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Determination of molecular weights by fluctuation spectroscopy: Application to DNA

(fluctuation spectroscopy/DNA molecular weight determination/T2 phage DNA/*Escherichia coli* DNA/*Drosophila melanogaster* DNA)

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ABSTRACT **A method for determining molecular weights of macromolecules by measuring spontaneous concentration fluctuations is described. The method is absolute, rapid, and requires no shearing forces on the molecules. We have applied this technique to the determination of molecular weight of DNA molecules. The molecular weight values obtained for T2 phage DNA (1.14×10^8) and replicating *Escherichia coli* DNA (3.9×10^9) agree with previous results. By monitoring individual molecules, an estimate of the molecular weight of nuclei and individual chromosomal DNA molecules of *Drosophila melanogaster* was obtained.**

There are many classical methods for measuring molecular weights (M_w) of macromolecules. DNA, because of its unusually large M_w and root-mean-square radius, poses special problems. Consequently, some of the standard methods (e.g., light scattering, sedimentation) become impractical. Several techniques that have been used for DNA (e.g., viscoelasticity, autoradiography, electron microscopy) have been critically reviewed by Freifelder (1) and Roberts *et al.* (2). Their work shows that there remains a need for a precise, simple, preferably absolute technique to measure the M_w of large DNA. In the present work, we discuss a method that endeavors to meet these criteria and demonstrate its application to the M_w determination of DNA from T2 phage and *Escherichia coli*.

In this method, one measures the spontaneous, inherent, thermodynamic concentration fluctuations of solute molecules in a given volume. These fluctuations are related in a simple way to the number of molecules, n, per unit volume. By measuring independently the concentration (weight per unit volume) of solute molecules, C, the M_w is obtained from the relation $M_w = C/n$. The method was suggested in an earlier work on fluctuation spectroscopy (3), and its feasibility has been demonstrated in a preliminary report (4).

BASIC PRINCIPLES

In an ideal gas or solution, the number of molecules, N, in independent equal volumes fluctuates randomly around the equilibrium value $\bar{N}$. The magnitude of the fluctuations, δN, is given by (5)

$$\left(\frac{\overline{\delta N}}{\bar{N}}\right)^2 = \left(\frac{\overline{\delta C}}{\bar{C}}\right)^2 = \frac{1}{\bar{N}} \qquad [1]$$

where the bars indicate time or ensemble averages and where C is the (wt/vol) concentration of the molecules. The fewer the number of molecules, the larger the fractional fluctuations. Therefore, for a given concentration $\bar{C}$, the size of the fluctuations increases with molecular weight.

By measuring these fluctuations (via any parameter that is sensitive to concentration), one can determine from Eq. 1 the number of molecules N in a given volume $\mathcal{V}$ within which the fluctuations are measured. By knowing the average concentration $\bar{C}$, the molecular weight is given by

$$M_w = \bar{C}\left(\frac{\delta C}{\bar{C}}\right)^2 \mathcal{V} A \qquad [2]$$

where A is Avogadro's number. One may easily verify, in analogy with classical light scattering theory, that for polydisperse solutions, this formula gives a weight-average M_w.

A simple "thought experiment" illustrates the method: if 1 liter of DNA, having a concentration of $\bar{C} = 10^{-6}$ g/ml is pipetted into one thousand 1-ml cuvettes, C (as measured, for example, by the optical absorbance A_{260}) will vary slightly from sample to sample. If the standard deviation (i.e., fluctuations) of absorbance $\overline{\delta A_{260}}/\bar{A}_{260} = 10^{-3}$, then $(\delta C/\bar{C})^2 = 10^{-6}$. This corresponds to 10^6 molecules per ml (Eq. 1) and to a M_w of 6×10^{11} (Eq. 2).

TECHNIQUE APPLIED TO DNA

In order to utilize the ideas of the preceding section, one needs to find a convenient parameter to monitor concentration, a method for obtaining statistically independent volumes, and a technique for electronically processing the fluctuations in a manner that minimizes the effect of unwanted, spurious fluctuations (noise sources).

Monitoring the Concentration. We use the fluorescence of the dye, ethidium bromide (EtBr), to monitor DNA concentrations (6, 7, ‡). The observed fluorescence intensity $\bar{I}_T$ is the sum of the intensities due to the bound dye, $\bar{I}_B$, and the free dye, $\bar{I}_F$. The observed fluctuations of the fluorescence intensity $\delta I_T = \delta I_B$, since δI_F is negligible with respect to δI_B. The fractional fluctuation of the DNA concentration, C, equals the fractional fluctuation of I_B,

$$\left(\frac{\delta C}{\bar{C}}\right)^2 = \left(\frac{\delta I_B}{\bar{I}_B}\right)^2 = \left(\frac{\delta I_T}{\bar{I}_T}\right)^2\left(\frac{\bar{I}_T}{\bar{I}_B}\right)^2 = \left(\frac{\delta V}{\bar{V}}\right)^2\left(\frac{\bar{I}_T}{\bar{I}_B}\right)^2 \qquad [3]$$

the last equality was obtained by converting the fluorescence intensity $\bar{I}_T$ into a voltage $\bar{V}$ by means of a photomultiplier tube. It is convenient to define a DNA concentration, C_0, for which $\bar{I}_B = \bar{I}_F$. In the limit of low fractional occupancy of binding sites or of a small ratio of bound to unbound dye, one can write

$$\frac{\bar{I}_B}{\bar{I}_F} = \frac{\bar{C}}{\bar{C}_0}; \quad \text{therefore} \quad \frac{\bar{I}_B}{\bar{I}_T} = \frac{\bar{C}}{C_0 + \bar{C}}. \qquad [4]$$

Abbreviation: M_w, weight average molecular weight; EtBr, ethidium bromide.

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‡ Ref. 7 relates fluorescence fluctuations to the chemical properties of the EtBr–DNA complex. These fluctuations are several orders of magnitude smaller than the ones observed in this work.

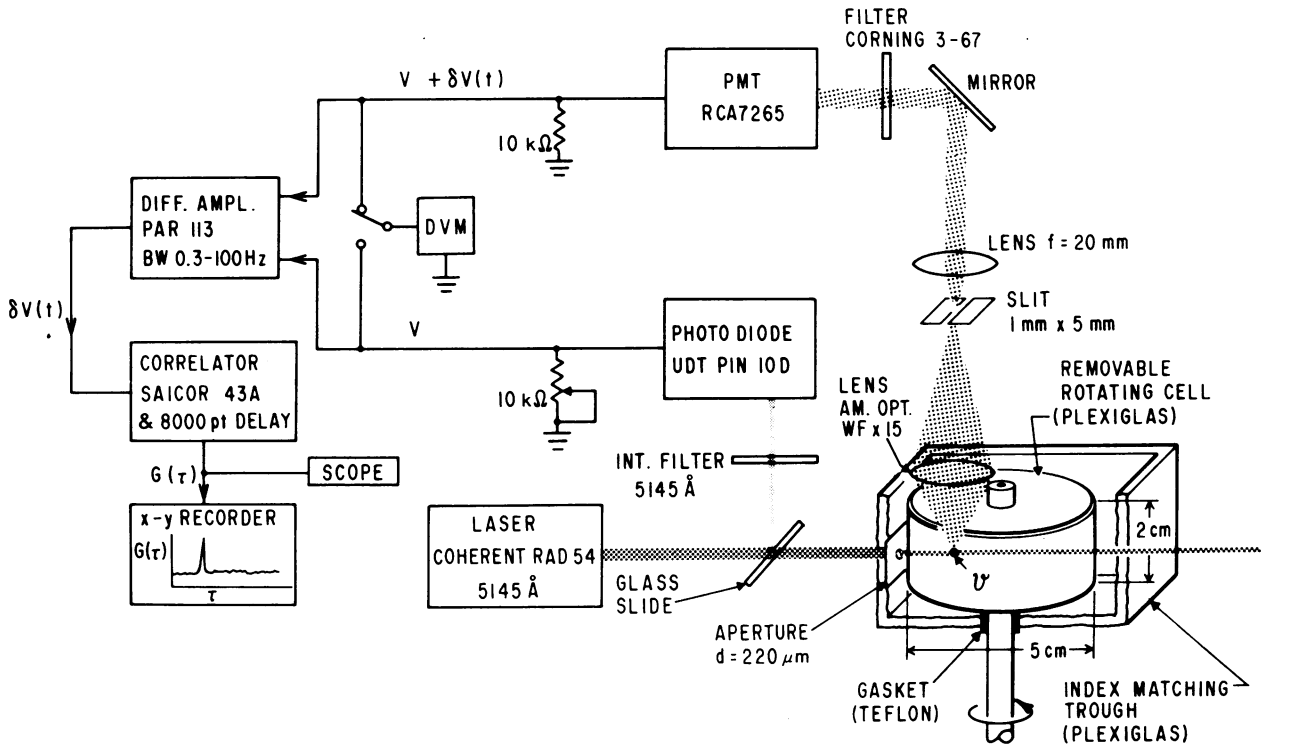

FIG. 1. The experimental arrangement used to measure concentration fluctuations of solute molecules (DNA) in a volume $\mathcal{V}$, defined by the light beam and associated optics. During the calibration with polystyrene spheres the Corning 3-67 filter was replaced with an Edmund Scientific no. 874 green filter. The following abbreviations are used: DIFF. AMPL., differential amplifier; PAR, Princeton Applied Research; BW, band width; pt, point; RAD, radiation; d, diameter; f, focal length; PMT, photomultiplier tube; DVM, digital voltmeter; INT, interference; AM. OPT., American Optical; UDT, United Detector Technology.

For the general case $\bar{I}_B/\bar{I}_T$ in Eq. 4 equals $(\bar{C}/\bar{C} + C_0)[1 + \theta(\bar{C} - C_0)/(RC_0)]^{+1/2}$, where θ is the fraction of occupied binding sites and R is the fluorescence enhancement factor when the dye binds to DNA [$R \approx 20$ for EtBr (6)]. Substituting Eqs. 4 and 3 into 2, one obtains for the M_w

$$M_w = \overline{\left(\frac{\delta V}{\bar{V}}\right)^2} \frac{(C_0 + \bar{C})^2}{\bar{C}} \mathcal{V}A. \qquad [5]$$

At $\bar{C} = C_0$, the expression $(C_0 + \bar{C})^2$ of Eq. 5 goes through a broad minimum as a function of $\bar{C}$. Thus, once C_0 is determined, inaccuracies in the measurement of $\bar{C}$ do not affect significantly the M_w determination (e.g., from $\bar{C}/C_0 = 2/3$ to $3/2$, the measured M_w changes only by ~4%). Working with $\bar{C} \approx C_0$ has also the related advantage of maximizing $(\delta V/\bar{V})^2$. The condition $\bar{C} = C_0$ (i.e., $\bar{I}_B = \bar{I}_F$) is easily determined experimentally by monitoring the fluorescence $\bar{I}_0$ of the solution with the dye alone and the total fluorescence $\bar{I}_T$ after adding the DNA. It is easily shown that for $\bar{I}_B = \bar{I}_F$

$$\bar{I}_T = 2\bar{I}_0(1 + 1/R)^{-1}. \qquad [6]$$

Sampling Independent Volumes. A beam of light, together with an optical system, defines a subvolume $\mathcal{V}$ of a cylindrical cell (see Fig. 1). As the cell rotates, different, equal and statistically independent volumes are exposed to the light beam and monitored. Other methods, utilizing, for example, the flow of solutions through a stationary cuvette, are also, in principle, usable. Their disadvantage, however, is that they may break or aggregate shear-sensitive molecules. The rotating cell technique avoids this problem, allows one to choose a convenient time scale for the fluctuations and in addition provides a way of separating the desired fluctuations (noise) from other noise sources, as described below.

Measuring the Concentration Fluctuations via the Autocorrelation Function. In order to obtain the M_w, one can, in principle, simply measure the mean square component of the time-varying component $\overline{\delta V^2}$ (with a root-mean-square voltmeter) and the dc voltage $\bar{V}^2$ (see Eq. 5). Unfortunately, there may be other unwanted noise sources present that can contribute to $\overline{\delta V^2}$. These can be eliminated to a large extent by using the autocorrelation function, $G(\tau)$, defined by (5)

$$G(\tau) = \overline{\delta V(t)\, \delta V(t + \tau)}. \qquad [7]$$

For $\tau = 0$, $G(0) = \overline{\delta V^2}$, which still includes the noise from all sources. The desired concentration fluctuation noise, however, is periodic in the cell rotation time T, since each detected molecule returns to the detected region after one turn of the cell (diffusion being negligible), i.e., $\delta V_P(t) = \delta V_P(t + T)$. Consequently, $G(T) = G(2T) = G(3T) = \overline{\delta V_P{}^2}$ where $\delta V_P(t)$ is the periodic noise component. On the other hand, most unwanted noise components (e.g., laser noise, shot noise, dust particles floating in front of the beam, etc.) are not periodic and therefore their autocorrelation function $G(T) = 0$.

The essence of the scheme is schematically represented in Fig. 2. The top trace shows the concentration fluctuations periodic in T. The lower trace shows the autocorrelation function $G(\tau)$, including the nonperiodic component near $\tau = 0$. The periodic component peaks with equal amplitudes after each cell rotation T.

Using the autocorrelation function, we can rewrite Eq. 5

$$M_w = \frac{G(T)}{\bar{V}^2} \frac{(C_0 + \bar{C})^2}{\bar{C}} \mathcal{V}A. \qquad [8]$$

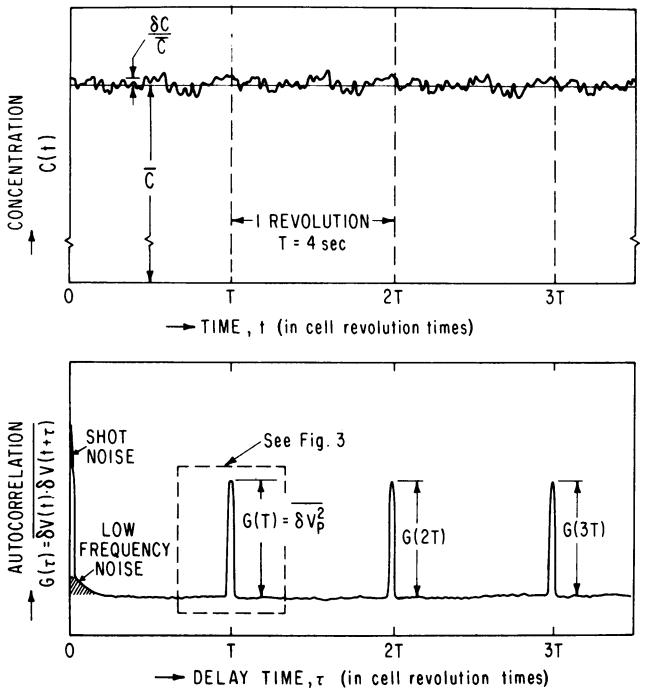

FIG. 2. Schematic representation of the concentration fluctuations (top) and the autocorrelation function (bottom). Note the near periodicity of the fluctuations that results in autocorrelation functions $G(T)$, $G(2T)$, and $G(3T)$ of equal amplitudes. The unwanted noise sources appear only in $G(0)$.

MATERIALS AND METHODS

The Cell. The rotating cell (Fig. 1) is made of polished plexiglas tubing, with a black plexiglas bottom, and a screw-on polished plexiglas top which allows for easy filling with a minimum of handling of the material. There is a small overflow chamber at the top. The cell is mounted on a ball-bearing supported shaft, which is driven by a synchronous motor through a metal chain drive at one revolution per 4 sec. A sample volume passes through the beam in about 10 msec. Thus, in one revolution about 400 statistically independent volumes are being sampled. The cell is immersed in water to match approximately the cell's index of refraction, thereby preventing small scratches on the surface of the cell from producing a detectable periodic signal.

The Optics. The fluorescence gathering lens is mounted on a micrometer screw and is adjusted to form a magnified image of the laser beam on a rectangular slit. The light passing through the slit is collimated by a second lens and filtered to remove scattered light.

The Electronics. The light is detected by a photomultiplier tube whose output contains a dc component $\bar{V}$ and the randomly fluctuating ac component $\delta V(t)$. In order to reduce the noise in the output of the laser, a small fraction of the light is deflected, detected with a photodiode and fed together with the photomultiplier output into a differential amplifier. The output of the amplifier is connected to the correlator. (For a description of a simple correlator see for example ref. 8.)

Materials. T2 phage, obtained from Miles Labs and from R. Adam (U. California, San Diego) (the latter used no nuclease in the purification procedure) (9), was digested at 50° for 40 hr in 0.15 mg/ml of Pronase, 0.5% of sodium decyl sulfate, 1 M NaCl in standard buffer containing 10 mM Tris, 10 mM Na-EDTA of pH 8.0. The DNA concentration during digestion was either 130 μg/ml or 13 μg/ml. DNA extracted at the lower concentration was dialyzed against standard buffer for 1 day before final dilution. DNA obtained by the phenol extraction method (10) used for the determination of C_0 of T2 phage DNA was donated by R. Adam. Folded chromosomes from *E. coli*, strain TAU-bar, were donated by B. Bowen (U. California, San Diego), who prepared them according to ref. 11. Calf thymus DNA (Sigma, Type I) was used as a standard for the determination of C_0 of *E. coli* DNA. Nuclei from *Drosophila melanogaster* were donated by S. Stanfield (U. California, San Diego). DNA concentrations were determined spectrophotometrically with $\epsilon = 0.0181\ \mu g^{-1}\ cm^2$ at 260 nm for phage T2 DNA (12) and $0.0198\ \mu g^{-1}\ cm^2$ for nonglucosylated DNA (13). Polystyrene spheres (quoted diameter = 1.011 μm, density = 1.05 g cm^{-3}, number = $1.76 \times 10^{11}\ cm^{-3}$) were obtained from Dow Chemical (Midland, Mich.); nuclease-free Pronase from Calbiochem; EtBr from Sigma Chemical, it was assayed spectrophotometrically using $\epsilon_M = 5.6 \times 10^3\ M^{-1}\ cm^{-1}$ at 480 nm (14). Water was double quartz distilled, filtered through a 0.22 μm Millipore filter.

EXPERIMENTAL RESULTS

Calibration of $\mathcal{V}$ with polystyrene spheres

Several of the experimental parameters that define the effective volume $\mathcal{V}$ (Eqs. 2 and 8) (e.g., the intensity profile of the laser beam, diffraction of the aperture, etc.) are difficult to determine with high accuracy. Consequently, $\mathcal{V}$ was determined empirically by measuring the fluctuation in the scattered light intensity from a known concentration of polystyrene spheres, n, and using the relation $1/\mathcal{V} = nG(T)/\bar{V}^2$. By evaporating the solvent from a known volume and measuring the residual dry weight n was determined. A value of 10.0 ± 0.1% (wt/vol) corresponding to $(1.76 \pm 0.02) \times 10^{11}$ spheres per ml was obtained, in perfect agreement with the quoted value (L. B. Bangs, Dow Chemical Co., personal communication). The solution was diluted to give a concentration of 1.00 ppm (wt/vol) in polystyrene spheres and poured into the cylindrical cell. The correlation functions $G(T)$, $G(2T)$, $G(3T)$ were found to be of equal intensities (see Fig. 2) indicating the absence of any significant convection currents within the cell. After making a small (4.5%) correction in $\bar{V}$ for scattered background light, the value of $G(T)/\bar{V}^2$ was found to be $(3.75 \pm 0.10) \times 10^{-2}$. This corresponds to $\mathcal{V} = (1.51 \pm 0.05) \times 10^{-5}\ cm^3$.§

DNA from T2 phage

To the standard buffer, 1 μM of EtBr was added and the fluorescence $\bar{I}_0$ of the solution measured. The stock solution of T2 phage DNA was diluted until the fluorescence $\bar{I}_T$ was 1.9 $\bar{I}_0$. This corresponds to the condition at which $\bar{C} = C_0$ (see Eq. 6). The concentration of DNA measured spectrophotometrically agreed with C_0, calculated from the known values of the binding constant k and enhancement factor R (6). The autocorrelation function $G(T)$ of a typical experiment is shown in Fig. 3. At least 20 such traces were averaged per sample. Experiments were performed on three independently extracted DNA samples obtained from the T2 phage that was purified in the absence of nuclease. The average M_w (of the Na salt) obtained from Eq. 8 was found to be

$$M_w = (114 \pm 5) \times 10^6 \qquad [9]$$

where the main error is due to the uncertainty in $\mathcal{V}$ (±3%) and C_0 (±3%). The error in the value of ϵ for DNA (12) has not been quoted and has, therefore, not been included. The above value

§ Errors are quoted as standard deviations of the mean.

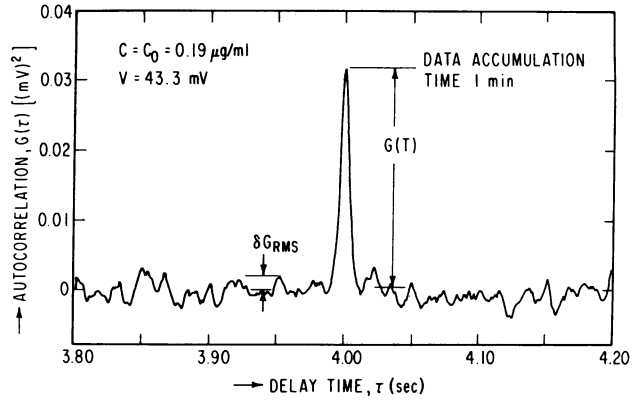

FIG. 3. The autocorrelation function $G(T)$ of the fluorescence intensity from a solution of T2 phage DNA and EtBr. From the measured values of $G(T)$, $\bar{V}$ and C_0, the M_w is obtained from Eq. 8. The noise of the baseline has two main components. One is due to the finite number of volumes (about 400) sampled per revolutions [this component is symmetric with respect to T, i.e., $G(T+t) = G(T-t)$]; the other (lacking symmetry) is due to the shot noise in the photomultiplier tube and the digitation scheme of the autocorrelator. RMS refers to root-mean-square.

of the M_w is in agreement with most published values which range between 106×10^6 and 130×10^6 (1, 2). Samples were also run at concentrations from 0.6 C_0 to 1.5 C_0 with no detectable concentration dependence of the apparent M_w. R. Kavenoff inspected all samples by electron microscopy. The three samples used to obtain the M_w (Eq. 9) showed little or no aggregation or degradation. One sample that was exposed to a shorter Pronase treatment showed aggregation and gave a 7% higher M_w; two other samples prepared from the commercial T2 phage showed degradation and gave a few percent lower M_ws.

DNA from *E. coli.*

The experimental procedure used to measure M_ws was the same as that used for T2 phage except that calf thymus DNA was used to obtain C_0. Three samples obtained from *E. coli*, harvested in the log phase (doubling time 45 min), were equilibrated after dilution in low-salt Pronase solutions. The average M_w was found to be:

$$M_w = (3.9 \pm 0.3) \times 10^9. \qquad [10]$$

When the DNA was sheared, a drastic (about ten-fold) reduction in M_w without a change in $\overline{I_B}/\overline{I_T}$ was observed. Since $\overline{I_B}$ depends on the degree of supercoiling (15) these findings show that we were dealing with free non-supercoiled DNA which was not supercoiled. For *E. coli* replicating their DNA continuously at a constant elongation rate, (16), the expected weight average M_w calculated from the size distribution (17) equals $2(1/\ln 2 - \ln 2) \approx 1.50$ genome M_w. If we assume a genome M_w of 2.7×10^9 (2, 17), then the replicating DNA should have a M_w of 4.05×10^9, in agreement with our results.

Nuclei from *D. melanogaster*

When the size of the DNA molecules becomes very large and their number in the measuring volume $\mathcal{V}$ is small, one can detect individual events (instead of fluctuating numbers). This was accomplished using the same apparatus (Fig. 1) and replacing the autocorrelator with a storage scope that displayed the individual events. As each nucleus passed through the detected region, voltage spikes, δV, about 10 msec long and with amplitudes up to $\delta V/\bar{V} \simeq 0.25$ were observed. Using the relation $M_w = (\delta V/\bar{V})\, C_0\, \mathcal{V}\, A$,¶ we obtained an estimate of 3×10^{11} daltons for the DNA content of the nuclei. This number agrees within about 40% with the published value (2, 18). Lysing the cell with 0.75% of lauryl dimethyl amine oxide and Pronase reduced $\delta V/\bar{V}$ to 0.03 corresponding to a M_w of about 4×10^{10}, in agreement with the accepted value for single chromosomes (2, 18).

DISCUSSION AND CONCLUSION

We have demonstrated the applicability of fluctuation spectroscopy (Flusy) to the determination of M_ws of macromolecules, and have used it to measure the M_ws of DNA of T2 phage and *E. coli*. The advantage of the method is that it is absolute (i.e., does not require indirect theoretical interpretations) and that it gives rapid and precise results. Thus, in one minute of data collecting the precision is about ±5%; this can be easily improved by increasing the collecting time or repeating the measurements. The method is, therefore, ideal when rapid and precise comparisons of M_ws of DNA are required (e.g., studying replicating DNA in order to differentiate between modes of replication). The absolute values are less accurate and depend on the calibration of the effective sample volume and the determination of C_0 with its attendant uncertainty in the extinction coefficient ϵ_{260}. We estimate the absolute accuracy to be at present ± (5–10)%. Fluctuation spectroscopy has the additional advantage that it requires no shear forces and does not depend whether DNA is linear or circular as long as the appropriate C_0 is used. It measures the weight-average M_w in distinction to the viscoelastic method which measures the maximum M_w (18).

The fluctuation method can be extended by several orders of magnitude beyond the 10^8 to 5×10^9 dalton range used in this work. The lower limit of M_w determination is inversely proportional to C_0, i.e., the product of the binding constant k and enhancement factor R. With EtBr the lower practical size limit of DNA is at present about 10^7 daltons. Dyes with kR several orders of magnitude greater than that of EtBr may soon be available (21, 22). Of particular advantage would be to have dyes with large kR in the presence of high salt concentration and detergent. This would allow the lysis to be performed in the rotating cell, thereby eliminating the handling of the DNA. The extension to higher M_w poses no inherent problem as long as one insures the ideality of the solution (by lowering the wt/vol concentration) and one keeps the linear dimensions of the detecting region large in comparison with the molecular dimensions. However, for very large M_ws, it may be advantageous to detect single molecules as has been done with the chromosomal DNA of *D. melanogaster* in this work. The advantage of this mode of operation is that it offers the possibility of obtaining distributions of M_ws as has been done with whole cells (23).

Although we have used fluctuation spectroscopy to measure the M_w of DNA only, the method is, in principle, applicable to any macromolecules whose concentrations can be monitored and whose weight per unit volume can be determined.

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¶ This relation follows from the fact that the spike height is proportional to DNA size. The $\mathcal{V}$ that appears in this relation is not identical, although roughly equal to the $\mathcal{V}$ in Eq. 8. The difference arises from the different weighting of the variations in intensity and fluorescence gathering efficiency within the detected region (19).

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