of 85 to 95 X 10^6 daltons by direct visualization in the EM.[143,144] In *Euglena*, EM measurements of relaxed molecules also show open circles of 92 X 10^6 daltons.[145] However, the kinetic complexity of this molecule was measured by Stutz,[146] using optical methods at 180 X 10^6 daltons. Notably, the only possible error in Stutz's experiment is that he did not seek the temperature optimum for the renaturation. (Experiments in both 0.195 and 1 *M* Na^+ were carried out at 60°C.) Stutz explained the difference between the two numbers (i.e., 180 and 92 X 10^6 daltons) by saying that a correction had to be made for the low (26%) G + C content of the *Euglena* chloroplast DNA. However, this correction is probably not valid.[71,147] More recently, Slavick and Hershberger[148] repeated the work of Stutz. They argued that strict attention had to be paid to the problem of finding the temperature optimum, due to the extremely heterogeneous base sequence composition of *Euglena* chloroplast DNA. Working at the temperature optimum, they found a kinetic complexity of 92 X 10^6 daltons with the use of optical methods. Now, returning to the situation with *Chlamydomonas* chloroplast DNA, we find that, as yet, no one has measured an intact circle (if, indeed, such molecules exist). Though there is no reason to expect that *Chlamydomonas* chloroplasts have the same size genome as *Euglena* and higher plants, it is at least tempting to speculate whether the present measurements of the kinetic complexity for *Chlamydomonas* chDNA may be too high. Remember that in *Chlamydomonas*, as well as in *Euglena*, the chDNA shows a great deal of base sequence heterogeneity. Moreover, no one of the three groups did careful studies on the effect of temperature on renaturation. Such studies appear to be especially important for DNA of heterogeneous sequence.

It is interesting to compare the kinetic complexity of the chDNA of *C. reinhardtii* with estimates of the amount of DNA per chloroplast. Chemical estimates by diphenylamine reaction indicate that the gametes of *Chlamydomonas* (which has but one chloroplast) contain 1.23 + 0.06 X 10^{-13} g of DNA, of which 6.9% is chloroplast DNA.[149,150] This corresponds to 5.16 X 10^9 daltons of DNA per chloroplast. Hence, the chloroplast should contain a minimum of 25 copies of its genome. Of course, it is still open to question whether each of these 25 copies has exactly the same informational content, since a few percent base substitution would alter the renaturation kinetics in an undetectable manner. More information on this question (though perhaps not a definitive answer) might be obtained by studying the restriction map of the chDNA.

Unfortunately, though much work has been done to study the effects of various drugs (acting on either chloroplast or nuclear function) on the replication of chloroplast or nuclear DNA, there are no findings on the physical nature of the replication apparatus (i.e., number of replicons, Cairns form, or rolling circle mode of replication, etc.); nor is there much information on the protein bound to the chloroplast or nuclear DNA. However, reports on histones from several green algae do show them to be significantly different from those of higher organisms.[151,152]

We will not discuss the DNA replication papers in general; however, two are worthy of special

note. First is a report by Blamire et al.[153] By studying various drugs which influence chloroplast, but not cytoplasmic or nuclear function in the cell, these authors have found that chloroplast function directly influences nuclear DNA replication. Second is a report by Chiang et al.,[154] which shows that radiolabeled thymidine is incorporated into the nuclear DNA only during zygote germination (when meiosis occurs), but not in vegetative growth or during gamete formation. The implication of this last finding for the study of meiosis at the DNA level is obvious.

Finally, before discussing the nuclear genome, it is necessary to mention two fascinating fields of study currently being pursued on the chloroplasts of *Chlamydomonas*. The first line of inquiry concerns the phenomenon of uniparental inheritance of genetic traits of the chloroplasts. The term "uniparental inheritance" refers to the fact that in genetic crosses the maternal parent transmits its own chloroplast genetic markers to all four cells of the meiotic tetrads resulting from at least 90% of the zygotes. In the remaining 10% of the cases paternal markers in the progeny are found. In the case of "biparental zygotes," genes from both parents are found in the meiotic products. In the case of "paternal zygotes," all genetic markers in progeny cells come from the paternal gamete. This process can be studied by physical and chemical as well as genetic methods. Unfortunately, the data from both types of study are conflicting and do not lead to any clear-cut model for the process. Since any critical discussion of the experiments would involve an examination of the genetic studies clearly beyond the scope of this review, we have decided not to go into the matter in detail. We will simply state the two leading hypotheses.

The first hypothesis is that of Sager, who proposes that uniparental inheritance is based on a DNA restriction and modification system which acts on the plastid DNA. In this model the male parent chloroplast contains an inactive restriction enzyme, and the female parent chloroplast contains an inactive DNA modification enzyme. In the process of zygote formation, at some time prior to the fusion of the parental chloroplasts, these enzymes are activated. Therefore, by the time of chloroplast fusion, the maternal chloroplast DNA is modified in such a manner as to protect it from the restriction system which has already destroyed the male chloroplast's DNA. An alternative model to explain uniparental inheritance has been put forward by Gillham. This hypothesis assumes that chloroplast DNA behavior in *Chlamydomonas* mating can be likened to the process of mating bacteria with incompatible plasmids. In this model there is a competition for a fixed and limited number of attachment sites. DNA which cannot become attached is not replicated and hence is either diluted out or destroyed. For a review of the data on uniparental inheritance and extensive further reference on the subject, the reader can see articles by Gillham[155] and Sager.[156] These reviews also touch on the second major field of study mentioned above: segregation and recombination of chloroplast genes in *Chlamydomonas*. Once again, Sager and Gillham present somewhat conflicting models, both rather too complex to detail here. Gillham interprets experimental results in terms of a multicopy plasmid theory. Sager, on the other hand, suggests that the vegetative cell is diploid in its chloroplast genome. In neither the case of uniparental inheritance nor the case of segregation and recombination does it appear that either Sager's or Gillham's model has a clear advantage in explaining all relevant data. The one point that can be made using the subject matter of this review is that it seems that multicopy models for the chloroplast genome are much more in keeping with the data from renaturation kinetics.

The sequence organization of the nuclear DNA of green algae is, potentially, an extremely important topic in the study of the evolution of DNA sequence organization. The green algae have, as we shall see, rather small genomes – on the order of 20 to 40 times the size of the *E. coli* genome. Moreover, the amount of repeated DNA is small. This is certainly consistent with the already mentioned[90] idea that in plants, at least, the amount of repeated DNA decreases along with decreasing genome size. However, the important key issue can be stated as a series of three interlocking questions.

1. Does the nature of repeated DNA in the green algae simply represent one end of a continuum with higher plants and animals at the other end (with the presence of interspersed short repeats and longer unique sequences bearing structural genes being the common trait of all eucaryotes)?

2. Does the small amount of repeated DNA

in the green algae represent a situation more akin to bacteria where though there obviously is a small amount of repeated-sequence DNA, there is no pattern of interspersed short repeated elements and longer gene bearing unique sequence elements?

3. Does the situation in green algae represent the elusive intermediate position between the two arrangements already mentioned?

Parenthetically, it might be mentioned that no qualitative difference might exist between bacteria and higher eucaryotes with respect to the organization of their repeated DNAs. This paradoxical statement could be true if the mode of evolution of higher eucaryotic repeats had been via the process of lengthening by recopying tandemly ancestral procaryotic repeated sequences – sequences too short to be detected by present methods. For example, if the bacterial repeats were 20 base pairs or less in length, they might not bind HAP and hence might not be detected even at long fragment lengths. Such short sequences, if present in low quantity, would also be undetectable by optical methods.

Four papers on three separate organisms will be discussed in an attempt to detail the somewhat nebulous present state of knowledge concerning the nuclear DNA sequence organization of the green algae. The first published renaturation kinetics of a green alga was presented by Wells and Sager[139] on *C. reinhardtii* nuclear DNA. This work was done optically and followed only the first 40% or so of the renaturation (see Figure 25A). They found a repeated component corresponding to some 30% of the nuclear DNA and a more slowly renaturing component which had an apparent complexity of 4.5×10^{10} daltons (though, of course, at the time the renaturation was stopped only 10% of this DNA had actually renatured). The analytical complexity of the *C. reinhardtii* nucleus is about 6.7×10^{10} daltons.[157] Hence, the slowly renaturing fraction was apparently unique-sequence DNA, with the question of whether the organism is haploid or diploid still unresolved kinetically. The problem presented by this work (which, it must be remembered in fairness to Wells and Sager, was but a minor point in a study concerned primarily with the chloroplast DNA) is the true amount of repeated DNA. Since the authors make no mention of correcting their data for formation of secondary structures within single strands, it seems probable that some 10% out of the observed 30% of reaction was due to first-order processes. An educated guess made with the use of these data might place the amount of repeated DNA in *Chlamydomonas* at 20%. More recently, Howell and Walker[140] have repeated the renaturation kinetics of *C. reinhardtii* nuclear DNA using HAP, a method we favor for quantitating kinetic components (see Figure 25B). Notably, Howell and Walker find no detectable amounts of repeated DNA – a limitation which we feel in practice must be taken to mean that no more than 5% of the DNA is repeated. (Remember that the zero-time binding fraction, 1 to 3% in this case, adds to an uncertainty of 3% or so inherent in the method.) One problem in considering these data is that the nuclear DNA, probably due to its high G + C content (64%), did not elute from HAP in its single-stranded state until the phosphate molarity was raised to 0.21 *M* instead of the 0.12 *M* phosphate generally used to elute single-stranded DNA. Howell and Walker follow the renaturation of the single kinetic component of the nuclear DNA out to 80% of completion. The kinetic complexity measured for this component is 6.0×10^{10} daltons – almost exactly equal to the analytical complexity of the nucleus, a fact which suggests that the organism is haploid. It still remains a problem to reconcile the HAP data of Howell and Walker with the optical data of Wells and Sager, i.e., is the amount of repeated DNA 5% or less of genome or is it 20 to 30%. Howell and Walker have repeated the renaturation optically and found no evidence of repeated DNA; however, they did notice an "extensive concentration-independent temperature-dependent chromatic effect." Thus, it is possible that the apparent presence of a repeated DNA fraction observed by Wells and Sager was entirely the result of the formation of secondary structure in single strands – that the contribution of single-strand structure should be so high, rather than the 10% contribution common in *E. coli* or *B. subtilis*, may be the effect of the higher G + C content of *C. reinhardtii*. Certainly the formation of single-strand structure should be temperature dependent and concentration independent. (However, it should be remembered that the two groups used different strains of the organism.) Finally, as Howell and Walker point out, other workers[154] find no evidence of repeated DNA in *Chlamy-*

25a

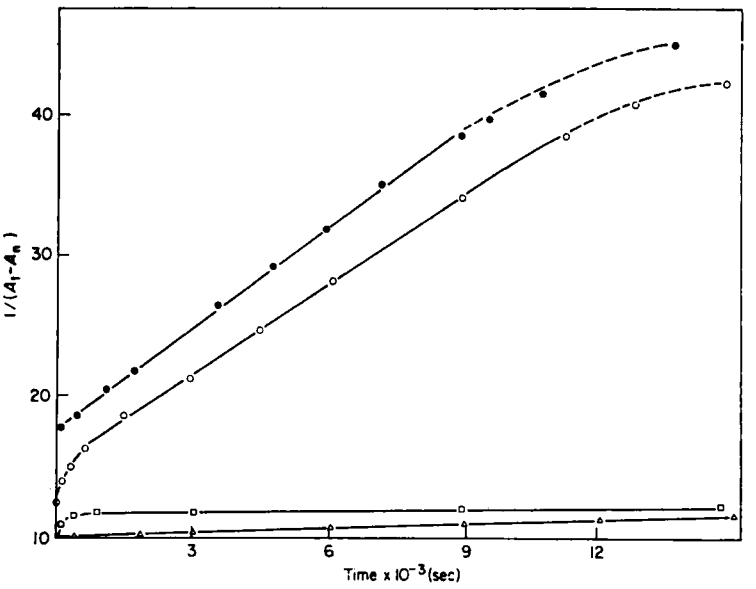

FIGURE 25. (a) Reciprocal k_2 plot of *E. coli* DNA, 15 μg/ml (–Δ–Δ–); *Chlamydomonas* nuclear DNA, 15 μg/ml (–□–□–); T4 DNA, 10 μg/ml (–●–●–); and chloroplast DNA, 12.5 μg/ml (–○–○–). All DNAs were sonicated to single-stranded size of 3 X 10^5 daltons. Measurements were taken at 1-min intervals or less for at least 120 min. Only a few points are shown for clarity of presentation. (From Wells, R. and Sager, R., *J. Mol. Biol.*, 58, 611, 1971. With permission.) (b) Combined C_0t curves for the reassociation of sheared $^{32}PO_4$-labeled chloroplast DNA, F_α (■–■); ^{3}H-labeled *E. coli* DNA (Δ–Δ); and $^{32}PO_4$-labeled chloroplast DNA, F_β (●–●) as analyzed by HAP column chromatography. Percent single strand (ss) DNA at termination of reassociation reaction is plotted vs. log C_0t in mol ℓ^{-1} sec. $^{32}PO_4$-labeled F_α and F_β were renatured separately, each with the standard ^{3}H-labeled *E. coli* DNA. Percent ss-DNA was corrected for the percent zero-time DNA binding (DNA not eluting at 0.12 or 0.20 *M* PB, but eluting at 0.48 *M* PB). Zero-time binding for F_α was 2.8%, for F_β was 10.2%. Single-component second-order reassociation curves were fitted to the data point by least squares analysis. (From Howell, S. H. and Walker, L. L., *Biochim. Biophys. Acta*, in press. With permission.) (c) Reassociation curves for the nuclear (●–●) and chloroplast (■–■) DNA of *Chlorella pyrenoidosa*. Renaturation was carried out in 0.12 *M* phosphate buffer and monitored by HAP chromatography. Temperature of renaturation was 56°C for chloroplast DNA and 66°C for nuclear NNA. The arrows mark the points of 50% renaturation. (From Bayen, M. and Dalmon, J., *Biochim. Biophys. Acta*, 395, 213, 1975. With permission.) (d) Second-order plot of the renaturation of the nuclear DNA of *Chlorella pyrenoidosa*. The renaturation was carried out in 2 X SSC at 71°C with a DNA concentration of 54.5 μg/ml and was monitored optically at 260 nm. The letters A, B, and C represent, respectively, the highly repeated, moderately repeated, and unique-sequence DNA. The arrow marks the point of 40% renaturation. (From Bayen, M. and Dalmon, J., *Biochim. Biophys. Acta*, 395, 213, 1975. With permission.)

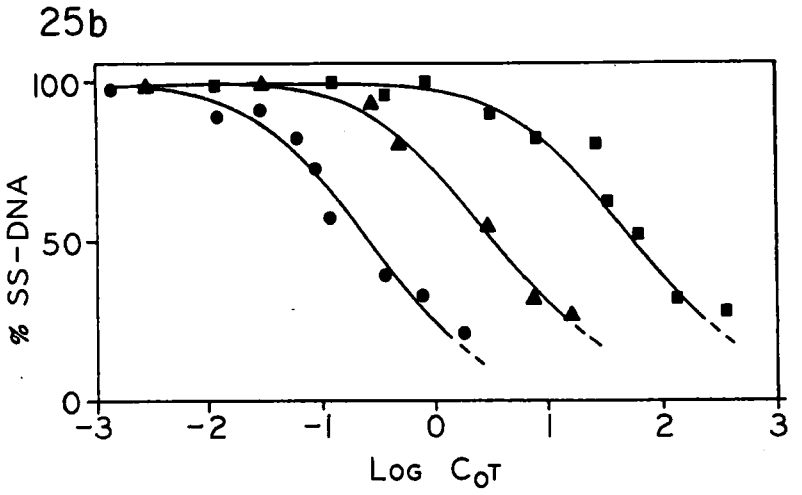

FIGURE 25B.

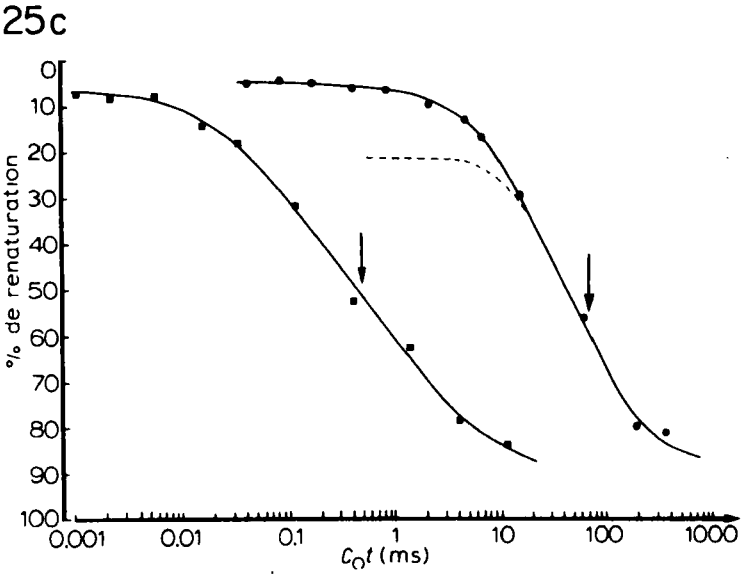

FIGURE 25C.

domonas nuclear DNA. Indeed, it is reported that by simply denaturing whole-cell DNA and allowing it to renature to a C_0t of 6 mol ℓ^{-1} sec and then separating out the renatured material on HAP, pure chloroplast DNA is obtained, as monitored by CsCl density gradients. This implies either that no nuclear DNA has renatured by a C_0t of 6 mol ℓ^{-1} sec (where the repeated DNA of Wells and Sager would be renatured) or that the nuclear DNA which has renatured cannot be found in the CsCl gradients either due to its having a lighter buoyant density than bulk DNA or due to technical reasons.

The work of Bayen and Dalmon is extremely interesting in light of the above discussion. These workers have measured the renaturation kinetics of the nuclear DNA of *Chlorella pyrenoidosa* both optically and by HAP.[158] As can be seen in Figure 25C, the HAP curve presents evidence for almost no repeats, although about 5% of the DNA renatures faster than can be measured (perhaps a zero-time component due to the presence of

25d

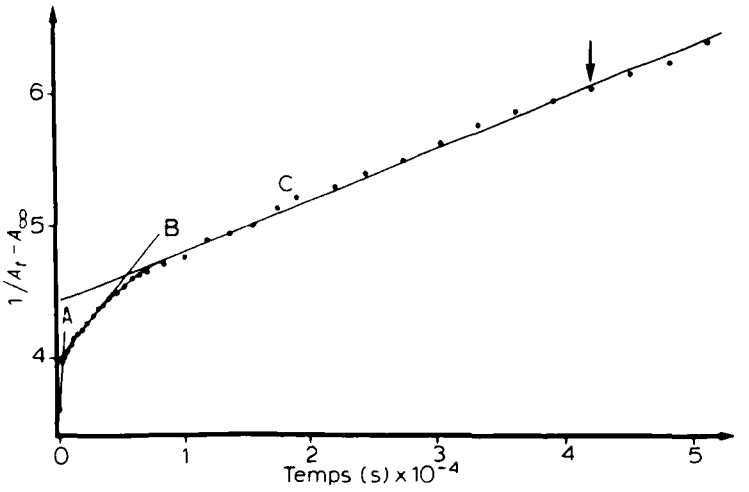

FIGURE 25D.

hairpin structures formed within a single strand). The optical renaturation (Figure 25D), on the other hand, suggests that some 20% of the DNA renatures faster than the unique-sequence DNA. This number, 20%, is obtained from analysis of the curvature in their RSO plots. The authors attribute part of it to a 5% component of highly repeated DNA and the remaining 15% to a fraction which renatures as if it were only three to four times repeated. This is very important, since this represents a most unlikely level of repetition. Indeed, it seems likely to us that the authors may have erred in interpreting their data. Some part of the observed curvature may be due to the formation of intrastrand secondary structure, and some or all (we are unable in this case to make exact calculations) of the remaining effect may be due to the expected deviation from linearity for nonrepeated sequences (see renaturation kinetics section of this review).

The data in these papers on *Chlamydomonas* and *Chlorella* suggest that a large discrepancy can exist between the HAP and optical renaturation results obtained on the nuclear DNA from the same green alga. However, as discussed above, this difference might be artificial, due to the differences between data obtained by HAP or optically. These differences might be eliminated, in part or fully, if optical data are analyzed according to Rau (Equation 46 in this review).

Apparently, however, this discrepancy is not mandatory. Siu et al. report the renaturation of the DNA of a colorless alga (*Polytoma*, an organism closely related to *Chlamydomonas*).[159] As can be seen in Figures 26A and 26B, both optical and HAP renaturation suggest that some 20 to 25% of the nuclear DNA renatures as repeated DNA. The HAP data, which the authors suggest should be used to quantitate repeated-sequence measurements, reveal a 6% zero-time fraction and a 20% fraction repeated roughly 1,000-fold. In this instance the authors have shown that the zero-time binding fraction appeared at extremely low C_0t (values as low as 8.8×10^{-7} mol sec l^{-1}) and appeared to form with concentration-independent kinetics. Since ribosomal DNA accounts for only 1.6% of the genome, most of the repeated DNA must not be ribosomal. Moreover, CsCl density gradient analysis of partially renatured DNA indicates that nuclear DNA of all density fractions contains a rapidly renaturing low-density satellite amounting to some 15% of the DNA. This, of course, not only gives further evidence of a repeated class of DNA, but also suggests that this repeated DNA is interspersed in the genome.

In summary, the renaturation kinetics of nuclear DNA from a variety of green algal species presents difficulties. In the case of *Polytoma*, the presence of interspersed repeated DNA seems likely and the presence of repeated DNA in quantities considerably in excess of ribosomal and transfer RNA cistrons seems definite. In the case

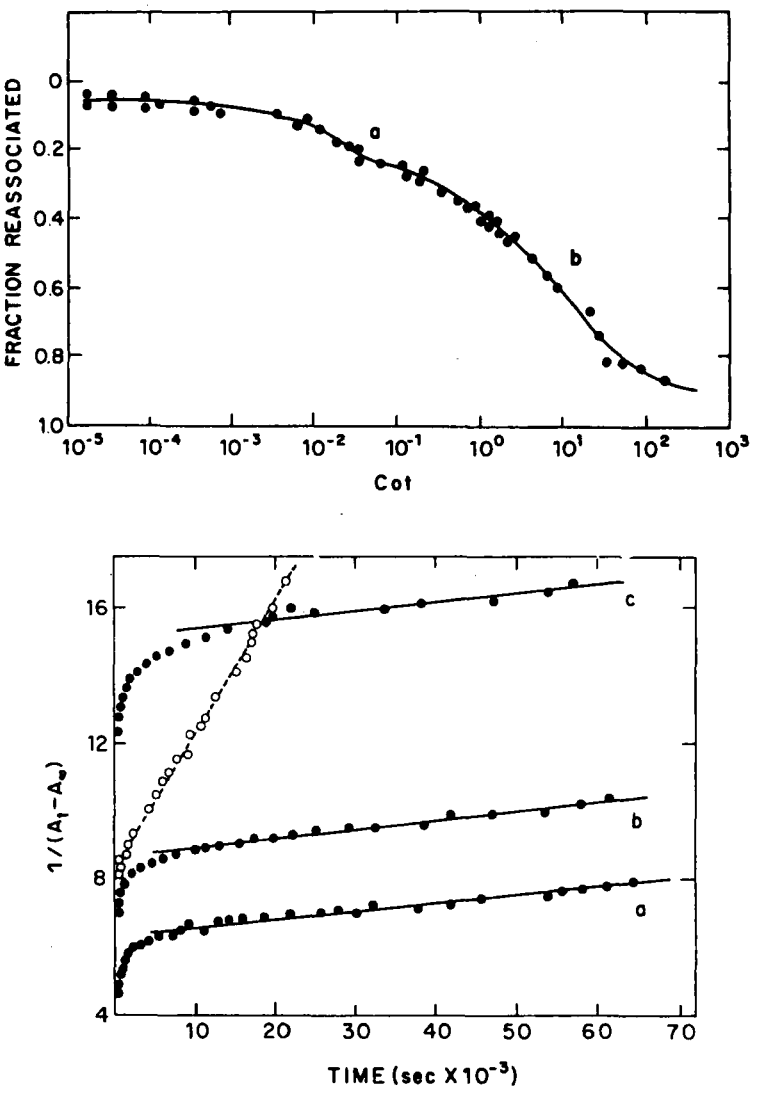

FIGURE 26. (a) C_0t plot for the nuclear DNA of *P. obtusum*. Purified ^{32}P-labeled nuclear DNA was sheared to a size of 350 base pairs ($S_{20,w}^{pH\,13}$ = 5.6S) and was concentrated and made to 0.68 *M* PB. Samples of 10 to 50 µl were placed in sealed capillary tubes and denatured by heating in an ethylene glycol bath at 110°C for 3 to 5 min. Renaturation was done by incubating samples in a 70°C water bath for various time points. Single-stranded DNA was eluted at 70°C from the HAP column by 3 ml of 0.14 *M* phosphate buffer, and reassociated DNA was eluted by another 3 ml of 0.4 *M* phosphate buffer. (b) Reciprocal kinetic plot of the renaturation of nuclear DNA. Nuclear DNA was sheared to a size of $S_{20,w}^{pH\,13}$ = 5.4S (1.2×10^5 daltons). The DNA was denatured with 0.2 *M* NaOH and then neutralized with NaH_2PO_4. Renaturation was done in 1 *M* Na^+ (ion concentration adjusted with 5 *M* NaCl) in three different concentrations: (a) 34.0 µg/ml, (b) 23.6 µg/ml, and (c) 13.7 µg/ml. (From Siu, C.-H. et al., *Chromosoma*, 48, 19, 1974. With permission.)

of *Chlorella* and *Chlamydomonas*, the situation is clouded with optical and HAP results in contradiction to each other, with the usually more reliable HAP data suggesting very little repeated DNA. However, a simple calculation reveals that the controversy over the repeat organization in these two species is occurring at the wrong level. In the green algae, nuclear genome DNA content seems to average around 7×10^{10} daltons. Assuming that the organisms contain 10^4 structural genes (a high estimate) and that each 1,200-bp structural gene has associated with it 300 base pairs of repeated DNA, one calculates that the total repeated content of the genome could be as low as 3%, of which the rDNA would constitute 1.5%. This low amount of repeated-sequence DNA could slip by detection. Even a tenfold excess of repeated DNA beyond that predicted in the simple model above would yield only 16% repeated DNA in the nucleus. Thus, it would seem premature to use the HAP data on *Chlamydomonas* and *Chlorella* to second guess the extent of generality of the Davidson model for repeated-sequence organization.[87] On the other hand, it would also be imprudent to stop the examination of the genomes of these algae at this point.

One final aspect of the DNA sequence arrangement of green algae concerns the structure of the ribosomal RNA (rRNA) cistrons. Using a technique developed by Miller and his associates,[160-162] the actual genes (rDNA) coding for rRNA can be visualized in the electron microscope as transcriptional complexes, with the nascent rRNA chains still attached. Three groups have utilized this technique to examine the rDNA of a number of green algae: *Acetabularia major, Acetabularia mediterranea, Chlamydomonas reinhardtii,* and *Batophora oerstedii.*[163-167] As in higher plants and animals, the rRNA genes have been found to be tandemly repeated. However, in contrast to the findings on animals, it appears that there is little, if any, conserved spacer in the primary transcription product in the two *Acetabularia* species.[166,167] Moreover, in one green alga (*Batophora oerstedii*) there appears to be little, if any, nontranscribed spacer between the supposed ribosomal RNA cistrons in a major part of the material visualized.[167]

Euglenids

For algae, the euglenids (represented almost exclusively by *Euglena gracilis*) are extremely well studied with respect to their DNA. The mitochondrial, chloroplast, and, most recently, nuclear genomes have been studied by renaturation kinetics. Though common members of the phylum Chlorophyta, along with the green algae, the euglenids bear small resemblance to the green algae in regard to their nuclear organization. Thus, while the green algae tend to have about 0.1 pg of DNA per nucleus, *Euglena* contains about 3 pg.[168] The chromosomes, numbering 45 to 50, are permanently condensed in *Euglena*, much as they are in the dinoflagellates.[169] However, recent reports indicate that unlike the dinoflagellates, chromatin from *Euglena* contains considerable amounts of basic protein,[170] though whether the typical eucaryotic subunit structure is present is not yet known. Histones or histone-like proteins from *Euglena* do exist, but seem to be different from those of higher organisms.[170] Information on the three known types of cellular DNA of *Euglena gracilis* (mitochondrial, chloroplast, and nuclear) is discussed below.

Various reports indicate some conflict concerning the size of the mitochondrial genome of *Euglena*. Manning et al., using the electron microscope, reported lengths ranging from 1 to 19 μm (2 to 38×10^6 daltons) with a mean length of 1.3 μm.[171] However, Nass et al., again using direct visualization in the electron microscope, reported a size of 1 μm using a variety of isolation techniques. More importantly, they report a small fraction of circles of 1.01 ± 0.05 μm.[172] Renaturation kinetic measurements using S1 nuclease to monitor renaturation suggest a kinetic complexity of 40×10^6 daltons.[173] Notably, these renaturation kinetics were determined before it was generally recognized that kinetics monitored by S1 digestion do not, and indeed should not, follow simple second-order kinetics. The observed divergence from second-order kinetics bothered the authors. However, they appear to have done an admirable job of controlling their experimental findings using the DNA of several viruses, and it is extremely hard to fault their conclusion. Also germane to this problem are the results of Crouse et al.,[174] who hybridized mitochondrial rRNA in excess to mitochondrial DNA. They found a saturation plateau at 3.7% of the DNA hybridized. Assuming a total rRNA cistron size of 1.6×10^6 daltons, one can calculate a mitochondrial genome size of 40×10^6 daltons, in good agreement with the renaturation data. This still leaves the problem

of reconciling this rather large genome size, calculated by indirect means, with the smaller sized DNA pieces (particularly the circles) actually seen with the electron microscope. One possible explanation for the rather uniformly small size of the visualized pieces and the occurrence of a small number of circles is the presence of restriction sites on the DNA. Thus, cuts by a restriction endonuclease could result in the small size, and short sticky ends left by the cuts could allow the DNA to circularize under certain conditions.

The only other source of information concerning the mitochondrial DNA of *Euglena* is a paper by Fonty et al.[175] They report a striking resemblance between the physical characteristics of the mitochondrial DNA of *Euglena* and that of the yeast *Saccharomyces cerevisiae.* They show that, as is the case in yeast, the mitochondrial DNA of *Euglena* elutes from HAP (as native double strand) at very high phosphate molarities. Also similar to the yeast situation is the fact that the buoyant density of the DNA in CsCl (1.690 g/cc) suggests a G + C content higher than that measured by direct chemical means. Indeed, the true G + C content of 25% (vs. the 31% estimated from buoyant density)[176,177] is extremely low. Since the anomolous HAP binding and buoyant density in yeast had been found to be due to A + T-rich stretches, the authors digested the *Euglena* mitochondrial DNA with micrococcal nuclease (an enzyme which preferentially attacks high A + T regions) and found evidence that 40% of the DNA is formed by stretches lower than 6.0% in G + C. Differential analysis of the melting curves provided more evidence for this component. In yeast (see later discussion) it has been found that A + T-rich spacers alternate with G + C-rich genes. That organisms as evolutionarily diverse as *Euglena* and *Saccharomyces* have similarly structured mitochondrial DNA is, of course, extremely interesting. This similarity is further suggestive evidence that the true genome size of the *Euglena* mitochondrion is around 40×10^6, since this is close to the measured size for yeast mitochondrial DNA. Moreover, it seems to agree with the trend that primitive organisms have rather large mitochondrial genomes – much closer in size to those of plants than to the 10 μm size common in animals.

The arguments detailing our knowledge of the size (92×10^6 daltons) and shape (circular) of the chloroplast DNA molecule have already been given. The following serves as a summary of the other known facts about the chloroplast DNA from *Euglena.* This DNA has a native buoyant density in CsCl of 1.685 g/cc.[178-180] Ribosomal RNA isolated from *Euglena* chloroplasts hybridizes with the chloroplast DNA, although there is disagreement about the number of rRNA copies per 92×10^6 daltons of DNA – the number varies between one and three.[181-183] Higher plants' chloroplasts, by comparison, have two copies of the rRNA per DNA molecule.[184] The chDNA of *Euglena* is separable into two peaks in alkaline CsCl, with the heavier peak binding ten times more rRNA than the light peak. In neutral CsCl, a satellite of density 1.692 to 1.700 which binds an inordinate amount of rRNA (up to 6.9% of the heavy peak hybridizes with chloroplast rRNA) is reported by some workers.[181,182,185] Crouse et al.[174] give proof that this heavy component is not mitochondrial DNA, which has the same density. Zablen et al.[186] characterize the sequence organization of the 16S rRNA species by obtaining its ribonuclease T1 fingerprints. The results of this characterization suggest a strong phylogenetic relationship (i.e., high numbers of coincident oligonucleotides) between this rRNA and the 16S rRNA of the blue-green alga *Anacystis nidulans,* the bacteria of the *Bacilli* group, and the red alga *Porphyridium.* As mentioned earlier, this sequence similarity of the chloroplast 16S rRNa with procaryotic 16S rRNA species proves the procaryotic origin of the chloroplast but not the endosymbiotic hypothesis. Finally, Rawson[187] has studied the RNA excess hybridization of whole-cell RNA with I^{125}-labeled DNA. His results, controlled for the contribution of cytoplasmic RNA to the overall hybridization, suggest that some 32% of the chDNA is transcribed (assuming that only one strand of the duplex is transcribed in any one region of DNA).

Once again, in the case of *Euglena,* there is considerable literature on the effects of various drugs on DNA synthesis in chloroplasts and nuclei,[188] and once again we will overlook this literature, calling attention only to a report by Manning and Richards.[189] Using the traditional Meselson and Stahl technique, these workers demonstrated semiconservative synthesis of both nuclear and chloroplast DNA. Notably, however, they found that chloroplast DNA replication occurred 1.5 times faster than nuclear replication. A stable chloroplast-to-nuclear DNA ratio is

apparently maintained by the degradation of chloroplast DNA with a DNA half-life of 1.6 generations.

The last topic of discussion on *Euglena* is the renaturation kinetics of the nuclear DNA. This work has been reported by Rawson,[190] who used HAP to follow the kinetics. As can be seen in Figure 27, Rawson found evidence of three kinetic fractions: a fraction composing 18% of the DNA (which renatured too rapidly to measure in the experiments performed), a middle repetitive fraction with an apparent complexity of 6.6×10^8 daltons and repetition frequency of 270-fold which made up 40% of the DNA, and a non-repetitive fraction which made up 36% of the DNA. The kinetics suggested that *Euglena* is diploid. As the author points out, chloroplast sequences (whole-cell DNA was used) should have renatured as middle repeated DNA, but should have represented no more than 15% of this DNA. Isolated DNA from each kinetic fraction was melted optically, with the observed melts looking much like similar melts for DNA from higher eucaryotes, i.e., the middle repeated DNA displayed lowered hyperchromicity and T_m and a rather broader melt compared to sonicated native DNA, while the nonrepetitive fraction melted much like sheared native DNA.

Several comments seem in order concerning this paper. First, and most important, it must be noted how similar the kinetics of renaturation and the renatured product of *Euglena* are to those of the dinoflagellate *C. cohnii*, as well as to those of higher eucaryotes. Once again, the trend showing increased amounts of repeated DNA as DNA per cell increases has been obeyed. Second, the large amount of DNA which renatured faster than the experimenter could follow deserves comment. This DNA, 18% of the genome, might indeed be highly repeated or it might be a "zero-time" component due to the formation of hairpin duplex within single DNA strands. If highly repeated, this DNA would compare in its level of repetition to the centromeric satellites of higher organisms and would constitute the first such satellite found in an alga (assuming the small amounts of rapidly binding DNA in *Chlamydomonas* and *Chlorella* were true "zero-time binding" fractions as the similar DNA in *Polytoma* was proven to be). On the other hand, if this DNA is truly "zero-time" DNA, it represents an unusually large fraction of the total DNA when assayed at 350 base pairs, as was done by Rawson.

YEASTS

Almost all physical studies on the DNA of yeasts have been done on *Saccharomyces cere-*

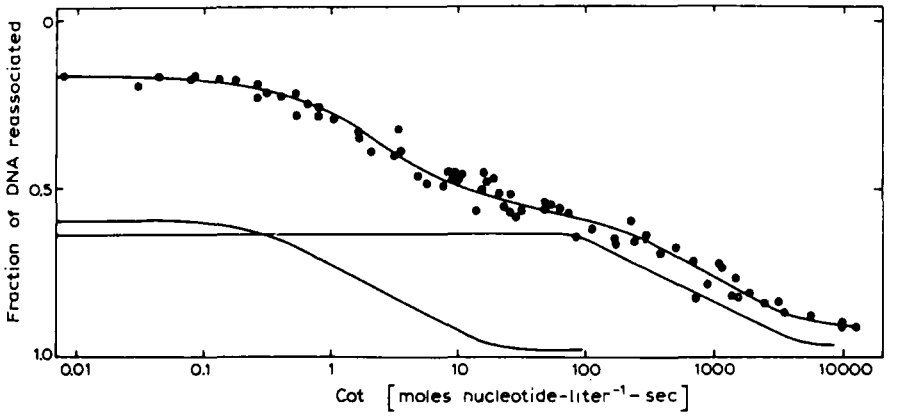

FIGURE 27. The kinetics of reassociation of total cell DNA from *E. gracilis*. Samples of DNA with a single-stranded molecular weight of 1.2×10^5 and of varying concentrations were dissolved in 0.12 *M* sodium phosphate (pH 6.8) buffer and denatured by boiling at 100°C for 5 min. The DNA samples were then allowed to renature for various times at 60°C. The fraction of DNA reassociation was measured by separating single- from double-stranded DNA on HAP columns. The curve drawn through the experimental points represents a least squares fit for two second-order components. The lower curves represent the predicted reassociation kinetics of these components if they were to exist individually. (From Rawson, J. R. Y., *Biochim. Biophys. Acta*, 402, 171, 1975. With permission.)

visiae. Therefore, in this review the only yeast which will be discussed in detail is *S. cerevisiae,* and it can be assumed that when the word yeast is used it refers to *S. cerevisiae* unless another yeast is specifically named. This organism is especially nice for any physical biochemical or molecular genetic work because so much genetic study has been done on it. Genes in *S. cerevisiae* are on 17 linkage groups which have genetic lengths ranging from 5 map units to approximately 400 map units.[191] Both DNA per cell and renaturation kinetic experiments have yielded various values for the DNA content of the *S. cerevisiae* nucleus, but all measured values fall within the range from 5×10^9 to about 1.3×10^{10} daltons, a very small nuclear DNA content for a eucaryote. Assuming an average value of 9×10^9 daltons, one calculates an average of 5 to 6×10^8 daltons per linkage group. Thus, yeast DNA is very amenable to physical studies, such as sizing, which are very hard to do on the larger DNA of most eucaryotes.

Yeasts may be considered primitive eucaryotes because they possess some characteristics which are more like those found in procaryotes than in eucaryotes. Yeasts do contain mitochondria as do other eucaryotes; however, *S. cerevisiae* can survive without respiring and without mitochondrial DNA.[192] The rRNA genes for 18S, 28S, and 5S RNA are physically closely linked together in *S. cerevisiae*[193,194] as is found in bacteria[195] but not in higher organisms.[196-198] Finally, the overall cell cycle of yeast is different from that of most eucaryotes in that both haploid and diploid cells undergo mitosis, and cell division occurs by budding and nuclear migration.[199]

One problem encountered when working with *S. cerevisiae* DNA is difficulty of isolation. The small DNA content which makes yeast good for many physical studies also makes isolation of DNA difficult. The ratio of protein:RNA:DNA is 200:100:1.[200] Therefore, extra deproteination steps must be added to the basic Marmur isolation.[201]

S. cerevisiae cells have tough cell walls so cells do not lyse easily. Successful lysis can be obtained by use of the French press, by freeze-thawing in detergent, or by use of the enzymes glusulase and zymolase.[201] Preparation of spheroplasts is the most gentle lysis procedure and therefore the preferred method if large DNA is desired.

DNA can also be isolated by first isolating nuclei. From the size and purity of the DNA obtained from isolated nuclei, this method seems good, although it is not as gentle as the isolation of DNA from spheroplasts.[202]

Isolated yeast nuclear DNA has a density of 1.699 g/cc,[202,203] as determined by CsCl density gradient centrifugation; this corresponds to a G + C content of 39%.[204] A high G + C component of the nuclear DNA presents itself as a heavy shoulder.[203,205] This DNA is mainly ribosomal.

It has been noticed that when purified yeast DNA is melted, the baseline can be slanted,[207,208] indicating that some contaminant probably isolates along with the DNA. Multiple purifications by chloroform:isoamyl alcohol extraction and ethanol precipitation do not remove the contaminant, nor does HAP chromatography.[208] The contaminant is not likely to be polysaccharide because HAP removes most polysaccharides. However, there have been reports that preparations of fungal DNA contain a polysaccharide banding in CsCl in the region corresponding to a density of 1.680 to 1.685 g/cc[209] and that polysaccharide can be removed from the DNA by digestion with α-amylase.[202]

The DNA content of *S. cerevisiae* has been determined both by DNA per cell measurements and by renaturation kinetic studies. DNA per cell experiments yield values of 1.2 to 1.3×10^{10} daltons for DNA content,[210-212] while renaturation kinetic studies have yielded values from about 5.2 to 5.8×10^9 daltons[213] to about 9×10^9 daltons.[214]

In each of the DNA per cell papers, the method used was one for which it is impossible to do the proper control, namely a control for positively interfering substances by doing the experiment on cells minus their DNA. In addition, the values obtained by DNA per cell measurements must be corrected for 5 to 20% mitochondrial DNA and 1 to 2% 2 μm DNA.[215] Renaturation kinetic experiments have neither of these problems. Therefore, values for nuclear DNA content obtained from renaturation kinetics should be more accurate than those obtained by DNA per cell measurements.

However, there exists almost a twofold difference between the values for the nuclear DNA content of *S. cerevisiae* found by different laboratories using renaturation kinetics. The reason for the discrepancy between the genome size found by Whitney and Hall[213] and that found

by Bicknell and Douglas[214] is not yet understood, and more work on this is necessary.

More work is also necessary to determine the pattern of interspersion of unique and repeated DNA in the nuclear DNA of *S. cerevisiae.* The only published work on this is an abstract by Whitney and Hall.[216] They found that yeast nuclear DNA contains 95% unique DNA with a complexity two times that of *B. subtilis* DNA and 5% repeated DNA (which is repeated 25 times), yielding a total genome content of 4.8×10^9 daltons. Lowering the renaturation temperature 10°C did not change the amount of the repeated DNA. The amount and complexity of the repeated DNA were not altered by increasing the size of the DNA fragments up to 1,500 nucleotides. These data suggest that the repeated sequences are long and/or clustered.

However, more recently Whitney and Hall[213] have found that the DNA used in the above studies had been selectively depleted (apparently during the process of DNA isolation) of a repeated-sequence component representing some 15% of the entire genome. Very careful checks show that no unique DNA was lost. Thus, these workers now report 20% repeated DNA and a total genome content of 5.2 to 5.8×10^9 daltons. Since such a large proportion of the repeated sequences was lost from the DNA used in the previous studies, more work must be done before conclusions can be drawn as to whether there is interspersion of repetitive and single-copy sequences in the yeast genome.

Taking an average value for nuclear DNA content and the existence of 17 linkage groups, the average size of a yeast chromosome is about 5×10^8 daltons, a size amenable to study. Sedimentation experiments aimed at measuring the sizes of yeast chromosomes by spheroplasting cells, lysing the spheroplasts on top of a sucrose gradient, and sedimenting the DNA through the gradient have been done by several groups. Blamire et al.[37] found DNA between 4 and 6×10^8 daltons, while Petes and Fangman[11] measured values between 5×10^7 and 1.4×10^9 daltons. However, sedimentation experiments suffer from the previously mentioned fact that because of the known strong dependence of sedimentation coefficient on rotor speed and molecular weight,[15,217,218] measured sedimentation coefficients may be too low because of too high rotor speeds. The difference between the true S and the measured S is proportional to RPM^4 and M^4.[219]

More recently, viscoelastic experiments have shown the largest pieces of DNA in the yeast nucleus to be about 2×10^9 daltons.[13] The two groups that measured the molecular weights of yeast chromosomes by sedimentation adjusted their rotor speeds so that they would get accurate S values for DNA of about 5×10^8 daltons. However, if the 2×10^9 dalton value is correct, then it can be calculated that the S values, and thus the molecular weights, measured by the sedimentation experiments would be too low because of too high rotor speeds (see discussion in Lauer and Klotz).[13] The viscoelastic technique does not suffer from the drawbacks that sedimentation does. Therefore, 2×10^9 daltons can be accepted as an accurate value for the size of the largest piece(s) of DNA in the yeast nucleus with three reservations.

1. Although the viscoelastic technique is a very gentle method for measuring molecular weights of large DNA, the DNA may be broken and the true size of the largest piece of DNA in the yeast nucleus may be larger than 2×10^9 daltons.
2. Pieces of DNA smaller than 2×10^9 daltons may be joined by protein linkers which are insensitive to the detergents and proteinases used in the experiments.
3. Viscoelastic measurements are sensitive to DNA aggregation. While aggregation cannot be ruled out completely, control experiments make its existence highly unlikely.

Assuming 2×10^9 daltons to be the true size of the largest piece of DNA in the yeast nucleus and comparing this value to the DNA content of the nucleus allow various interpretations concerning yeast chromosome structure. Assuming an average content of about 8 to 9×10^9 daltons, we then conclude that the largest piece of DNA contains about 25% of the DNA in the nucleus. At first glance this seems surprising considering the genetic evidence for 17 linkage groups in yeast. However, the two results are not incompatible. If one assumes that the amount of DNA in a chromosome is proportional to its map length, then the largest chromsome in *S. cerevisiae* should contain 13% of the DNA in the nucleus.[191] However, it may be that the largest chromosome is larger than is indicated by genetics, and we may just be

measuring the largest chromosome. Of course, if this is so, the chromosome contains an unusually large proportion of the DNA (25% of the DNA is a large percentage to be in one chromosome if there are 17 chromosomes). A second possibility is that yeast nuclear DNA may consist of chromosomes linearly connected by nongene-containing DNA lengths longer than 50 map units. In other words, there may be more than one linkage group per piece of DNA. Then, genes on two chromosomes connected by a linker would appear to assort randomly because of frequent crossing-over between them, just as "characters due to factors placed far apart on a chromosome assort almost at random due to crossing-over between them."[220]

The main problem encountered when trying to make interpretations concerning yeast chromosome structure is the lack of a good value for nuclear DNA content. Once this value is known, interpreting the DNA size data will be more straightforward.

In addition to sedimentation and viscoelastic measurements, the molecular weight of yeast nuclear DNA has also been measured in the electron microscope.[221] These experiments resulted in the measurement of linear molecules ranging in size from 1.2 to 8.4 $\times 10^8$ daltons. The most probable explanation for why these values are smaller than values measured by other methods is that DNA, which is very sensitive to shear, breaks when it is being manipulated for visualization in the electron microscope.

In *S. cerevisiae*, DNA synthesis is restricted to the first quarter of the cell cycle.[222-224] Williamson and Fennell[225] have done a "Meselson and Stahl"-type[226] experiment in which they have found that yeast nuclear DNA replicates semiconservatively.

Newlon et al.[221] and Petes et al.[227] have used electron microscopy to show that the replication of yeast chromosomal DNA occurs by multiple internal replication bubbles, as is true of the DNA of higher eucaryotes[93,94] (see Figure 28). Initiation occurs at multiple sites along a chromosome. These sites are spaced about 25 μm (0.5 $\times 10^8$ daltons) apart. More recently, Petes and Williamson[228] have shown by fiber autoradiography that yeast nuclear DNA contains multiple initiation sites for DNA synthesis and that DNA is replicated bidirectionally from these sites at a rate of approximately 7 μm min/replication fork. Thus, in the number of replication units, the size of replication units, the directionality of replication, and the rate of replication, the replication of yeast DNA is more like that of eucaryotes[93,94] than that of procaryotes.[229,230]

Studies involving induction of mutation in *S. cerevisiae* by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine have shown that for given genetic markers, distinct peaks of reversion occur at specific times in the cell cycle.[231] It has been shown for *E. coli* that the peak of mutation induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for a given genetic marker occurs at or near the time of replication of the marker as determined by independent physical techniques.[232] Assuming that this relation is also true for yeast, the above experiments allow the conclusion that in yeast individual genes replicate at discrete times during the S period.

Because of their small sizes, yeast chromosomes have been very hard to visualize in the light microscope. The best light micrographs, those of Tamaki,[233] show condensed regions presumed to be chromosomes. Light microscopy has been more useful for visualizing grosser features of mitosis – bud formation, nuclear migration and fission, and cell division. From such studies and the knowledge that DNA synthesis occurs in the first quarter of the cell, it seems that the separation of chromatin may occur well in advance of nuclear fission. That this may be the general rule in fungi receives support from the observations of Robinow.[234]

More details of mitosis have been elucidated by Williamson[235] from examination of serial sections in the electron microscope. In resting cells the chromatin is aggregated into a single mass occupying a small part of the nuclear volume. Within 5 min of inoculation of the cell into growth medium, the aggregated chromatin breaks up. However, the separated pieces do not disperse throughout the nucleus, but stay on one side of it. Toward the end of the phase of DNA synthesis, the chromosomes move toward the end of the nucleus nearest the developing bud, and the chromosomes seem to become broader and slightly better defined. Immediately following the period DNA content of about 8 to 9 $\times 10^9$ daltons, we then restricted to one end of the nucleus but instead occupy the whole nucleus, although they are segregated into two roughly equal masses separated by a zone of seemingly homogeneous nucleoplasm. At this stage the nuclear membrane has developed a pronounced equatorial furrow, so that the nucleus is dumbbell shaped. Following this

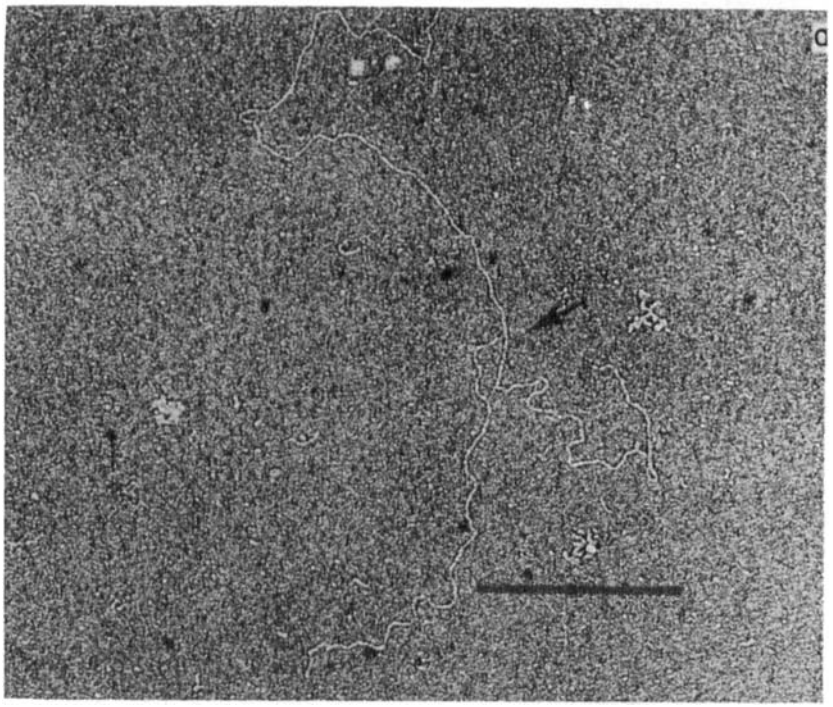

FIGURE 28. Electron micrographs of portions of yeast DNA replication intermediates. DNA from CsCl gradients or sucrose gradients was spread using a modification of the aqueous procedure of Davis, Simon, and Davidson which has been described in detail. Molecules were photographed using a Philips EM 300 electron microscope. Contour lengths in centimeters were determined using a Keuffel and Esser map measurer and were converted to microns by comparing the contour lengths of yeast DNA to the contour length of intact bacteriophage T4 DNA spread under the same conditions. T4 DNA has a well-characterized molecular weight (1.2×10^8) and contour length (52 μm long). (a) A Y-shaped fork with arms 2.2 μm long. (b) A bubble with branches between the forks 5.5 μm long. The bars represent 1 μm. The arrows indicate forks. (From Newlon, C. S., Petes, T. D., Hereford, L. M., and Fangman, W. L., *Nature*, 247, 33, 1974. With permission.)

the nucleus migrates into the neck of the bud. Finally, the nuclear membrane invaginates and nuclear fission occurs.

Various aspects of mitosis in yeast are unusual for a eucaryote. In contrast to higher cell nuclei, but in common with at least some other fungi,[234,236,237] the nuclear membrane remains intact throughout the division process. No structure resembling a nucleolus has been observed in *S. cerevisiae*, although structures resembling nucleoli have been clearly observed in other fungi.[234,238-243] The chromosomes undergo no process of condensation and coiling comparable to that seen in the mitotic chromosomes of higher cells. Of course, because yeast chromosomes are so small, the amount of condensation needed to make the yeast chromosome into a manageable packet for the purposes of mitosis may be very slight.

However, *S. cerevisiae* does have a mitotic spindle, as do other eucaryotes. From electron microscope studies of mitosis in *S. cerevisiae*, Peterson and Ris[244] have reported:

> The spindle microtubules originate from a plaque-like structure called the spindle pole body (SPB) embedded within the nuclear envelope. Early in mitosis the single SPB duplicates. A spindle containing both continuous and non-continuous microtubules is formed between these structures as they move apart on the nuclear periphery. Chromosomes do not condense and are not individually visible . . . . In diploid mitosis the number of microtubules issuing from a SPB generally ranges between 40 and 45. Most are non-continuous and often they are visibly associated with a chromatin fiber. Similar spindle structure and microtubule-chromatin attachments are seen in haploid mitosis, but here the number of microtubules issuing from a SPB is about half that in the diploid and the SPB is smaller. The pole-to-pole microtubules vary in number from spindle to spindle but in each case enough

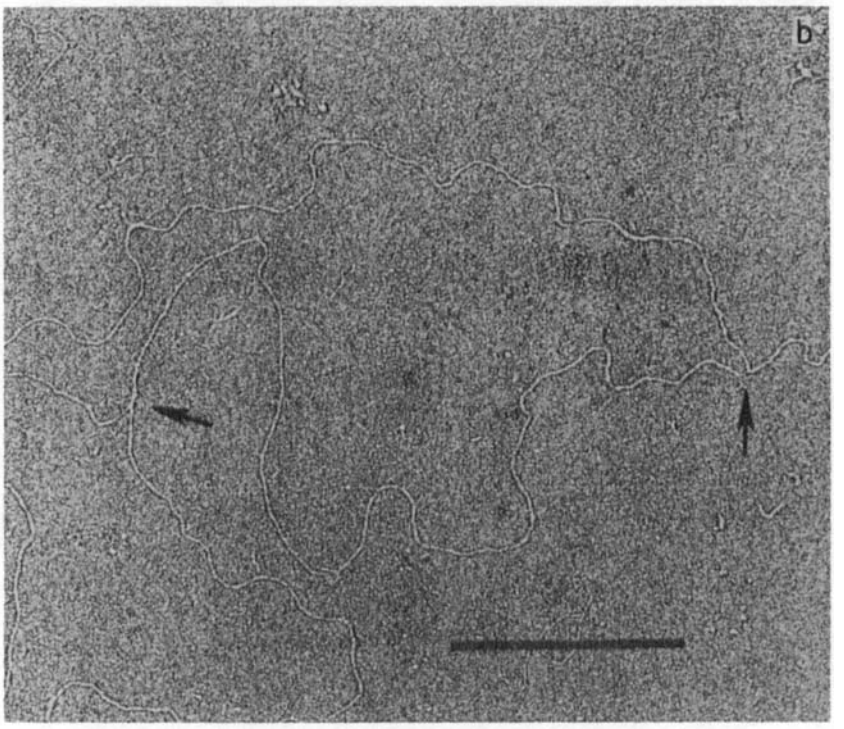

FIGURE 28B.

microtubules are associated with a spindle pole body to account for each linkage group (34 in diploid, 17 in haploid) being associated with a single non-continuous microtubule.

A final point to be made about the nuclear DNA of *S. cerevisiae* concerns yeast chromatin. Polyacrylamide gel electrophoresis of the histones of *S. cerevisiae* has shown that F1 is clearly absent, and it is questionable whether there is F3.[245,246] In addition, nuclease digestion has shown that the basic subunit of yeast chromatin contains about 135 base pairs of DNA compared to the 200 base pair subunit found in the chromatin of higher eucaryotes.[247] Thus, despite differences in histones, yeast chromatin does have a subunit structure comparable to that found in the chromatin of higher eucaryotes.

As mentioned above, in addition to nuclear DNA, yeast contains both mitochondrial and 2 μm DNA. Yeast mitochondrial DNA is so interesting that as much practical work has been done on it as on yeast nuclear DNA. Electron micrographs of DNA released by osmotic shock from isolated mitochondria of *S. cerevisiae* show the mitochondrial DNA to be a closed circular duplex with contour length of about 25 μm[248] (see Figure 29). Attempts to isolate the 25-μm circles intact have been unsuccessful. DNA isolated by direct lysis followed by CsCl equilibrium gradient centrifugation consists of linear molecules 2 to 24 μm in length.[248]

Yeast mitochondrial DNA contains 18% G + C by base analysis,[249] but neither the T_m (74.5°C)[250] nor the buoyant density (1.684 g/cc)[250] are what would be calculated for DNA of 18% G + C.[204,252] Yeast mitochondrial DNA contains extended (poly dA) (poly dT) and (poly dA·dT)(poly dA·dT) sequences,[252,253] and the discrepancy between the G + C content as calculated from the buoyant density and the T_m was assumed to be due to the presence of such sequences.[254] More recently, studies involving fractionation of mitochondrial DNA by either HAP[255,256] or CsCl gradient centrifugation[257] have demonstrated that relatively G + C-rich regions of molecular weight about 1.5×10^6 alternate with A + T-rich sequences. For more

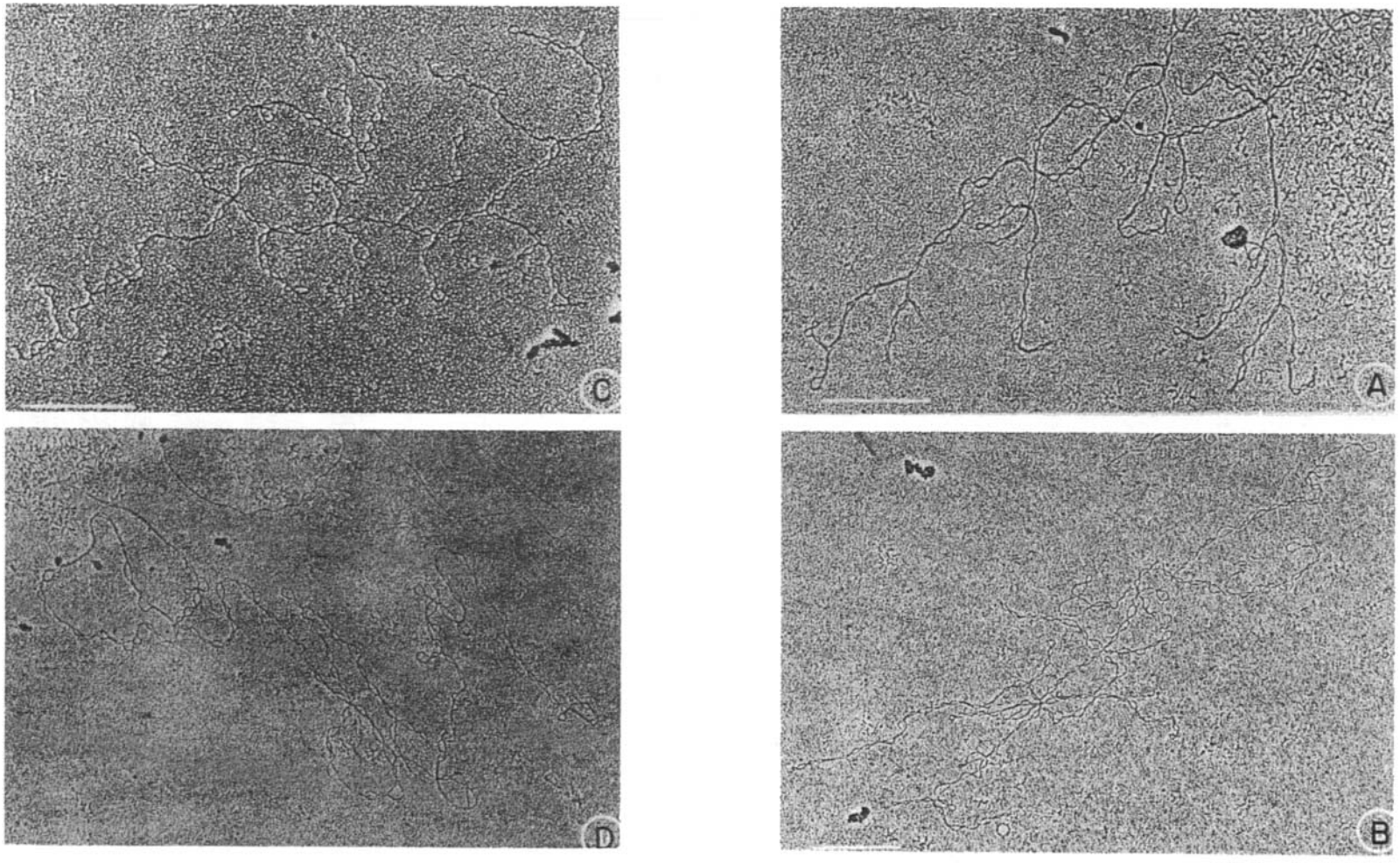

FIGURE 29. Electron micrographs of twisted circular DNA molecules released from yeast mitochondria, lysed by osmotic shock. The scale line is 0.5 μm (a to c) S. *cerevisiae*, length, respectively: 26, 28, and 25 μm. (d) *S. cerevisiae* Iso-N, length 24 μm. (From Hollenberg, C. P. et al., *Biochim. Biophys. Acta*, 209, 8, 1970. With permission.)

detailed analysis of the pyrimidine tracts of the mitochondrial DNA, see Ehrlich et al.[258]

The complexity of *S. cerevisiae* mitochondrial DNA as determined by renaturation kinetics is equivalent to that of a unique 25-μm piece of DNA.[248] However, in another renaturation kinetics study, Christiansen et al.,[259] working with *Saccharomyces carlsbergensis*, found a very marked influence of the molecular weight of reacting single-strand pieces on the genome size. The complexity of the DNA ranged from about 5×10^7 to 2.5×10^8 daltons when DNA fragments of molecular weights 2×10^5 and 1.5×10^6 daltons, respectively, were used. Christiansen et al., again working with *Saccharomyces carlsbergensis*,[260] have since extended this work, varying the renaturation temperature and the pH and observing how the rate constant depends on DNA fragment lengths. They found that the rate constant is smaller when longer fragments are used (see Figure 30). In other words, the DNA renaturation reaction occurs more slowly when longer DNA fragments are used, contrary to theory for simple renaturations. This allowed them to conclude that the pieces of denatured mitochondrial DNA with a molecular weight above 2 to 3×10^5 daltons are not fully extended random coils in 1 *M* NaCl at T_m -25°C. Presumably, single-strand interaction between adenine- and thymine-rich sequences, which are clustered at certain distances

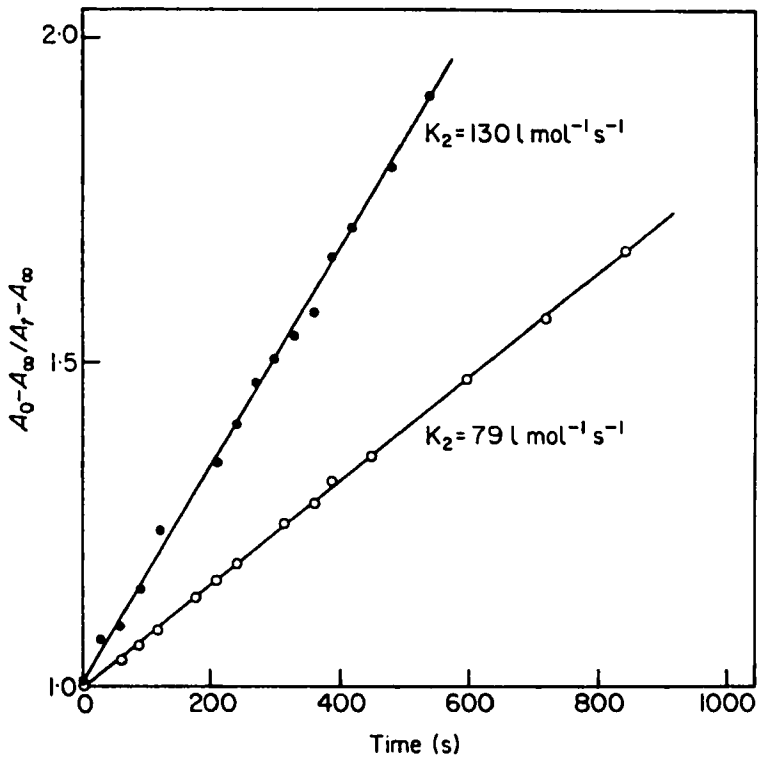

FIGURE 30. Second-order rate plots of the renaturation of yeast mitochondrial DNA. The DNA was denatured and degraded by heating to 100°C in 0.01 × SSC. After preheating to the renaturation temperature, 5 *M* NaCl was added to a final concentration of 1 *M*. The O.D. at 260 nm was recorded every 30 sec. The native absorbency was recorded before denaturation, and after accounting for the dilutions this value was used as A_∞. A_0 was the absorbancy at zero time (extrapolated) and A_t the reading at time t. The rate constants were calculated from the slopes of the straight lines and the concentrations of the DNAs. Two reactions are shown. Both were at a renaturation temperature of 59.5°C. (–●–●–) ($K = 130$ l mol^{-1} s^{-1}) DNA of a single-stranded molecular weight of 1.7×10^5. (–○–○–) ($K = 79$ l mol^{-1} s^{-1}) DNA of a single-stranded molecular weight of 8.7×10^5. The molecular weights were measured by sedimentation analysis in 1 *M* NaCl, 0.05 *M* Na_2HPO_4, pH 7.0. (From Christiansen, C., Christiansen, G., and Bak, A. L., *J. Mol. Biol.*, 84, 70, 1974. With permission.)

within the molecules, is the molecular basis for these observations.

From the above experiments, the size of the *S. cerevisiae* mitochondrial DNA is calculated to be 49×10^6 daltons, which corresponds to 25.5 μm (taking 1.92×10^6 daltons as equivalent to 1 μm).[261] Thus, the measure agrees closely with the length and complexity found by Hollenberg et al.[248]

Recently, Prunell and Bernardi[262] further investigated the mitochondrial genome of *S. cerevisiae* by degrading mitochondrial DNA with micrococcal nuclease. By using conditions so that the enzyme degrades the A + T-rich stretches very strongly whereas it inflicts only a limited number of breaks in the G + C-rich stretches, they have been able to show that the A + T-rich sequences are very homogeneous in base composition and have a G + C content lower than 5%, that the G + C-rich sequences are very heterogeneous in base composition with the G + C content ranging from 25 to 50% when the average size of the fragments is 1.2×10^5 daltons, and that the A + T-rich and G + C-rich sequences are present in about equal amounts and have comparable average sizes. These authors postulate that the G + C-rich sequences correspond to genes, while the A + T-rich sequences correspond to spacers.

An enormous amount of physical study has been done on the mitochondrial DNA of petite mutants of yeast, that is, respiratory-deficient mutants which are unable to grow on nonfermentable carbon sources. Many studies have shown that the mitochondrial DNA of petites differs from that of grande yeast in buoyant density, base composition, thermal denaturation and renaturation, nearest neighbor distribution, pyrimidine tracts distribution, and hybridization characteristics.[249,253,257,263-278] Experiments have shown that the way in which the mitochondrial DNA is altered varies from one petite strain to another. However, in general the mitochondrial DNA of suppressive petite mutants (that is, petite mutants in which the petite phenotype is due to a change in the mitochondrial DNA, not to the complete absence of mitochondrial DNA nor to a nuclear mutation) is higher in A + T-rich sequences than the mitochondrial DNA of wild-type strains. Studies have shown that the altered DNA contains tandem inverted repeats,[279] presumably clusters of repeated A + T-rich sequences. In sedimentation studies, Michels et al.[280] have shown that in some petite strains the mitochondrial DNA is of the same molecular weight as mitochondrial DNA from the parental grande strain. Thus, it seems that some G + C-rich sequences are lost while some A + T-rich sequences are amplified. Mechanisms for this process which may involve excisions, duplications, inversions, and amplifications have been postulated.[279] That petite mitochondrial DNA seems to have lost some G + C-rich regions while some of its A + T-rich regions have been amplified and the fact that petite mutants lack some genes found in grande yeast support the hypothesis put forth by Prunell and Bernardi[262] that G + C-rich sequences correspond to genes and A + T-rich sequences correspond to spacers.

This discussion of the mitochondrial DNA of *S. cerevisiae* is very brief considering the amount of experimentation that has been done on this DNA. Unfortunately, because of space considerations, further discussion of yeast mitochondrial DNA must be omitted.

The last major class of yeast DNA is 2 μm DNA, also called omicron DNA. This DNA consists of closed circles about 2 μm in length and with the same density in CsCl equilibrium gradients as yeast nuclear DNA (1.701 g/cc).[248,281] Actually, these circles range in length from about 0.3 to 7 μm, but the great majority are about 2 μm in length.[248,281] Two micrometer DNA comprises 1 to 5% of the total DNA in a yeast cell.[281]

Whether 2 μm DNA is of nuclear or mitochondrial origin is still in question. To investigate this question, Clark-Walker and Miklos[282] have used a petite whose mitochondrial DNA consists of small covalently closed circles. Using this altered mitochondrial DNA as a marker for cytoplasmic contamination, they found that purified nuclei do not contain DNA. Thus, they concluded that the 2 μm circles are of cytoplasmic origin. However, Zeman and Lusena[283] have shown that in vivo labeling of the 2 μm DNA is inhibited by cycloheximide in analogy to the behavior of nuclear and not mitochondrial DNA.

Some studies have shown that resistance determinants for certain inhibitors of aerobic (mitochondrial) metabolism are located on a cytoplasmic DNA species other than the mitochondrial DNA.[284] Griffiths et al. have suggested that the location of these markers may be on 2 μm DNA, but this is just a possibility, and experiments directed at investigating this have not been com-

pleted. The idea of such an episome is certainly interesting.

Recently, Petes and Williamson[285] have shown by electron microscope studies that the 2-μm circles replicate via a single bidirectional replication bubble, similar to those observed during replication of bacteriophage and *E. coli* chromosomes (see Figure 31).

Physical studies on the DNA of yeasts other than *S. cerevisiae* have been few. Christiansen et al.[259] have done a renaturation kinetic study of nine yeasts and found that all have between 5 and 16% of their nuclear DNA in repeated sequences and that the genome sizes vary from about 6×10^9 to about 1.3×10^{10} daltons. However, not enough data are presented in this paper to critically evaluate the results.

Dutta[286] has done the renaturation kinetics of several fungi in each of the three fungal groups—basidiomycetes, ascomycetes, and phycomycetes. Each species was found to contain 10 to 20% repeated DNA, some of which was probably mitochondrial DNA. Thus, fungi seem to contain a small percent of repeated DNA.

SLIME MOLDS

The only two slime molds whose DNA has been extensively studied are *Physarum polycephalum*, a true or acellular slime mold, and *Dictyostelium discoideum*, a cellular slime mold. Therefore, in this review the discussion of physical studies on the DNA of slime molds will be confined to these two organisms.

Physarum polycephalum is an especially nice organism for DNA studies because in the plasmodium all of the nuclei are synchronized so that the different stages of the cell cycle (i.e., DNA synthesis, G2, and mitosis) occur at the same time in all nuclei.[287] The organism is also very nice because it has two vegetative stages during which growth but not differentiation occurs (plasmodium and amoeba) and three developmental stages during which differentiation but not growth occurs (sclerotization, sporulation, and transformation between flagellated and nonflagellated stages in the amoebae). Thus, growth and differentiation are temporally separate so that growth can be studied without differentiation occurring and differentiation can be studied in the absence of growth.[287] At the present time, most studies on the DNA of *P. polycephalum* have been done in one of the two vegetative stages, plasmodium and amoeba.

Strains of *P. polycephalum* are generally classed

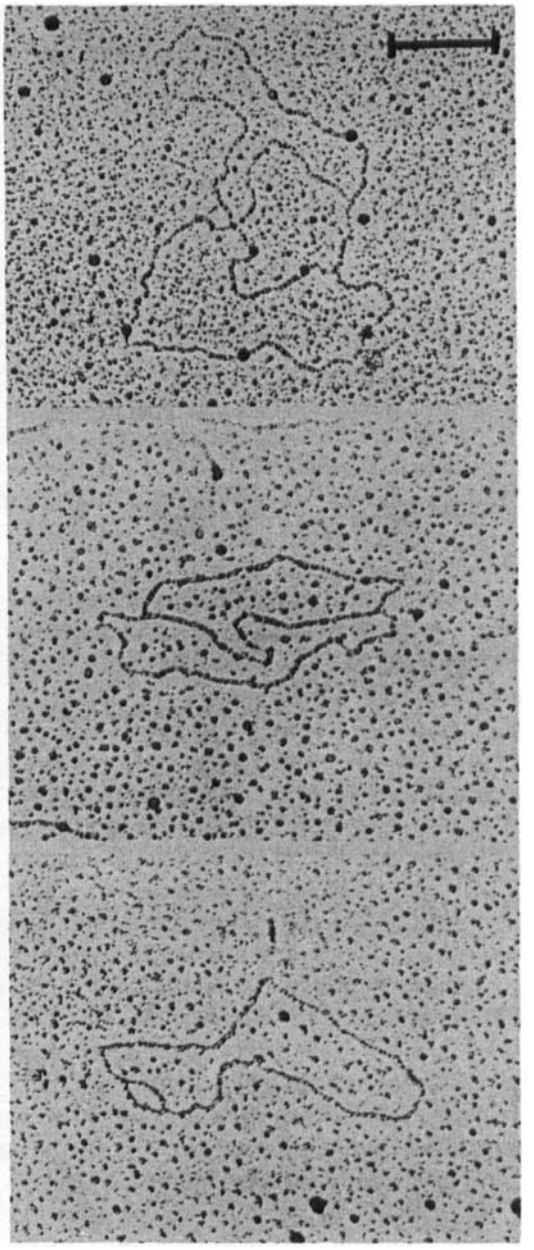

FIGURE 31. Replicating 2 μm circular DNA molecules. These molecules were observed in DNA isolated from cdc 8 after cells had been incubated for 3.5 hr at the restrictive temperature. The calibration bar represents 0.2 μm. All molecules were photographed at the same magnification. (From Petes, T. D. and Williamson, D. H., *Cell*, 4, 250, 1975. With permission.)

as either heterothallic (sexual) or colonial (asexual), depending on how plasmodia are formed from amoebae. In heterothallic strains two haploid amoebae of different mating types fuse, and karyogamy occurs to form a diploid zygote which develops into a diploid plasmodium.[288] In colonial strains one or several identical haploid amoebae develop asexually into a haploid plasmodium; it is unknown whether one amoeba can develop into a plasmodium or whether several amoebae must unite before plasmodium formation occurs.[289]

However, it has recently been shown that this classification of strains into two groups is somewhat artificial, because asexual plasmodium formation can occur in strains which are classed as sexual. The distinction is that the rate of asexual plasmodium formation is 10^{10} greater for colonial strains, being $\frac{10^{-1}}{hr}$ for colonial strains and less than $\frac{10^{-12}}{hr}$ for sexual strains.[289-291] This distinction between sexual and asexual strains would be unimportant for this review except for the fact that the number of chromosomes is different in the two types.

The exact number of chromosomes in *P. polycephalum* is not known. Not enough genetics has been done on the organism to determine the number of genetic linkage groups. Mohberg et al. have determined the number of chromosomes by microscopy,[292] but variation exists in the numbers of chromosomes seen, both within strains and between different strains.

In heterothallic strains, the diploid chromosome number is about 50. However, some plasmodia have been found to contain 75 to 80 chromosomes, others 175, others 240, and still others only 25 to 30.[292] The very high chromosome counts were often found in plasmodia containing nuclei of high ploidy.

Because of their haploid number of chromosomes, it should be easier to count chromosomes in amoebae of heterothallic strains. Unfortunately, no method is known for synchronizing the cell cycle in amoebae. Thus, chromosome counts have not been done in amoebae because of the difficulty in obtaining a number of good metaphase spreads from unsynchronized cultures.

The prototype asexual strain of *P. polycephalum* is Colonia. Chromosome counts on the haploid plasmodia of Colonia have yielded 35 to 40 for the number of chromosomes per nucleus.[292]

DNA per nucleus measurements have been done at various stages of the life cycle on both sexual and asexual strains of *P. polycephalum*.[292,293] Measurements on plasmodia from heterothallic strains have been done in G2 phase when the plasmodial nuclei, which are diploid, have the 4C DNA content. Plasmodial nuclei in G2 from heterothallic strains contain about 1 to 1.2 pg of DNA (about 6 to 7 $\times$ 10^{11} daltons).[292,293] Thus, the diploid DNA content is about 0.5 to 0.6 pg of DNA (about 3 to 3.5 $\times$ 10^{11} daltons). Rapidly growing haploid amoebae of heterothallic strains also appear to be almost entirely in G2, for they contain 0.6 pg of DNA (3.5 $\times$ 10^{11} daltons).[292,293]

By microdensitometry it has been shown that both plasmodia and amoebae of colonial strains are haploid and thus contain equal amounts of DNA per nucleus.[289] The DNA content of colonial nuclei is intermediate between that found in plasmodia and amoebae of heterothallic strains.[292]

It should be emphasized here that the DNA content of *P. polycephalum* nuclei is a number which is frequently misquoted. People generally state that *Physarum* nuclei contain 6 $\times$ 10^{11} daltons of DNA and 50 chromosomes, but they neglect to state that DNA per cell measurements are done in G2 in *Physarum*. Thus, this way of presenting the data is misleading for it implies that each chromosome contains twice as much DNA as it really does. It would be better to state that in a diploid cell, the 2C DNA content is about 3 to 3.5 $\times$ 10^{11} daltons of DNA and the chromosome number is 50.

Unfortunately, all the analytical determinations of DNA content in *P. polycephalum* were done by the diphenylamine method, for which a control for positively interfering substances cannot be done. Some indication of positive interference has even been found – plasmodia grown on agar yielded higher DNA per nucleus measurements than did plasmodia grown on filters,[293] presumably because of interference from the polysaccharide in agar. However, the DNA per nucleus measurements certainly yield a value which is close enough to the true value to be useful.

By comparing the DNA per nucleus values to those of other organisms, it is seen that *P. polycephalum* falls between yeasts and most higher organisms in DNA content. Dividing 3.5 $\times$ 10^{11} daltons (the 2C DNA content) by 50 chromosomes (the diploid chromosome number)

yields about 7×10^9 daltons of DNA, on the average, per chromosome. Thus, the chromosomes of *P. polycephalum* are of a size which could be examined by viscoelastic retardation-time experiments. At this date, no one has tried to size these chromosomes by any physical method.

From renaturation kinetic studies, the DNA of *P. polycephalum* seems to be that of a typical eucaryote. The nuclear DNA consists of 55% unique and 45% repeated sequences[294] (see Figure 32). From these data the complexity of *P. polycephalum* nuclear DNA is 130 times that of *E. coli*.[294] Taking 2.7×10^9 daltons for the complexity of *E. coli*, the complexity of *P. polycephalum* DNA is then 3.5×10^{11} daltons per haploid genome. Therefore, the DNA content calculated from renaturation kinetics agrees, within a factor of two, with the analytically determined DNA per nucleus. However, it is unclear from this paper whether the *E. coli* standard mentioned in the paper was actually run by the authors. If the standard was not run, the calculated complexity of *P. polycephalum* DNA may be in error by a factor of three to four.

Over 90% of the plasmodial DNA consists of nuclear main band DNA. This DNA has a density of 1.700 g/cc in CsCl,[294-296] which corresponds to a G + C content of 41%.[204] This agrees well with the G + C contents determined by T_m (39.8%)[296] and by base analysis (41%).[296]

In addition to the main band DNA, the nuclear DNA also contains a heavy satellite with density in CsCl of 1.714 g/cc.[295,297,298] This satellite accounts for about 2% of the total DNA in the organism and has been found by hybridization to contain rRNA cistrons.[299] Thus, *P. polycephalum* is like *S. cerevisiae* in that in both organisms the genes for rRNA are located on a heavy nuclear satellite.

Besides nuclear DNA, the only other DNA found in *P. polycephalum* is mitochondrial DNA, which accounts for about 5 to 10% of the total plasmodial DNA,[297,300] is synthesized throughout the division cycle,[301,302] and bands as a light satellite in CsCl.[302] This DNA has been identified as mitochondrial (1) by autoradiographic studies coupled with CsCl centrifugation,[300,303] (2) by the observation by Brewer et al.[304] that isolated *Physarum* mitochondria incorporate deoxyribonucleoside triphosphates into a DNA fraction whose buoyant density is the same as that of the light satellite, and (3) by direct preparation of the satellite DNA from purified mitochondria.[296]

The mitochondrial DNA of *Physarum* has a density of 1.686 g/cc in CsCl,[296] which corresponds to a G + C content of 26%.[204] As with the nuclear main band DNA, the percent G + C as determined by T_m(25%)[296] and by base analysis (26%)[296] agrees with that calculated from CsCl equilibrium density gradient centrifugation.

The renaturation kinetics of *Physarum* mitochondrial DNA has not been done. It has been suggested that this DNA contains repeated sequences or great homogeneity,[296] but not enough data have been presented to support this conclusion.

Sonenshein and Holt[305] have measured the size of *P. polycephalum* mitochondrial DNA by sedimentation and have found a broad band with a peak at an S value corresponding to about 18 μm of DNA. No closed circles have been isolated. The heterogeneity of this DNA in the centrifuge suggests that it may have been broken and that the true size is larger than 18 μm. At any rate, *P. polycephalum* mitochondrial DNA is definitely larger than the 5 μm circular mitochondrial DNA found in most higher eucaryotes thus far studied and is closer in size to the 20 to 25 μm mitochondrial DNA of *S. cerevisiae*,[248,260] *Tetrahymena*,[306] and *Paramecium*.[307]

Light and electron microscope work has shown that the mitochondria of *P. polycephalum* contain an elongated chromosome-like body, located in a central portion of the inner matrix.[308-312] This body is Fuelgen positive[312] and undergoes division similar to that of bacterial nucleoids.[311] Cytochemical studies have indicated that a large amount of the DNA in the mitochondria may be condensed in the central bond.[310,312] Experiments involving osmotic shock of the mitochondria followed by enzymatic digestion have demonstrated the presence of a large amount of DNA in the central bodies.[313]

By autoradiography it has been shown that synthesis of RNA occurs on the rodlike body.[312] This synthesis occurs nonrandomly on the body although the DNA is distributed homogeneously throughout the body.[314] These findings suggest that the activity of the DNA in the body may be selectively regulated.

As mentioned above, the mitochondrial DNA of *P. polycephalum* is synthesized throughout the cell cycle.[297,300,302] No studies have been done

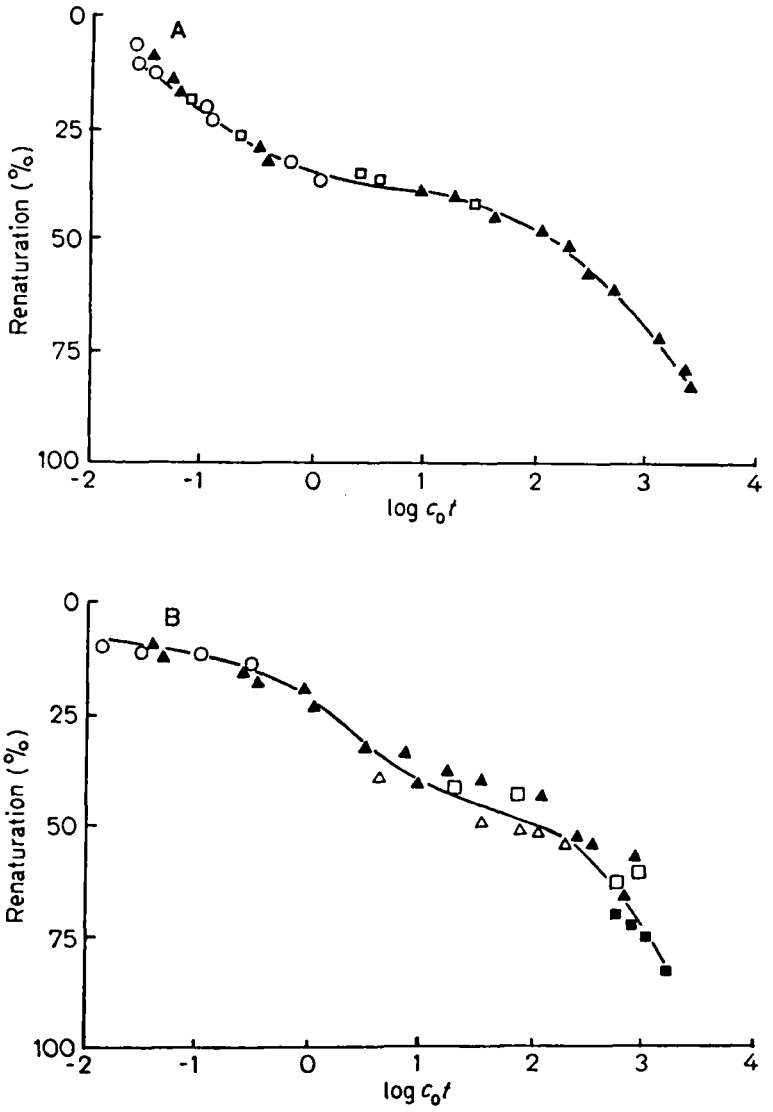

FIGURE 32. Renaturation of main band DNA from *Physarum*. (a) Hyperchromicity was continually measured at 260 nm (0% reassociation corresponds to 38% hyperchromicity of heat-denatured DNA). (O) 235 μg DNA/ml, (□) 270 μg/ml, (▲) 396 μg/ml. (b) For each point 5 μg DNA was analyzed on a 0.5-ml HAP column. (O) 100 μg DNA/ml, (Δ) 250 μg/ml, (▲) 500 μg/ml, (□) 900 μg/ml, (■) 1.000 μg/ml. (From Fouquet, H. et al., *Eur. J. Biochem.*, 44, 408, 1974. With permission.)

on the actual physical method of replication of this DNA.

Like the mitochondrial DNA, the heavy nuclear satellite, which contains the rRNA cistrons, is also synthesized throughout the cell cycle.[295,297,298,315] This continual synthesis is very unusual for a nuclear satellite DNA. Recently, Bohnert et al.[315] have demonstrated by electron microscopy circular DNA molecules the size of a ribosomal cistron (3.9 ± 0.2 μm contour length) and multiples thereof, as well as possible rolling circles in nucleolar DNA from plasmodia of *P. polycephalum*. These workers were unable to positively identify the DNA circles they saw as DNA-containing ribosomal cistrons because the major portion of the nucleolar DNA was made up of linear molecules 2 to 50 μm long. Twenty-five percent of the circular DNA seen was composed of lariats, which could be replication intermediates according to the rolling circle mechanism. How-

ever, as the authors point out, the possibility that small DNA circles were artifactually produced from linear DNA segments of discrete size classes during manipulation of the DNA cannot be excluded. Rolling circles have been demonstrated to occur in *Xenopus* oocyte nucleolar rDNA.[316,317] In *Xenopus* oocytes the rDNA cistrons are known to be undergoing amplification,[316,317] but in *P. polycephalum* amplification of rDNA is not occurring.[315]

In contrast to the continual replication of the two satellite DNAs, the main band nuclear DNA is synthesized in a specific S period.[318,319] The mitotic cycle of *Physarum* lacks a G1 stage;[298,318,319] the whole cycle lasts 8 hr, and chromosomal DNA is replicated during the first 3 hr following mitosis.[297,318,319] The timing of nuclear DNA synthesis has been studied to a finer degree. By experiments involving isotopic pulse labeling in one S period, density labeling in the following S period, and isopycnic centrifugation of DNA, it has been shown that DNA molecules replicated in the first fifth of one S period are replicated during the first fifth of the following S period.[320]

Much work has been done on the replication of *P. polycephalum* DNA. However, since DNA replication is not the subject of this review, only those studies which yield information on the physical structure of the DNA and its initiation sites and replication intermediates will be covered here.

The initiation and replication sites of DNA synthesis in the nuclei of *Physarum* have been studied by Kuriowa.[321] Examination of nuclei by thin sectioning and electron microscopy reveals that the nuclei contain two types of regions other than the nucleoli – electron-dense regions which contain chromatin-like fibrils about 300 Å in diameter and electron-transparent regions containing finer fibrils about 100 Å in diameter. By labeling nuclei with [H^3]thymidine at beginning and mid S phases and doing electron microscope autoradiography, it was observed that the initiation sites for DNA synthesis are in the boundary region between the electron-dense and electron-transparent regions, that within a few minutes replication sites migrate from the boundary region to the electron-transparent region, and that almost all of the nuclear DNA is synthesized in the electron-transparent region.

Sucrose sedimentation has been used by Brewer[322] to study the mechanism of DNA replication in *P. polycephalum*. When he uniformly labels the DNA of *Physarum*, isolates the nuclei in G2, lyses the nuclei on top of a neutral sucrose gradient, and sediments the DNA, the double-standard DNA has an S value corresponding to 2.3×10^8 daltons. When he follows the same procedure but makes the gradient alkaline instead of neutral, the single-stranded pieces have a molecular weight of 4×10^7 daltons, which is about 1/6 of 2.3×10^8 daltons and implies that each strand in each double-stranded fragment actually contains three pieces. Brewer also does the same two experiments on nuclei which are pulse-labeled and isolated in S phase. In this case he finds that the single-stranded pieces are 1.5×10^7 daltons but increase to 4×10^7 daltons as the nuclei are isolated at later times in S and in G2. He also finds that the double-stranded fragments isolated in S are 3.6×10^8 daltons but decrease to the G2 value of 2.3×10^8 daltons as nuclei are isolated later in S and G2. From these experiments he concludes that he is isolating DNA replication units. He also postulates that his results could support a model of DNA replication, first suggested by Taylor,[323] in which single-strand gaps or nicks or alkali-labile bonds occur alternately on the two DNA strands and serve as initiation and termination sites for DNA replication. The model postulates that the nicks could be repaired by ligase after strands are synthesized.

Although Brewer is interested in replication, his experiments are applicable to the study of the size(s) of DNA in the *Physarum* nucleus. Brewer calculates that if each G2 nucleus of *P. polycephalum* contains 6×10^{11} daltons of DNA and 50 chromosomes, and if the pieces of DNA in the nucleus are 2.3×10^8 daltons in size, then each replicated chromosome should contain about 50 double-stranded DNA molecules. This is of interest with respect to the question of the number of pieces of DNA in a eucaryotic chromosome.

In a more recent paper, Brewer et al.[324] extend this work to show that although the initiation of synthesis of most double-stranded molecules occurs early in S, the synthesis of the single-stranded subunits is initiated at least throughout the first 2 hr of the 3-hr S period. This is shown by a combination of pulse labeling the DNA with several different radioactive isotopes and sedimenting the DNA in either neutral or alkaline sucrose gradients.

Most recently, Brewer has studied DNA synthesis in vitro in homogenates and isolated nuclei of *P. polycephalum*.[325] In this paper he only looks at single-stranded DNA and sizes the replicating DNA by alkaline sucrose sedimentation. However, in these experiments he finds two discrete size clases of DNA – a heavier one which behaves similarly to the DNA strands previously found[322,323] and a lighter species which sediments at 10S no matter when the cultures are homogenized. Brewer suggests that these experiments could support a "continuous-discontinuous" mode of DNA replication and that possibly an enzyme active in joining the intermediate DNA fragments is inactive or missing in the cell-free system.

It is possible that all of the above work by Brewer and co-workers may be valid. However, a major problem exists in all of this work: An assumption was evidently made that the pieces of DNA in *Physarum* nuclei are of a size which can be measured by sedimentation at the very high rotor speed (35,000 rpm) that was used in all of the above experiments. In order to prove that the measured S values are not artifacts of the techniques used, Brewer's experiments should be repeated as functions of rotor speed. (For a discussion of possible artifacts associated with sedimentation experiments, see the sedimentation section of this review.)

The last aspect of DNA replication in *P. polycephalum* that we wish to mention is the finding of Wagar and Huberman that nascent DNA in *Physarum* is covalently linked to RNA.[326] The experiments which show this were done by a modification of the modification of the nearest neighbor analysis used by Sugino and Okazaki[327] to determine whether RNA is covalently linked to nascent DNA in *E. coli*. The result in *Physarum* is very interesting with respect to the current controversy over whether RNA is linked to nascent DNA in all, some, or no organisms.

The last aspect of *P. polycephalum* DNA which deserves mention is that the chromatin seems to be that of a typical eucaryote with respect to its chromosomal proteins. *Physarum* contains six histones – three arginine rich and three lysine rich.[328] These histones occur in isolated *Physarum* nuclei in about equal weight to the DNA.[329] By polyacrylamide dodecylsulfate gel electrophoresis, only five bands are present because one arginine-rich protein and one lysine-rich protein have the same molecular weight. The molecular weights of *Physarum* histones are different from but comparable to the molecular weights of calf thymus histones.[328] By polyacrylamide dodecylsulfate gel electrophoresis, about 30 different protein fractions can be resolved in the acidic protein fraction from *Physarum* chromatin.[328] Thus, in both its histone and nonhistone chromosomal proteins, *Physarum* seems to be a typical eucaryote.

The other slime mold on whose DNA many physical studies have been done is *Dictyostelium discoideum*. This organism is one which has often been used to study development because of its unusual life cycle. When food is present, myxamoebae grow and divide, but when the food is exhausted, the amoebae aggregate into a motile slug. This slug moves around for a while, then stops moving, rounds up, and forms a fruiting body in which some of the cells become stalk cells and others become spores of the fruiting body. Thus, the originally alike amoebae have developed into two different types of cells in the slug.[330]

In the past 5 years, much good physical study has been done on the DNA of *D. discoideum*. This work is of course interesting from the viewpoint of DNA structure and sequences and the study of evolutionary relationships between organisms. However, it is also useful in that it will make possible the study of control of development in *Dictyostelium* by a molecular biological approach.

For DNA studies, *D. discoideum* has several advantages as an experimental organism. First, in this slime mold, as in *P. polycephalum*, cell division and cell differentiation occur at different times.[330] The second advantage is that in *Dictyostelium*, recombination occurs via a parasexual cycle.[331-333]

The myxamoebae of *D. discoideum* are predominantly haploid[334] and contain seven chromosomes.[335] Genetic studies have only just begun on this organism, but mutants have been easily isolated,[336] and it is clear that genetic studies are possible.

CsCl equilibrium density gradient centrifugation of the DNA of *D. discoideum* has been done by Sussman and Rayner[337] and by Firtel and Bonner.[338] The results of these two groups are in agreement, except that Sussman and Rayner did not find the peak for the heavy nuclear satellite which was seen by Firtel and Bonner. These experiments revealed three DNA bands. Using a

density of 1.7035 g/cc for *E. coli* DNA as standard,[339,340] main band nuclear DNA has a density of 1.676 g/cc which corresponds to 23% G + C,[204] a heavy nuclear satellite has a density of 1.687 g/cc which corresponds to 33% G + C,[204] and mitochondrial DNA has a density of 1.682 g/cc which corresponds to 28% G + C.[204]

The T_m's of nuclear and mitochondrial DNA are 78.5°C[338] to 78.8°C[337] and 80°C,[337,338] respectively. Base compositions calculated from T_m's[251] are 23% G + C for nuclear DNA and 26% G + C for mitochondrial DNA. Thus, the values for base composition determined by CsCl equilibrium gradient centrifugation and by T_m are in agreement for nuclear DNA and differ slightly for mitochondrial DNA, though the numbers are possibly within experimental error of each other.

Controversy exists over the percent of the total DNA which is nuclear. From the area under the peaks in CsCl equilibrium centrifugation, Sussman and Rayner[337] calculate that 40% of the whole-cell DNA is mitochondrial. From renaturation kinetics, Firtel and Bonner[338] determine that 28% of whole-cell DNA is mitochondrial. We are unable to judge which of these values is correct for the percent mitochondrial DNA.

Sussman and Rayner[337] have determined the DNA content of *D. discoideum* chemically in whole-cell preparations and in nuclear and mitochondrial fractions. Using a population of cells which was primarily haploid, they measured 1.3×10^{-13} g DNA per cell. Dividing by 1.3 because the cells were in continuous replication and subtracting out 40% of the DNA (they found 40% of the DNA to be mitochondrial) yielded 6×10^{-14} g DNA per haploid nucleus or 3.6×10^{10} daltons. Dividing this value by seven chromosomes (the haploid chromosome number) yields about 5×10^9 daltons as the average DNA content per chromosome or about twice the size of an *E. coli* chromosome.

The renaturation kinetics of *D. discoideum* has been done by Firtel and Bonner.[338] This work has been done very carefully. The renaturation kinetics has been done both optically and on hydroxyapatite, and renaturation experiments have been performed on whole-cell DNA, nuclear DNA, nuclear single-copy DNA, nuclear repetitive DNA, and mitochondrial DNA (see Figure 33).

From this work it is found that *D. discoideum* nuclear DNA consists of approximately 70% single-copy sequences characterized by a $C_0t_{1/2}$ (pure) of 68 and 30% repeated sequences characterized by a $C_0t_{1/2}$ (pure) of 0.28. The $C_0t_{1/2}$ of *E. coli* DNA under the same circumstances is 6.5. Thus, the complexity of *D. discoideum* single-copy nuclear DNA would be about 10.5 times that of *E. coli* DNA or about 28×10^9 daltons (using 2.7×10^9 daltons for the size of *E. coli* DNA).[10,229] The complexity of the nuclear reiterated DNA would be 1/23 that of *E. coli* DNA or about 1.2×10^8 daltons, and it would be repeated about 100 times.

However, Firtel and Bonner say that because of the difference in G + C content between *D. discoideum* DNA and *E. coli* DNA, the *D. discoideum* DNA should renature at a rate two thirds that of the *E. coli* DNA if these two DNAs were of the same complexity. Using 2.8×10^9 daltons for the size of *E. coli* DNA and multiplying the $C_0t_{1/2}$ ratios for the DNAs by 2/3, they conclude that the complexity of the nuclear single-copy DNA is about 7 times that of *E. coli* DNA or 20×10^9 daltons, and the complexity of the nuclear repetitive DNA is about 1/35 that of *E. coli* DNA or 8×10^7 daltons with this DNA repeated 120 times. The problem involved in making this kind of calculation is that controversy exists over the direction of the dependence of the rate of renaturation on G + C content, and recent work indicates that the dependence, if any, is so small that it can be ignored.[67,71,147,254,341,342,375] Therefore, the values calculated without adjusting for G + C content are the better ones to use until the effect of G + C content is well established.

From the complexity values uncorrected for G + C content, one calculates 4×10^{10} daltons for the nuclear DNA content of *D. discoideum*, a slightly higher value than is found by Sussman and Rayner[337] by chemical determination of nuclear DNA content (3.6×10^{10} daltons). Dividing 4×10^{10} daltons by 7 yields 5.7×10^9 daltons for the average DNA content per chromosome.

Firtel and Bonner[338] have also determined by renaturation kinetics that the mitochondrial DNA of *D. discoideum* has a complexity of approximately 35 to 40×10^6 daltons. This places *Dictyostelium* mitochondrial DNA in a size class with *Drosophila*, *Tetrahymena*, and *Paramecium* mitochondrial DNAs, which have complexities between 30 and 40×10^6 daltons (15 to 20 μm). This is larger than the 10×10^6 daltons (5 μm) DNA found in higher animal mitochondria and smaller than the 50×10^6 dalton (25 μm)

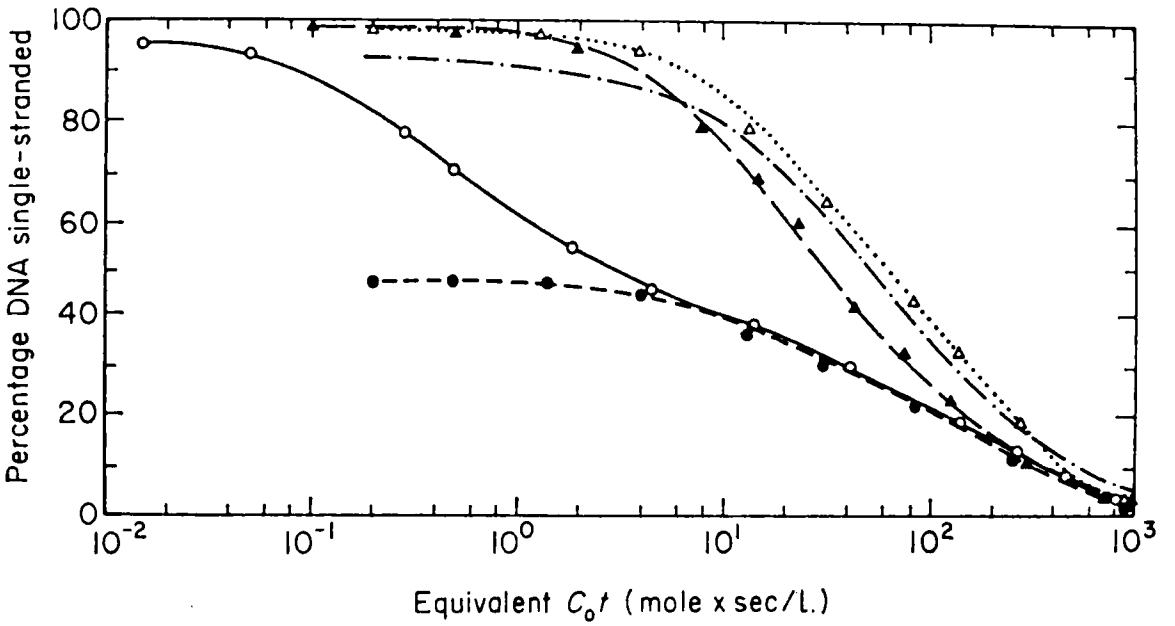

FIGURE 33. Optical and HAP C_0t plots of single-copy DNA and HAP C_0t plot of total DNA. (–O–O–) HAP curve of total-cell DNA. (···Δ···Δ···) HAP curve of tracer amounts of single-copy DNA reassociated with total-cell DNA. A ratio of 1,000:1, total-cell DNA to single-copy DNA (100,000 cts/min/μg), was used. (–●–●–) Data from single-copy curve single-copy DNA. (–·–·–·–·) Optical curve of single-copy DNA. This curve is a combination of curves seen at 112 μg/ml, 0.12 *M* phosphate buffer, 56°C and 1,160 μg/ml, 0.24 *M* phosphate buffer, 60°C. Conditions for HAP curves: for points below C_0t 0.4, 50 μg DNA (25 μg/ml) was used in 0.12 *M* phosphate buffer at 56°C; for points between C_0t 0.4 and 10, 100 μg at 100 μg/ml was used; for points above C_0t 10, 100 μg DNA at 500 μg/ml in 0.24 *M* phosphate buffer at 60°C. HAP C_0t curve of renaturation of total-cell DNA was done in parallel experiments using labeled total-cell DNA (2,000 cts/min/μg). Purification of single-copy DNA (fraction II). Sheared DNA was adsorbed to hydroxyapatite at 60°C in 0.03 *M* phosphate buffer, washed, eluted at 0.48 *M* phosphate buffer, and reassociated to an equivalent C_0t 75. The single-stranded material eluted from the HAP column at 0.12 *M* phosphate buffer was used as fraction II. (From Firtel, R. A. et al., *J. Mol. Biol.*, 66, 353, 1972. With permission.)

mitochondrial DNA of *S. cerevisiae* and *Neurospora crassa*, and the still larger mitochondrial DNA of higher plants.[343]

In this same paper, Firtel and Bonner[338] tried to determine whether *D. discoideum* mitochondrial DNA is closed circular by adding ethidium bromide to CsCl gradients. No evidence of circularity was found, but such closed circular DNA may be present but broken or nicked by nucleases.

The interspersion pattern of repetitive and single-copy DNA sequences in the genome of *D. discoideum* has been determined by Firtel and Kindle.[344] Renaturation kinetics was done both by hydroxyapatite binding and by S1 nuclease digestion of various length tracer DNA fragments renatured to low C_0t in a vast excess of sheared DNA (see Figure 34). This work appears very well done. The results show that about 50 to 60% of the single-copy sequences in DNA fragments 1,500 nucleotides long and 75% of the single-copy sequences in fragments 3,000 nucleotides long are linked to short interspersed repeated DNA sequences. The average length of the single-copy sequences is 1,500 nucleotides. The repeated DNA is of two types – one half is in short fragments 250 to 450 nucleotides long which are interspersed with the single copy DNA and the other half consists of sequences greater than 2,000 nucleotides long. These results indicate that *D. discoideum* has the same type of structural organization of the genome as do the sea urchin *Strongylocentrotus purpuratus*[345] and the frog *Xenopus laevis*.[346]

PROTOZOA

Oxytricha (A Hypotrichous Ciliate)

In the group of ciliates called hypotrichs, nuclear development and the nature of the DNA have been well studied mainly in two organisms,

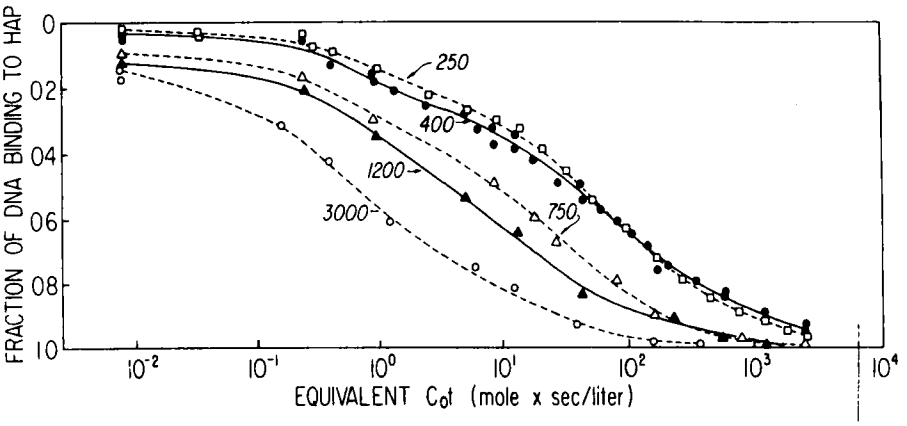

FIGURE 34. Renaturation of tracer DNA of various lengths to nuclear DNA. ^{32}P DNA was sheared to various lengths, sized, and renatured to a 200-fold excess of 350-nucleotide-long ^{3}H total nuclear DNA. The fraction of the DNA containing duplex regions was assayed as described in the authors' study. Zero C_0t binding material was removed from DNA with fragment lengths equal to and larger than 1,200 nucleotides prior to renaturation. The fraction bound (F) from fragment sizes 1,200 nucleotides long or smaller was calculated: $F = \frac{B_{ci} - B_0}{T - B_0}$ where B_{ci} = counts adsorbed to HAP at C_0t_i, B_0 = counts bound at $C_0t = 0$; T = total counts eluted in 0.12 and 0.48 *M* PB washes. The ^{3}H carrier DNA showed the same renaturation kinetics as the 400 nucleotide-long tracer and is not shown in the figure. (□) 250-nucleotide-long tracer. (●) 400-nucleotide-long tracer. (△) 750-nucleotide-long tracer. (▲) 1,200-nucleotide-long tracer. (○) 3,000-nucleotide-long tracer. (From Firtel, R. A. et al., *Cell*, 5, 403, 1975. With permission.)

Stylonychia and *Oxytricha*. Most of the work on *Stylonychia* has been carried out by Ammermann.[347-351] Prescott and coworkers[352-357] have done extensive physical studies on the DNA of *Oxytricha*. Both organisms are closely related and quite similar. Arbitrarily, it is mostly the work from Prescott's laboratory we outline here. This work serves as an excellent example of the large amount that can be learned about nuclear arrangement from DNA physical studies. Much of what is outlined below is taken directly from a recent review by Prescott and Murti.[355] This review should be consulted for details and further references.

Oxytricha and related ciliates are of special interest because of their highly unusual nuclear arrangement.

The ciliated protozoa contain two kinds of nuclei: a micronucleus and a macronucleus. The micronucleus possesses clearly visible chromosomes, is diploid, divides by mitosis, and synthesizes only a trace of RNA. The macronucleus contains no discernible chromosomes, has many times the diploid amount of DNA, divides amitotically, and provides virtually all of the RNA needed to run the vegetative life of the cell.

During conjugation in ciliates, the micronucleus undergoes meiosis, the two cells exchange haploid micronuclei, and then separate. An exchanged micronucleus fuses with a resident, haploid micronucleus to produce a new diploid micronucleus in both exconjugant cells. The new micronucleus then divides by mitosis without division of the cell. Accompanying these events, the old macronucleus starts to disintegrate and eventually disappears completely. A new macronucleus grows from one of the new micronuclei, the principal event being the production of the large amount of DNA characteristic of the macronucleus. When formation of the new macronucleus is complete, the ciliate resumes its vegetative life of cell growth and reproduction.[355]

Oxytricha contains four genetically identical micronuclei and two macronuclei; the mature macronuclei contain about 600 times more DNA than the micronuclei. An *Oxytricha* cell with stained micronuclei (MI) and macronuclei (MA) is shown in Figure 35.

In *Stylonychia*, a series of events precedes the formation of a mature macronucleus.

1. Banded, polytene chromosomes similar in appearance to those of *Drosophila* are formed in the nucleus -- presumably the synthesis of these polytene chromosomes is totally directed by the micronucleus.
2. The polytene chromosomes are cut in the

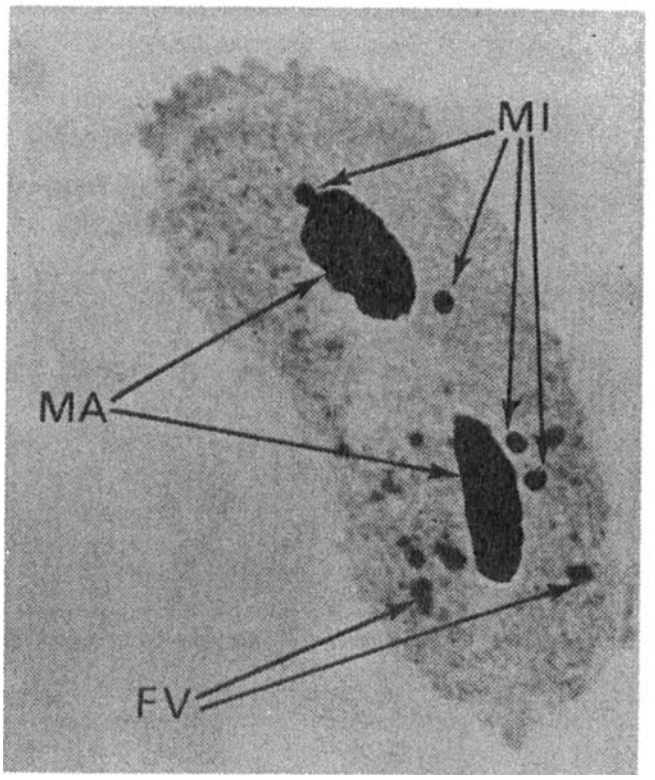

FIGURE 35. An *Oxytricha* cell stained with the Feulgen technique and with fast green. The two macronuclei (MA) and four micronuclei (MI) are visible. The cell contains a few food vacuoles (FV) that stain with fast green. (After Prescott, D. M., Murti, K. G., and Bostock, C. J., *Nature*, 242, 576, 1973. With permission.)

interband region and then each band is surrounded by a membranous vesicle.

3. About 93% of the DNA is degraded in the vesicles.

4. The remaining DNA is replicated many times to obtain a mature macronucleus.

The DNA content as a function of time of development of the macronucleus is shown in Figure 36 for *Stylonichia*. The events in the formation of the mature *Oxytricha* nucleus are presumed to be the same since both organisms are very closely related.

Physical studies of the DNA of the micronuclei and macronuclei of *Oxytricha* have helped elucidate many of the details of the events in mature macronucleus formation. We outline here only the main findings.

An important question is whether any genetic information or complexity is lost upon macronucleus formation. Early experiments[357] indicated qualitatively that some complexity is lost. They measured buoyant density and thermal denaturation profiles for both macronuclear and micronuclear DNA; the data are presented in Figures 37a and 37b. Both the buoyant density and thermal denaturation profiles are broader for the micronuclear DNA, indicating the presence of heterogeneous DNA sequences (AT rich and GC rich) not present in the macronuclear DNA.

This loss of complexity has recently been quantitated using DNA renaturation kinetics[358] shown in Figure 37c. The micronuclear DNA is seen to renature over a wide range of C_0t values, indicating DNA sequences of varying degrees of repetitiveness and DNA of very high complexity, both typical traits of eucaryotic nuclear DNA. The macronuclear DNA, however, renatures over a narrow range of C_0t values, similar to *E. coli* and T4, indicating all the different DNA sequences are repeated about the same number of times. The macronuclear DNA is also less complex. These are

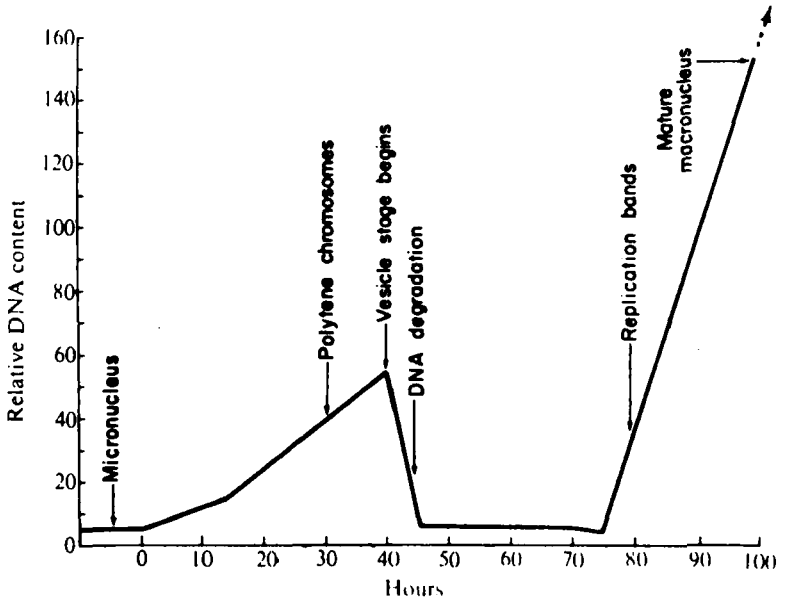

FIGURE 36. The course of DNA changes during the development of the macronucleus from a micronucleus. The initial DNA build-up results in the formation of polytene chromosomes. The polytene chromosomes are cut into individual bands, and most of the DNA is then degraded. Finally, the remaining DNA replicates many times to produce the mature macronucleus. (From Ammerman, D., *Arch. Protistenk.*, 108, 108, 1965, appears modified as above in **Prescott, D. M., Murti, K. G.,** and **Bostock, C. J.,** *Nature*, 242, 576, 1973. © Macmillan. With permission.)

the results one would expect if the macronuclear DNA were derived from micronuclear DNA, all sequences polytenized to the same degree, and then some of the sequences selectively destroyed.

The authors note a few problems with this renaturation kinetics: The $C_0t_{1/2}$ value for *E. coli* DNA is only 11 ± 2 times that of T4 DNA, compared to the expected value about 20. This may be due to GC composition effects as the authors suggest or due to slow base pair zipping under the conditions of their experiments.[69,76] Also, the slowest renaturing portion of micronuclear DNA renatures 4.5 times slower than expected for unique DNA from DNA per cell measurements. This is certainly unusual because unique DNA is theoretically the slowest renaturing DNA. Despite these discrepancies, the renaturation data still substantiate the idea that DNA sequence complexity is lost during macronuclear development. Ammerman et al. have obtained similar renaturation kinetic results.

An interesting experiment would be to drive the renaturation of radioactively labeled micronuclear DNA (of varying fragment lengths and at low concentration) with a vast excess of unlabeled macronuclear DNA. From the extent of reaction, one can then directly determine the percent of sequences in the micronuclear DNA which are represented in the macronuclear DNA; also from fragment length studies, information on interspersion of macromolecular DNA sequences in the parent micronucleus may be obtained.

Molecular weight measurements on the macronuclear and micronuclear DNA using sucrose sedimentation and electron microscopy have led to interesting results. About 90% of the macronuclear DNA sediments at 10S, corresponding to a molecular weight of about 1.5×10^6 daltons (2,000 base pairs), pieces of DNA easily big enough to code for single polypeptides. The rest of the macronuclear DNA sediments at about 14S, corresponding to a molecular weight of about 3.6×10^6 daltons; from hybridization studies this DNA appears to code for ribosomal DNA.[354] Lengths of DNA measured in the electron microscope correspond well to the 1.5×10^6 dalton molecular weight. Sedimentation and electron microscopy of micronuclear DNA yield pieces

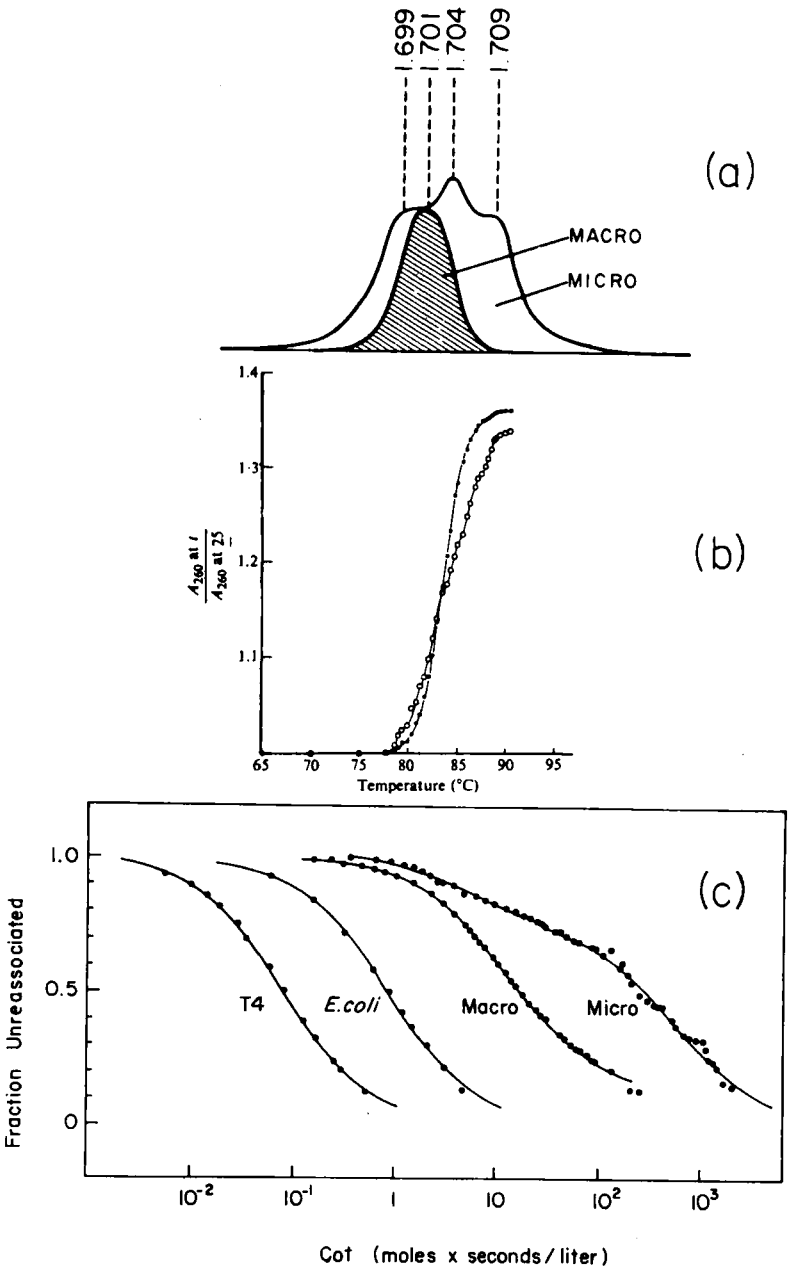

FIGURE 37. Evidence for reduction in complexity of macromolecular DNA compared to that of micronuclear DNA in *Oxytricha*. (a) Buoyant density profiles in CsCl for micronuclear DNA and macronuclear DNA. Micronuclear DNA consists of at least four density components with peaks at about 1.699, 1.701, 1.704, and 1.709 $g \cdot cm^{-3}$. (From Prescott, D. M., Murti, K. G., and Bostock, C. J., *Nature*, 242, 576, 1973. With permission.) (b) Melting curves for micronuclear DNA (○) and macronuclear DNA (●). Micronuclear DNA melts with a complex pattern, indicating the presence of several components. (From Prescott, D. M., Murti, K. G., and Bostock, C. J., *Nature*, 242, 576, 1973. With permission.) (c) Renaturation kinetics of T4, *E. coli*, and *Oxytricha* macronuclear and micronuclear DNA. All renaturations were carried out in 20% formamide, 0.5 *M* sodium phosphate buffer pH 6.8 and were monitored optically. The smooth lines are second-order curves computer fitted to the data (except for reassociation of micronuclear DNA where the fitted curve consists of two second-order components). Data shown have been corrected for effects of molecular weight and base components. (From Lauth, M. R., Heumann, J., Spear, B. B., and Prescott, D. M., *Cell*, submitted. With permission.)

hundreds of times larger than macronuclear DNA and ranging in size over an order of magnitude. This result is consistent with shear broken chromosomal DNA of original molecular weight greater than about 10^9 daltons.

From these studies, it appears that macronuclear DNA has been transected specifically into gene-size pieces of DNA. The strongest evidence that these gene-size pieces may indeed be single genes is that *E. coli* RNA polymerase binds specifically to one end of these pieces. Several electron micrographs showing RNA polymerase bound to pieces of macronuclear DNA are shown in Figure 38. As a result of these and other physical studies on *Oxytricha*, Prescott and co-workers have developed a very plausible model for the formation and nature of the macronucleus. This model is presented in Figure 39.

Ammerman and co-workers[374] disagree with Prescott and co-workers on one critical point. They find very large DNA molecules at all stages of the developmental cycle; that is, while Ammerman's group also finds "gene-sized" DNA molecules in the mature macronucleus, they find a large percentage of molecules many times larger. They suggest that this result may be due to differences in DNA isolation procedures. It is obviously important that the reason for this discrepancy be understood.

The presence of small, uniformly sized DNA in the macronucleus could be due to a restriction-type enzyme specifically cutting the DNA. Indeed, Wesley's[359] study shows that there are restriction-like cuts in the macronuclear DNA. However, it remains to be shown whether these restrictionlike cuts are of biological importance or due to artifactual action of a restriction enzyme during DNA isolation.

Tetrahymena and *Paramecium* (Holotrichous Ciliates)

Tetrahymena and *Paramecium* are ciliated protozoa which also contain both micronuclei and macronuclei. The nature of the DNA in the macronucleus and its relationship to micronuclear DNA have been studied in some detail by Allen and Gibson.[360] Their studies were carried out on several strains of both *Paramecium aurelia* and *Tetrahymena pyriformis*. What follows is mostly from their paper.

The average DNA content of the micronucleus for several strains of *P. aurelia* and *T. pyriformis* is 3.7×10^{11} daltons and 2.7×10^{11} daltons, respectively, for a G1-diploid genome. The average G1 macronuclear DNA content for these strains is in the neighborhood of 3×10^{14} daltons for *P. aurelia*, so the macronucleus contains about 800 times as much DNA as the micronucleus. For *T. pyriformis*, the average G1 macronuclear DNA content is about 1.2×10^{13} daltons, so the macronucleus contains only 45 times as much DNA as the micronucleus. (Some of the data for calculating these averages were obtained from Reference 362.) In comparison, the *Stylonychia* macronucleus contains about 30 times as much DNA as the micronucleus (see Figure 36). In

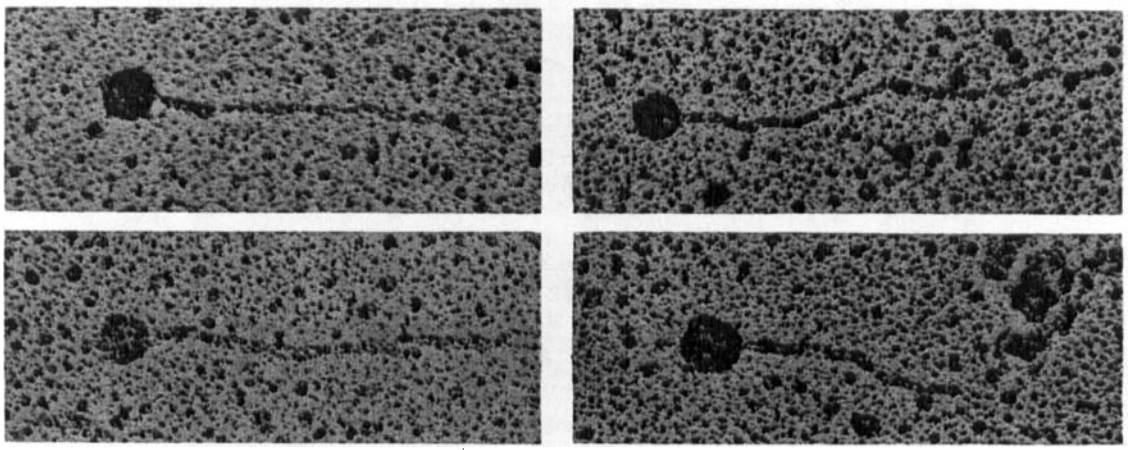

FIGURE 38. Electron micrography of *B. subtilis* RNA polymerase and *Oxytricha* macromolecular DNA complex. (From Murti, K. G., Prescott, D. M., and Pené, J. J., *J. Mol. Biol.*, 68, 413, 1972. With permission.)

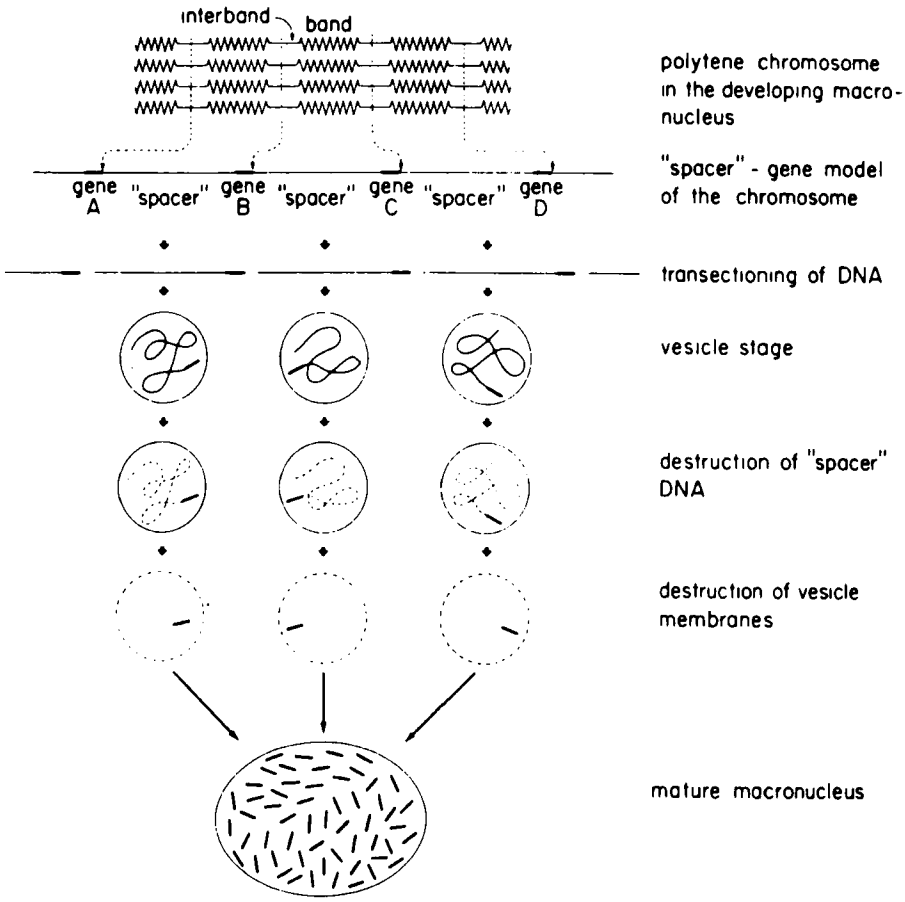

FIGURE 39. A scheme to explain the derivation of the gene-sized pieces of DNA in the mature macronucleus from the chromosomes of the micronucleus. The micronucleus goes through several rounds of DNA replication to produce the polytene chromosomes of the early macronuclear anlage. Each band of the polytene chromosomes in the macronuclear anlage is assumed to represent a single genetic locus. The polytene chromosomes are transected between successive bands. The polytenic copies of DNA in each band become enclosed in a vesicle. Only one copy is shown in each of the three vesicles in the drawing. Most of the DNA in each vesicle (band) is considered to consist of a "spacer" that separated the gene copy in one band of a chromosome from the gene copy in the next band. Destruction of the "spacers" occurs in the vesicle stage and is assumed to account for the degradation of 94% of the DNA in the anlage. Only the structural genes with control regions, accounting for 7% of the original chromosomal DNA, are preserved. Destruction of the vesicle membranes produces a macronuclear anlage containing gene-sized pieces of DNA. This DNA is replicated many times to produce the DNA-rich, mature macronucleus. (From Prescott, D. M. and Murti, K. G., *Cold Spring Harbor Symp. Quant. Biol.*, 38, 609, 1973. With permission.)

Oxytricha the G1 macronucleus has about 90 times the DNA of the G1 micronucleus.

One very unusual property of the DNA from both *P. aurelia* and *T. pyriformis* macronuclei is its unusually high A + T content, 72 and 75%, respectively.[360] In comparison, *Oxytricha* macronuclear DNA has 42% A + T.[358]

The complexity of the DNA in the macronucleus of these several strains of *P. aurelia* and *T. pyriformis* was determined by Allen and Gibson[360] using renaturation kinetics monitored by HAP chromatography. Some of their results are presented in Figure 40. The nonrepeated macromolecular DNA had average complexities of 2.2×10^{11} daltons for *P. aurelia* and 1.4×10^{11} daltons for *T. pyriformis*, which is close to the haploid micronuclear DNA content, 1.85×10^{11} daltons and 1.35×10^{11} daltons, respectively. Thus, it appears there is little or no DNA sequence diminution in the formation of the macronucleus.

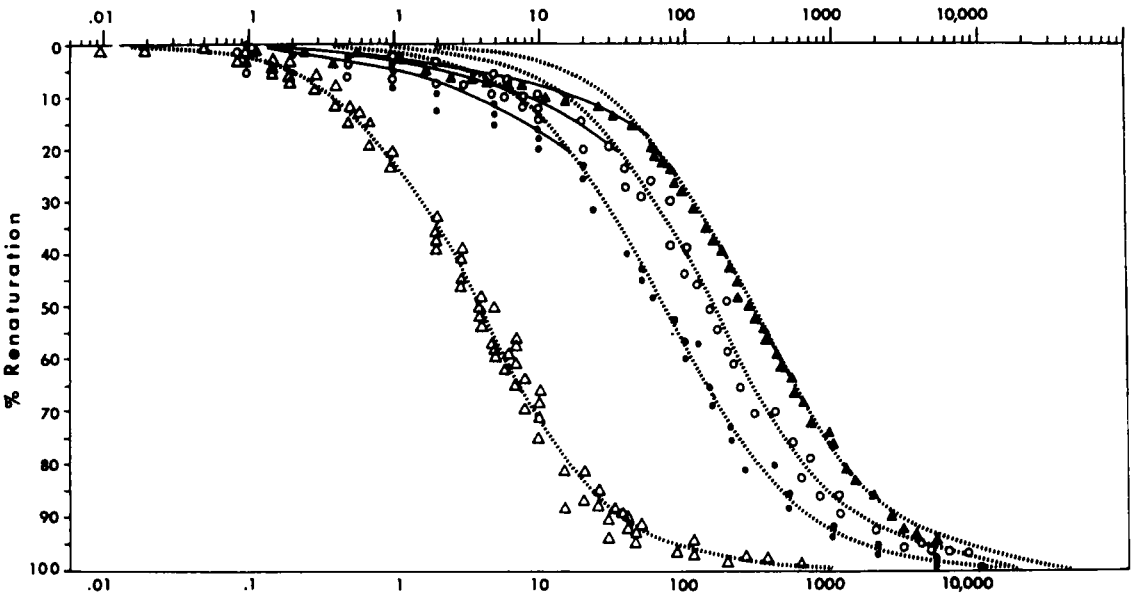

FIGURE 40. Renaturation kinetics of *E. coli* and ciliate (*Tetrahymena* and *Paramecium*) DNAs. A mixture of unlabeled ciliate DNA was made with radioactive *E. coli* DNA. Each mixture was sheared by sonication to pieces 500 nucleotide pairs in length, heat denatured, and incubated at 50°C in 0.12 *M* sodium phosphate buffer (pH 6.8) for different periods of time. The samples taken at different times were frozen and later adsorbed to HAP columns at 50°C, denatured DNA being eluted in washes with 0.5 *M* phosphate buffer. The fraction of each sample that renatured is plotted above. The concentrations of DNA which were incubated were as follows: *T. pyriformis* 1/7 (●), 80 and 800 μg/ml; 1/8 (○), 70, 90, 700, and 900 μg/ml. *P. aurelia* 8/138 (▲), 65 and 820 μg/ml. *E. coli* (△), 3, 5, 24, 49, and 45 μg/ml. The dashed lines drawn through the data at 50% renaturation represent the theoretical renaturation curves for a second-order reaction. The solid lines are drawn through the fast-renaturing samples. (After Allen, S. and Gibson, I., *Biochem. Genet.*, 6, 293, 1972. With permission.)

In contrast, from renaturation kinetics *Oxytricha* shows considerable sequence diminution, 50- to 100-fold (Figure 37c). Thus, there appears to be an important difference in the mechanism of macronucleus formation in *Oxytricha* as compared to that in *P. aurelia* and *T. pyriformis*. These renaturation kinetic results also show that *P. aurelia* has about 15% repetitive DNA, and *T. pyriformis* has between 20 and 30% repetitive DNA depending on the strain.

There is one problem with the renaturation kinetic results which the authors[360] have studied in some detail, but for which they can find no certain explanation. When renatured, nonrepeated macronuclear DNA is again melted and the amount of single-stranded DNA as a function of temperature is quantitated by elution from hydroxyapatite, a large percentage of the DNA elutes at temperatures lower than the native melting temperature. The usual explanation for this phenomenon is that the low-melting DNA contains mismatched bases which reduce its thermal stability. However, the renatured nonrepeated DNA of a "normal" organism usually does not have mismatches – mismatches would imply that different nuclei of the same organism contain different DNA sequences and thus potentially possess different genetic information. While this is unlikely for a normal nucleus, it may, however, be possible for a macronucleus since the real store of genetic information is the micronucleus. Perhaps the macronuclear DNA is not always copied faithfully from the micronucleus. On the other hand, since RNA (and presumably proteins from that RNA) is synthesized in the macronucleus, mutated macronuclear genes due to unfaithful copying of micronuclear DNA would probably be fatal to the organism.

Another explanation for this low melting temperature is that renatured DNA may be found in the unusually high A + T content (70 to 75%) of macronuclear DNA. At such A + T contents, especially if some single strands are A rich and others T rich, a fair percentage of stable, nearly complementary double-stranded helices might form. This could be happening in their experi-

ments, since their renaturation temperature (50°C) is not the most stringent for selecting against helices with mismatches. The authors claim, though not strongly, that they have not detected any base composition differences in this low-melting renatured DNA.

The mode of distribution of macromolecular gene copies between daughter macronuclei has been studied genetically by Orias and Flacks[362] in *T. pyriformis*. They found that different copies of the same gene are segregated randomly between daughter macronuclei. This result is illustrated schematically in Figure 41. There are several explanations which one can put forth for this random segregation. For example, very extensive recombination could occur to randomize the distribution of alleles. Perhaps the most likely explanation is that each gene is a separate piece of DNA, as Prescott and co-workers have found for *Oxytricha*, and these pieces are located randomly within the macronucleus and therefore segregate with about a 50-50 chance of going to each daughter nucleus. It would certainly be interesting to obtain molecular weights of macronuclear DNA of *P. aurelia* and *T. pyriformis* to see if gene-size pieces also exist in these organisms.

The mitochondrial DNA (mtDNA) of *T. pyriformis* has been studied in some detail. It is 15 μm (30×10^6 daltons) long and linear,[363] whereas most mtDNA is circular.[364] This lack of circularity, however, may be an artifact of isolation. Recent electronmicroscopic studies[365] indicate an unusual property of denatured, single-stranded mtDNA. The two ends of a single strand appear to be complementary, so that the single-stranded DNA would be circular in very dilute solutions under renaturing conditions.

The histones of *Tetrahymena* have been studied in some detail. Iwai and co-workers have determined the amino acid contents of several histone fractions. The most interesting result is that histone IV[367,368] of *Tetrahymena* varies markedly from that of calf thymus. It is this histone which has been the most conserved throughout evolution; for example, pea and calf thymus differ only in two amino acids[368] out of 102. An example of the way histone IV of *Tetrahymena* differs from that of other organisms is that it has about 10% glycine residues while calf thymus and pea histone IVs have about 16%. What these differences might mean in terms of histone evolution and the relationship of histone evolution to differentiation is a very interesting question.

Amoeba

The nuclear DNA of the small, free-living amoeba *Acanthamoeba castellanii* has been studied in detail by two groups.[369-371] The results of both groups agree for some experiments, but differ markedly for others. Some of the results raise important questions which the authors do not discuss.

Both groups find that the nuclear DNA has about 58% G + C as determined by CsCl buoyant density. Bohnert and Herrmann[371] find about 61% G + C from melting temperature. Thus, the nuclear DNA may contain some unusual bases or unusual sequences.

Renaturation kinetics on nuclear DNA has been carried out by both groups. Each group used somewhat different conditions for renaturation: 60°C and 0.33 *M* Na^+ for the Marzocco and Colli group and 80°C (our estimate) and 1 *M* Na^+ for the Bohnert and Herrmann group. Both groups followed the renaturations using optical density at 260 nm. The Marzocco and Colli group found a kinetic complexity of 1.42×10^{11} daltons for the single class of DNA observed in their experiments. They call this DNA unique. The authors note that only 17% of the original hyperchromicity is

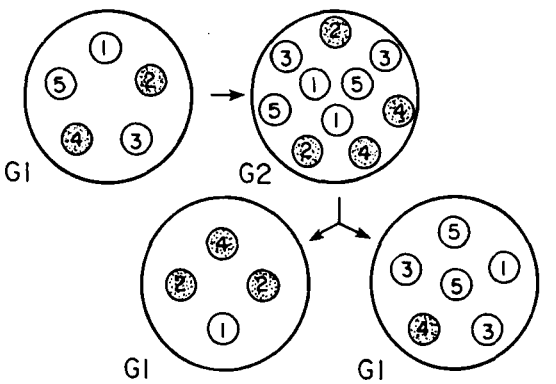

FIGURE 41. Illustration of the model of replication and random distribution of macronuclear gene copies during binary fission in syngen 1 of *T. pyriformis*. The largest circles represent the macronuclear membrane. Small circles represent haploid copies of the same gene in an *Aa* heterozygote. The open circles represent *A* alleles and stippled circles *a* alleles. The allele copies are numbered to show their fate. Only 5 copies are shown, instead of 45, for the sake of simplicity. G1 and G2 refer to stages in the cell cycle.

recovered during renaturation, a suspiciously low number, since it is usually possible to recover 80% or more of the original hyperchromicity. The authors have also measured the DNA content per nucleus and find it to be 6.4×10^{12} daltons (more DNA than man), a number 45 times larger than the kinetic complexity. Thus, if their numbers are correct, we conclude that either the whole genome is repeated 45 times in the nucleus (simple polyploidy) or that this 17%, low-complexity class is repeated DNA and the experiments have not been carried out far enough in time to observe the renaturation of the unique DNA.

The Bohnert and Herrmann group found several classes of DNA ranging in complexity from 1×10^7 to 4.1×10^8 daltons for the repeated DNA (20% of the total DNA) and a complexity of 2.1×10^{10} daltons for what they imply is unique DNA. This latter kinetic complexity value differs by 7-fold from the value found by the Marzocco and Colli group and is more than 300-fold less than the nuclear DNA content. It appears that it will take much more work to see which, if either, result is correct.

In the same paper, Bohnert and Herrmann studied the mitochondrial DNA of *A. castellanii* in careful detail and presented convincing results. The mtDNA studies were actually the main part of their paper. From electron microscopy they found that up to 80% of the molecules were circular with a circumference of 12.7 μm (25.4×10^6 daltons). Both CsCl buoyant density and thermal denaturation experiments yield 69.4% A + T, indicating that no unusual bases are present. Such a high A + T content for mtDNA is not unusual, for example, the mtDNA of the yeast *S. cerevisiae* has 82% A + T.

They present results for DNA renaturation kinetics carried out at both T_m-23°C (the usual condition) and T_m-10°C (more stringent conditions). Since DNA with such high percent A + T might form a sizable amount of stable, mismatched helices, the use of more stringent conditions where such helices should not form is a good idea. Their results at T_m-23°C do indicate either extensive single-strand structure (presumably A + T-rich structure) or mismatched double-stranded helices (A + T rich) forming early in the reaction. At T_m-10°C, this structure disappears. The results of several experiments using different fragment lengths and the two different temperatures yield a single class of DNA with kinetic complexity of about 2.6×10^7 daltons, a number close to the physical size of 2.5×10^7 daltons. Thus, it appears that the mtDNA is simple nonrepeated DNA.

Marzocco and Colli[369] have made limited studies of *A. castellanii* mtDNA also. They find the same A + T content as the other authors, but also find a complex renaturation pattern, with many classes of DNA ranging in complexity from 4×10^7 to 1×10^{10} daltons.[372] These values are difficult to believe because the mtDNA is physically only 2.5×10^7 daltons long.

Another amoeba which seems to be a popular organism for study is *Amoeba proteus*. It has an unusually large nucleus (29 to 60 μm in diameter) containing a large amount of DNA (33.6 pg or 2.02×10^{13} daltons).[372] While we suspect that some physical characterization of *A. proteus* DNA may have been done, we are unable to locate any studies.

SUMMARY AND DISCUSSION

We have now presented all "relevant" data on the techniques of physical study of DNA and on the presently known findings concerning the DNA of primitive eucaryotes. The use of quotation marks to set off the word "relevant" is, of course, advised, for it denotes the two most severe limitations of any review such as ours: (1) We have to some degree edited the material known to us. Though editing is almost inevitable in a review, we have no doubt employed the practice to a somewhat larger extent than is common in our attempt to maintain the critical nature of this review. (2) We almost certainly are not aware of all the material which exists on the subject reviewed. We lament this state of affairs, yet it is not surprising due to the extreme scope of the subject matter. To the best of our knowledge, this is the first review to deal with the DNA of such a wide variety of primitive organisms. Hopefully, neither our editing nor our ignorance will have allowed a truly fundamental fact to go unnoticed.

To close, we would like to make a brief point-by-point summary of what we feel to be the most important technical and organism-related comments and findings.

Technical Matters

1. In dealing with organisms of high A + T, high G + C content, or extreme sequence hetero-

geneity, DNA renaturation kinetics should be studied as a function of temperature to help pinpoint anomalous, sequence-produced effects.

2. In both optical- and HAP-monitored DNA renaturation, there may be a first-order component arising from the reactions within single DNA strands. The cause of these effects appears to be different in optical and HAP studies. Thus, any DNA (bacterial, viral, or eucaryotic) can show an initial first-order kinetic component (of 10% or more) when the renaturation is monitored optically at $T = (T_m\text{-}25^\circ C)$. This component is presumed to result from nonspecific formation of single-strand structure and is often mistaken for a repeated DNA fraction. In HAP-monitored renaturations, viral and bacterial renaturations show no such first-order or nonsecond-order component (or less than 1% of such a component, the practical limit of detection). However, HAP-monitored renaturation of most, if not all, eucaryotic DNA shows a zero-time binding fraction. This fraction, in cases where careful study has been made, has been proven to result from the formation of specific double helical structures within a DNA single strand. The danger exists that this zero-time component may be mistaken for satellite sequence DNA.

3. In constructing RSO plots for optical renaturations, one should plot the normalized form $\frac{A_0}{A_t} - 1$ vs. time instead of the form $\frac{1}{A_t - A_\infty}$ vs. time. The use of the normalized form makes it much easier for other workers to make calculations from data.

4. As the data for yeast so vividly illustrate, even much careful study can given conflicting results for such supposedly simple measurements as DNA per cell or kinetic complexities of DNA. Hence, it seems advisable, wherever possible, to make even the simplest of measurements in a number of independent ways.

Findings on the DNA of Primitive Eucaryotes

1. Although sufficient data are not available to make a definitive statement, it certainly seems that chromatin structure of primitive eucaryotes is not a carbon copy of that in higher organisms. The dinoflagellates appear to lack histones entirely and to order their DNA in space in a manner much more similar to bacteria than to higher eucaryotes. In other organisms, such as green algae and yeast, histones are present though they may differ considerably from the histones of cow or peas; in still other organisms, such as some protozoa, the histones are both present and quite similar to those of higher eucaryotes. In yeast, the only organism thus far studied, a subunit structure has been found in the chromatin which resembles but may not be identical to that in higher eucaryotes. Clearly more data are needed, but it seems quite likely that most (but not all) primitive eucaryotes will be found to have both histones and subunit-structured chromatin. Whether these subunit structures will be quantitatively different from higher forms is a most interesting question.

2. The sequence composition of the DNA of lower eucaryotes has been much more thoroughly studied than their chromatin, but once again the data available stop short of real answers. The questions these data raise are, however, fascinating. In the first place, though a rather large number of organisms have been examined, as yet no simple repeated-sequence satellite DNAs of the form found in mice or *Drosophila* have been detected. Of course, few of the studies done thus far would have detected very low levels of satellite DNA; however, if indeed no such satellites exist in lower forms, this would place some restrictions on any proposals concerning the function of satellite DNA. It certainly seems imperative to reexamine the DNA of lower eucaryotes using more sensitive techniques (perhaps endonuclease digestion would be appropriate) in an effort to resolve this question.

There is also the question of the presence and function of moderately repeated sequences. We have seen an overall trend, i.e., the smaller the genome, the smaller the amount of repeated-sequence DNA. Of course, this trend has yet to be proven in more sensitive experiments where the percent repeated DNA is measured as a function of fragment size via HAP chromatography. For *Chlamydomonas* there is even a question as to whether any moderately repeated DNA exists (beyond the small amount found in bacteria). But that question simply remains to be answered since a repeated fraction as small as 1.5% of the genome would be sufficient to place a 300 base pair repeat next to each structural gene. Finally, we note that in the case of *D. discoidium* (the most carefully studied organism) and to a lesser degree in *C. cohnii* and *Polytoma* there is proof that repeated DNA sequences exist interspersed with unique DNA.

3. The physical nature of the DNA replication apparatus in primitive organisms is, again, little studied. In yeast we see a typical higher eucaryotic mode of replication utilizing multiple replication bubbles moving bidirectionally. In *Physarum*, RNA has been found attached to Okazaki-type pieces in a discontinuous mode of replication. For the large majority of primitive eucaryotes, however, even simple fiber-autoradiographic studies of DNA replication have yet to be done.

4. Finally, the interior of a chloroplast seems procaryotic in nature. Whether or not the chloroplast arose from a procaryotic endosymbiont may be an unanswerable question.

Early in this review we suggested that study of the DNA of primitive eucaryotes might shed light on the evolution and function of the characteristic features of DNA of higher eucaryotes. It seems obvious that the answer to this question is a rather tantalizing "maybe."

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