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THE $S^{\circ}_{20,w}$ OF UNSHEARED DNA FROM WHOLE CELL LYSATES OF *Escherichia Coli*

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We present measurements of the sedimentation coefficients of DNA present in whole cell lysates of *E. coli*. The method used is a preparative version of the band sedimentation experiment of Bruner and Vinograd. We show that in order to obtain reliable data on the time dependence of sedimentation, it is necessary to accelerate and decelerate the rotor over much longer times than the standard centrifuge allows. We describe the necessary modifications to the preparative centrifuge and use them to determine the $S^{\circ}_{20,w}$ of unsheared *E. coli* DNA. The value for the fastest moving components in the lysate is 220 S. The molecular weight of the DNA corresponding to this sedimentation coefficient is probably 1.7×10^9 g/mole. However, alternative values cannot be ruled out.

1. Introduction

The molecular weight of the DNA of the *E. coli* chromosome is known to be $(2.6 \pm 0.3) \times 10^9$ g/mole [1–5]. By employing a gentle lysis procedure on stationary phase cells, Klotz and Zimm [6] have measured the intrinsic viscosity of the lysate and the relaxation time associated with the viscoelastic relaxation of the longest molecules in the lysate. By combining these two measurements and utilizing an empirical relationship between intrinsic viscosity and molecular weight they were able to determine a molecular weight of 2.7×10^9 g/mole for the largest molecules present. In addition, they conclude that these molecules represent 30 to 40% of the DNA present.

In the hope of utilizing this lysate for the purpose of extending the data for the dependence of $S^{\circ}_{20,w}$ and molecular weight for DNA into the range of 10^9 to 10^{10} g/mole, we have determined the distribution of sedimentation coefficients present in the lysate. (See Freifelder [7] and Schmid and Hearst [8] for reviews of $S^{\circ}_{20,w}$ and molecular weight of DNA.) The method of analysis which we used was a preparative version of the band sedimentation experiment in which a low density sample (the lysate) was layered on top of a high density solution. The resulting sample was centrifuged and the time dependence of the sedimentation of the DNA in the lysate was determined. The resulting distribution of sedimenting species exhibited a peak at about 140–160 S with 30% of the material having an average sedimentation coefficient of 220 S.

Two technical problems arose during the course of this work. The first problem grew out of the necessity to nullify the so-called anomolous speed effect. This anomoly which causes very large molecules to exhibit a lower sedimentation coefficient at high speeds than at low speeds has been observed by several workers [9–13]. The magnitude of the effect as well as properties on which it depends have been derived theoretically by Zimm [14] and by Zimm and Schumaker [15]. Briefly, Zimm proposes that since the friction factor for the end segments of any polymer is greater than for the middle ones, then on the average the ends will drag behind the molecule as it moves through the solvent. This distortion produces a segment distribution which exposes more segments to the solvent than the relaxed spherical distribution. This increased exposure results in a higher friction factor for the molecule as a whole and thus one observes a decrease in sedimentation coefficient associated with high molecular distortions [14]. In practice this distortion is not observed for DNA whose molecular weight is below 10^8 g/mole. A 10% decrease in sedimentation rate for T2 DNA has been observed in sucrose gradients at 60000 rpm [10]. The effect has also been observed in sedimentation studies of bacterial lysates in sucrose gradients where the dependence on speed is very great [11–13].

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The Zimm theory allows us to estimate the way in which the sedimentation coefficient depends on speed, molecular weight, et cetera. As we will show later, the magnitude of the effect depends on molecular weight, solution density, and centrifugal acceleration as $M^{3.11}(1-\bar{v}\rho)^2$ (rpm)^4 r^2 where $\bar{v}$ is the partial specific volume of the DNA, ρ is the solvent density, M is the molecular weight, rpm is the speed in revolutions per minute and r is the radial distance from the center of rotation. The strong dependence on speed and molecular weight is evident. It is also apparent that whereas increases in speed and size increase the effect, increasing the density decreases the effect. It was immediately obvious to us that we could perform a sedimentation measurement in high density CsCl and cancel out the effect of size and speed in this way. Thus we determined a density-speed combination which by Zimm's theory gave a 1% decrease in S-value.

The second technical problem arose from the experimental conditions dictated by the constraints described above as well as the necessity of working at very low concentration to avoid gels. Since we observed that gels became a serious problem at DNA concentrations of about 1 μg/ml, we were forced to use radioactive DNA and to do the sedimentation measurement in the preparative centrifuge. To get a reliable sedimentation coefficient one must take time points and plot log r versus time for the sample. While this procedure is trivial in the analytical centrifuge, in the preparative centrifuge the necessity of decelerating and accelerating the rotor for each point causes the gradients to mix (for speeds below 15 000 rpm). We have been able to overcome this problem by limiting the acceleration and deceleration of the rotor to very low values. With controlled acceleration and deceleration we can now get as many as six time points on successive tubes and obtain the log r versus time plot.

2. Materials and methods

2.1. Chemicals, enzymes and stock solutions

CsCl used was radiotracer grade from Harshaw Chemical Company. An amount greater than that needed to saturate 100 ml of glass distilled H_2O at room temperature was totally dissolved at 50°C. This solution was filtered through a 0.22 μ Millipore filter and allowed to cool at room temperature overnight. This procedure produced a saturated CsCl solution free of insoluble matter. Solutions for making gradients were diluted from this stock solution. Pronase was obtained from Calbiochem as "nuclease free" grade B and was used without further treatment. Grade B egg white lysozyme was obtained from Calbiochem. Both enzymes were kept refrigerated as solids until needed. Lysis of the bacteria was accomplished exactly as Klotz and Zimm describe. Their stock solutions are boric acid buffer (BA), 0.195 M NaCl and 10% Brij-58 detergent in BA. The BA was prepared as follows. 1.74 g B_2O_3, 1.46 g Na_2 EDTA, 4.12 g NaCl and 1.08 g NaOH were dissolved in less than 500 ml of glass distilled water. If necessary the pH was adjusted to 8.4 with concentrated NaOH. Finally the volume was brought to 500 ml.

All chemicals were reagent grade with the exception of Brij-58, a commercial preparation which was a gift from B.H. Zimm of the Department of Chemistry, University of California, San Diego.

2.2. E. coli growth, storage and labelling

The strain of *E. coli* B used was HF4733, the generous gift of Dr. A.J. Clark of the Molecular Biology Department, University of California, Berkeley. This strain requires thymine and vitamin B_1 for growth and it lacks endonuclease I activity [16]. The bacteria were grown up to stationary phase at 37°C in nutrient broth. The culture was made 10% in dimethyl sulfoxide and 1 ml portions were sealed into sterile ampoules. These were frozen and stored in liquid nitrogen until needed.

A typical culture for lysis was grown and labelled using the following procedure. The day before a lysis was to be done, a 1 ml ampoule of bacteria was thawed and a few drops were placed into 10 ml of the Klotz and Zimm [6] defined medium (per ml of culture: 5.66 mg $Na_2HPO_4 \cdot 7H_2O$, 1.0 mg NH_4Cl, 3.0 mg KH_2PO_4, 0.13 mg $MgSO_4$, 50 μg $CaCl_2$, 24 mg glucose, 0.14 mg L-tryptophan, 0.34 mg L-arginine, 0.6 mg dl methionine, 20 μg thymidine). This culture was incubated with shaking at 37°C to stationary phase. The OD_{650} of the culture (taken versus H_2O) was 3.2 ± 0.2 and the generation time was 48 ± 2 minutes. This culture was diluted approximately 1:100 and again allowed to grow up to stationary phase. Finally the second culture was diluted 1:1000 into medium containing 250 μc/ml ^{3}H-thymidine (Schwarz/Mann). The final specific ac-

tivity of the thymidine was 1.42 curie/mmole. The labelled DNA therefore contains 2.4×10^6 dpm/μg. The total volume of this culture was 1.0 ml and it was grown for 10–12 hours in a sterile 25 ml Erlenmeyer flask with shaking. On the day of lysis, the cells were harvested by centrifugation out of this 1 ml culture.

2.3. Lysis, gradient preparation, and transfer procedure

2.3.1. Harvest and lysis

The cells were harvested by centrifugation out of medium at 3000 rpm for 10 min in a refrigerated centrifuge precooled to 4°C. They were resuspended in cold BA and pelleted a second time. This procedure in BA was repeated twice more to remove all traces of unincorporated tritium. The cells were then counted for tritium by taking a 10λ aliquot and dissolving it in 10 ml of liquid scintillation cocktail (triton X-100, PPO-POP-OP-toluene) plus 1.0 ml H_2O. Typically there were about 130000 cpm ^3H in 10λ of cell suspension [$(1-3) \times 10^7$ cpm/ml]. Essentially all of these counts were incorporated into DNA. The cells were then diluted with cold BA to about 2×10^6 cpm/ml. This level gives a final DNA concentration of about 0.5 μg/ml in the lysate. The lysate was constructed in a chamber made from a 1 ml sterile disposable syringe as shown in fig. 1a. The tip of the syringe was sliced off at the 0.05 ml mark. With the cut end up, the syringe could be securely fitted in a small test tube rack. The end of the plunger was placed at the 0.25 ml mark and the lysis of the cells was carried out exactly as described by Klotz and Zimm [6]. The diluted cells were treated at 70°C for a maximum of 10 min. They were then lysed by addition of the following solutions in the specified order:

(1) 25λ heat treated cells in BA,
(2) 50λ 4 mg/ml pronase in 0.195 M NaCl,
(3) 5λ 10% Brij-58 in BA,
(4) 25λ 2 mg/ml lysozyme in BA.

The addition was carried out as quickly as possible at room temperature and the lysate was brought to 50°C immediately by immersion in a water bath to the 0.1 ml mark. Each lysate was capped with a small serum stopper and the whole was allowed to digest for 20 to 26 hours at 50°C (fig. 2b).

2.3.2. Gradient preparation

The experiment to be done is essentially a preparative version of the band sedimentation technique of Bruner and Vinograd [17]. In this experiment a low density solution containing the sample is layered on top of a high density solution. The large difference in density established (by diffusion) a rather steep density gradient throughout the analytical cell. This gradient is necessary for thermal stability as well as for maintenance of a stable macromolecular band [17,18]. The larger column length of the preparative tube and the resulting shallow diffusion gradient in the part of the solution distal to the sample layer would result in severe accelerative convection if not stabilized by an artificially created density gradient. Thus in all of our sedimentation runs the lysate was layered onto a linear density gradient of CsCl of 1.60 to 1.62 g/cm^3. It will be shown in a succeeding section that the preferred density for these experiments is 1.60 g/cm^3. Since the gradient volume was chosen to be 4.0 ml and the lysate volume (ρ = 1.12 g/cm^3) was 0.1 ml, the resulting average density in the tube was 1.60. Thus, just prior to the end of the digestion period described in the preceding section, the appropriate high and low density stocks are prepared and gradients are made in 1/2″ × 2″ Beckman polyallomer tubes using the Beckman gradient former. Contrary to the recommendations in the instruction manual for the machine, we consistently made gradients light first and heavy last. This technique produced a more linear gradient than making the gradient in the opposite way. As soon as the gradients were made, the samples were removed from the bath and layered on top as described in the next section.

2.3.3. Transfer procedure

Since we were working with giant polymers whose sensitivity to shear is well known, the procedure by which the samples were transferred to the tops of the gradients was of critical importance. Therefore we will describe it in detail in this section. The general precaution must be taken against any sort of uncontrolled flow. The cells have been lysed in the cut off syringes with this idea in mind. Fig. 1 illustrates the transfer process step by step. In 1b, the lysate is digesting at 50°C. Once all the gradients are made the lysates are removed one by one and the following procedure is carried out. Using gentle thumb pressure on the end of the plunger the lysate is transferred to the end so that the meniscus becomes flat (1c,d). The rate of motion should not exceed 0.01 ml/s. The syringe is then rotated 180° slowly and evenly (1e). Again using gentle finger pressure 1/4 to

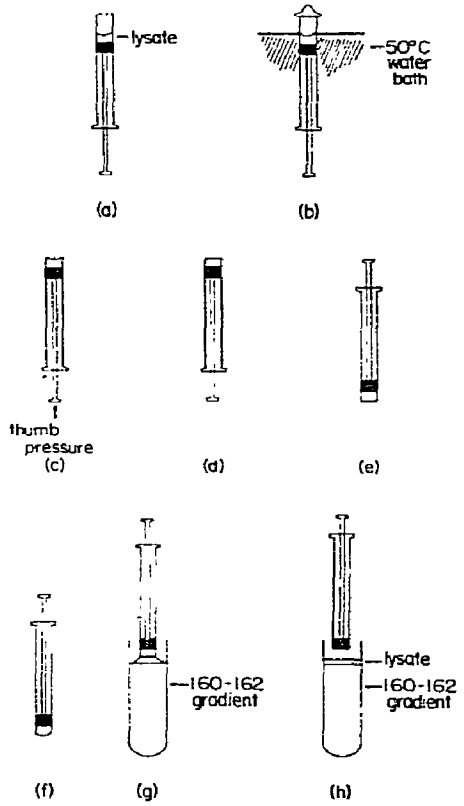

Fig. 1. Technique of lysate transfer. See Methods for explanation.

1/3 drop is formed at the tip (1f) and contact is made between this drop and the gradient (1g). At this point it is important to maintain contact with the gradient without tilting the syringe. If not, an air bubble may form at the syringe mouth and the lysate will run down the side of the syringe. Finally, the lysate is transferred to the gradient with finger pressure at the rate of 0.01 ml/s (1h). This whole procedure takes a little under 1 minute to perform and requires a little sensitivity to the surface tension of the substance being transferred (H_2O, salt solution or detergent). It is in fact the procedure generally used in our laboratory for handling giant DNA in many different aqueous environments. The rate 0.01 ml/s is about 1/10 of that suggested by Zimm (personal communication) to avoid shearing DNA of greater than 10^9 g/mole.

2.4. Choice of density and speed of centrifugation

The choice of the density at which to work was dictated by two considerations: We needed a high density to ensure that the tritium counts we saw were in essentially protein-free DNA and also we knew that high density would be necessary to nullify the anomolous speed effect. On the other hand one cannot raise the density indefinitely for two reasons. First, the rate of sedimentation decreases to zero as one approaches the buoyant density of 1.710. Second, one should stay as far as possible away from the buoyant density because the buoyancy factor is not known accurately close to buoyancy except in an equilibrium gradient. It is necessary to know this factor in order to convert the observed sedimentation coefficient to $S_{20,w}$. Thus, we chose to work at a density of 1.60 g/cm^3. This density is high enough to nullify the anomolous speed effect and to ensure the observance of pure DNA. There is also good buoyancy data at 1.60 g/cm^3.

Having chosen the working density, we estimated the proper speed at a given density and coil distortion from the Zimm theory. The theory for the decrease in observed S-value as derived by Zimm [14] gives the ratio of S_{obs} to S°_{obs} (the value at zero speed) as an infinite series:

$$S_{obs}/S^{\circ}_{obs} = 1 - D_2y^2 + D_4y^4 - \ldots . \quad (1)$$

Zimm points out that though the series converges, it does so slowly; the higher terms being important at moderate distortions [15]. Nevertheless, if we insist that $S_{obs}/S^{\circ}_{obs} \geq 0.99$, then $D_2y \leq 0.01$ and all higher terms are in fact negligible (at least for the purpose of speed estimation). Thus we choose $D_2y^2 = 10^{-2}$ and with $\rho = 1.60$ g/cm^3 we can compute a speed from a knowledge of D_2 and y. The coefficients D_n are products of combinationals and are given by Zimm [14]. The term y measures the molecular distortion:

$$y = 8.374 \times 10^{-24}\, M^2(\partial\rho/\partial c)^2_{\rho}(\text{rpm})^2 r/T\eta S^{\circ}_{obs}, \quad (2)$$

where

M = molecular weight of the Cs salt of the DNA under study,
$(\partial\rho/\partial c)_{\rho}$ = the three-component buoyancy factor for the working density,
rpm = rotor speed in revolutions per minute,
r = radial distance in cm,
T = absolute temperature,

η = solvent viscosity in poise,
S°_{obs} = the zero speed S-value of the DNA in the solution of density ρ.

This equation for y differs from Zimm's only in the buoyancy term. The Zimm theory was derived for a two component system and our system contains 3 components. Thus we have made the obvious substitution in Zimm's equation. While we have not shown that this substitution is the only change in the theory when applied to a 3 component system, that question is a refinement which does not concern us here. It is necessary to transform this equation by substituting for S°_{obs}, its dependence on M. First we convert S°_{obs} to $S^{\circ}_{20,w}$:

$$S^{\circ}_{20,w} = S^{\circ}_{obs}\,\eta_{rel}\left(\frac{(\partial\rho/\partial c)}{(\partial\rho/\partial c)_{\rho}}\right)\frac{M_{Na}}{M_{Cs}}, \qquad (3)$$

with
$\eta_{rel} = \eta^{23^{\circ}}_{CsCl}/\eta^{20^{\circ}}_{water}$,
$(\partial\rho/\partial c)$ = buoyancy factor of Na DNA at infinite dilution of salt,
$M_{Na}/M_{Cs} = 0.75$.
Solving for S°_{obs} and substitution into (2) gives

$$y = \frac{8.374\times10^{-24}M^2(\partial\rho/\partial c)_{\rho}(\partial\rho/\partial c)(\mathrm{rpm})^2 r}{1.33T\eta^{20}_{w}S^{\circ}_{20,w}}.$$

Using the following values and relations for the remaining variables in this expression we get y as a function of speed shown in eq. (5):

$M = 1.33 \times 2.6 \times 10^9$ g/mole $= 3.46 \times 10^9$ g/mole (Cs salt),
$(\partial\rho/\partial c)_{\rho} = 0.056$ (see section on data reduction),
$(\partial\rho/\partial c)^{\rho} = 0.46$ (Cohen and Eisenberg [19], extrapolated to infinite dilution of NaCl),
$r = 6.78$,
$T = 2.98 \times 10^2$ K,
$\eta^{20}_{w} = 10^{-2}$ poise,
$S^{\circ}_{20,w} = 0.01517\,M^{0.445}$ (see discussion); (4)

$$y = 1.85 \times 10^{-8}\,(\mathrm{rpm})^2. \qquad (5)$$

We want $D_2 y = 10^{-2}$ and since $D_2 = 2.887 \times 10^{-2}$ [14] we get the estimated speed for a 1% effect as 5600 rpm. We round this number off to 6000 rpm since it is easier to reproduce the latter speed on our machines.

2.5. Centrifugation

In order to carry out the required experiment of determining the position of the DNA as a function of time we typically centrifuged 5 to 6 identical gradients in the Beckman SW 50.1 rotor using the L2-65B preparative centrifuge. We have shown (see Results) that in order to avoid destruction of the gradients by convective mixing one must control the deceleration and the acceleration of the rotor at very low values compared to those encountered in normal usage of the machine. The modification of the centrifuge was suggested by Mr. K. Ishimaru of the Spinco Division of Beckman Instruments (Palo Alta, California) and consisted of a switch and a small potentiometer. These were wired into the speed control chassis across the drive armature. This gave manual control of the amount of current going to the drive.

It is also important to obtain reproducible deceleration and acceleration curves. For this purpose we connected a digital pulse counter to count the pulses on pin one of the tachometer board in the speed control chasis. Fig. 2 shows an example of deceleration and acceleration curves which were typical using these devices. We have used a value of about 200 rpm per minute for the deceleration and acceleration in the work reported here. This procedure yields gradients completely free of mixing and the Svedberg plots of the data are comparable in linearity to the data obtained from a boundary sedimentation experiment done in the analytical centrifuge.

Finally a note on temperature is pertinent. In order to avoid thermal gradients in the tubes we always set the refrigeration to hold at the ambient rotor temperature. This was usually (22 ± 1)°C.

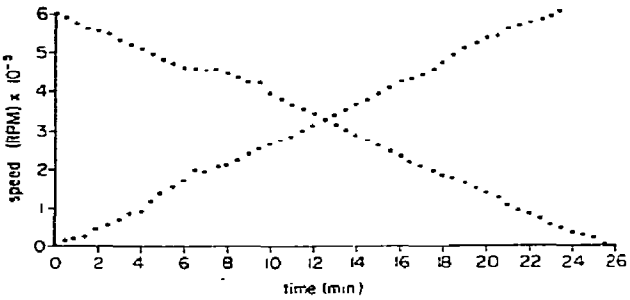

Fig. 2. Deceleration and acceleration schedule.

2.6. Gradient fractionation and tritium counting

The gradients were fractioned from the bottom by puncturing the tube with a stainless steel 23-gauge needle (about 2 1/2" long). Nine drops per fraction were taken directly onto a Whatman GF/A filter paper disks (2.4 cm) through the needle. A typical gradient yielded about 55 ± 1 fractions. Since the Brij-58 used to lyse the cells is soluble in CsCl, the drop size changes when the detergent is encountered. We compensated for this change by estimating at the time of fractionation and from experience, how many more drops were necessary to keep the fraction volume constant. This approximation procedure not only works quite well (cf. the sedimentation data in fig. 5) but also occurs in a part of the gradient which is rather unimportant for the purpose of this work. Therefore no more quantitative procedure has been used.

The filter papers were dried with a sunlamp and each paper was counted for tritium counts per minute (cpm) in 10 ml of omnifluor (New England Nuclear) dissolved in toluene (4 mg/ml). Typically each paper was counted for 10 minutes. The tritium present is expressed here as cpm per fraction. The background was 30 to 35 cpm and the efficiency was 26%.

2.7. Reduction of data and correlation of S_{obs} to $S_{20,w}$

The position of a fraction relative to the center of rotation was determined by the use of eq. (6). Here the maximum radius of the rotor is r_M, the total number of fractions is N and the fraction number is n.

$$r = r_M - 3.77(n/N). \quad (6)$$

This relation neglects the curvature at the bottom of the tube. 3.77 cm is the column height in the gradient. The time of centrifugation was taken as the total time between 4000 rpm on acceleration and 4000 rpm on deceleration.

Svedberg plots were constructed for the motion of two positions in the distribution: the fraction number corresponding to the maximum number of counts (r_{peak}) and the half height of the leading edge of the distribution ($r_{1/2}$). The half height is the fraction number which corresponds to 1/2 of the counts in the peak fraction (after background correction). The position of the half height provided more precise data than the peak position. The position-time data was plotted as $\log_{10}$ r(cm) versus t (hours). The best straight line was taken by eye through the data and the slope was equal to $3600\omega^2 S_{obs}/2.303$. S_{obs} was corrected to a solvent of the viscosity and density of water at 20°C as described below.

The correct form of the Svedberg equation for DNA in a concentrated binary solvent (salt = xy, solvent = H_2O) is

$$S_{T,xy} = M_x(\partial\rho/\partial c)_{\mu,T}/f_{T,xy}.$$

In this relation M_x is the dry molecular weight of the DNA with associated cation x, $f_{T,xy}$ is the molar friction factor at temperature T and for the experimental composition of xy, and $(\partial\rho/\partial c)_{\mu,T}$ is the expression of the buoyancy factor in the three component system. It is the change in density with DNA concentration at constant chemical potential of diffusable solutes at temperature T. This quantity has been very carefully measured as a function of solution density for DNA by Coher and Eisenberg [19]. Using the above form of the Svedberg equation one obtains eq. (3) for the conversion S_{obs} to $S^{\circ}_{20,w}$. The value of η_{rel} was interpolated from data of Bruner and Vinograd [17]. We find $\eta_{rel} = 1.015$. Implicit in eq. (3) is the assumption that $f_{20,NaCl}$ equals $f_{22,CsCl}$. The values of the two density increments were obtained in the following way.

The value of $(\partial\rho/\partial c)_{20,w}$ presents no particular problem. The measurements of Cohen and Eisenberg [19] in NaCl – water solutions can be extrapolated to a density of 1.0 to give 0.46 for the density increment. The value of $(\partial\rho/\partial c)_{\rho}$ however depends entirely on the slope of the $\partial\rho/\partial c$ versus ρ curve at buoyancy. By fitting the Cohen and Eisenberg data for $\partial\rho/\partial c$ versus ρ for DNA in CsCl with various functions one may arrive at values of $d(\partial\rho/\partial c)_{\mu}/d\rho$ at $\rho = 1.710$ which vary from –0.47 to –0.55. There is no reason to choose one value over another. A more useful approach is to estimate the slope from a three component theory of sedimentation equilibrium. Schmid and Hearst [20] have calculated a value of –0.49 using data on the net hydration. We have calculated a value of –0.53 based on the ratio of the effective density gradient and the composition density gradient. Since there is some uncertainty in both numbers we choose to average them to –0.51 ± 0.02 for the required slope. This number is well within the range of values obtainable from Cohen and Eisenberg's data and represents a more rational approach to the problem than that of curve fitting. The reason for this

assertion lies in the fact that the data from equilibrium density gradients is more reliable close to buoyancy than differential density measurements.

Therefore, close to buoyancy we use

$$(\partial\rho/\partial c)_{\mu} = -0.51\,(\rho - 1.710)$$

as the dependance of $(\partial\rho/\partial c)_{\mu}$ on ρ. 1.710 is the buoyant density of *E. coli* DNA at 1 atmosphere pressure [21]. Thus we find that $(\partial\rho/\partial c)_{\mu}$ at $\rho = 1.60$ is 0.056 ± 4%. Using eq. (3) for correction to standard conditions we get eq. (7).

$$S^{\circ}_{20,w} = 6.26\, S_{obs}\ (\pm 4\%). \tag{7}$$

We would like to point out that although the data of Bruner and Vinograd [17] for $S_{20,w}$ versus density in CsCl apparently extend through the region of buoyancy, they do not in fact report data sufficiently close to buoyancy to determine the required factor. Thus we expect our factor of 6.26 to be more accurate than the data of Bruner and Vinograd for obtaining $S^{\circ}_{20,w}$'s from sedimentation at densities near 1.71 g/cm^3.

2.8. Analysis of the sedimentation coefficient distribution

The distribution of counts per minute over fraction numbers can be transformed into a distribution of molecules over $S^{\circ}_{20,w}$ by associating an $S^{\circ}_{20,w}$ with each fraction number and then normalizing the counts per minute per fraction to the total area. For each gradient considered a scale of $S^{\circ}_{20,w}$ was constructed using the best value obtained from the Svedberg plots (fig. 5) and eq. (6). That is we determined the constant K in the equation,

$$S_{20,w} = K \log\,(r_M - 3.77\, n/N)/r_m. \tag{8}$$

Here r_m equals the apparent meniscus as determined by extrapolating the Svedberg plot of fig. 5 to zero time. Other variables are as previously defined. The normalization of count per minute was accomplished using eq. (9).

$$y(n) = H_n / \sum_{i=1}^{53} H_i\, \Delta S_i, \tag{9}$$

where

H_n = ^{3}H-counts per minute at fraction n,

ΔS_i = increment in $S^{\circ}_{20,w}$ between fraction i and $i+1$ as determined by eq. (8),

$y(n)$ = normalized distribution function.

Note that the sum is taken to fraction 53, the fraction number corresponding to r_m in eq. (8).

In addition, another distribution was determined for some of the data reported here. It was useful to know the fraction of molecules having a certain sedimentation coefficient or greater. This distribution function called $Y(n)$, is given in eq. (10).

$$Y(n) = \sum_{i=1}^{n} H_i \Delta S_i / \sum_{i=1}^{53} H_i \Delta S_i. \tag{10}$$

Both of these distributions are invariant in time.

Eqs. (8), (9) and (10) were computed for each fraction and then the fraction number was removed by matching the resulting tables. In this way we arrived at the two desired distributions.

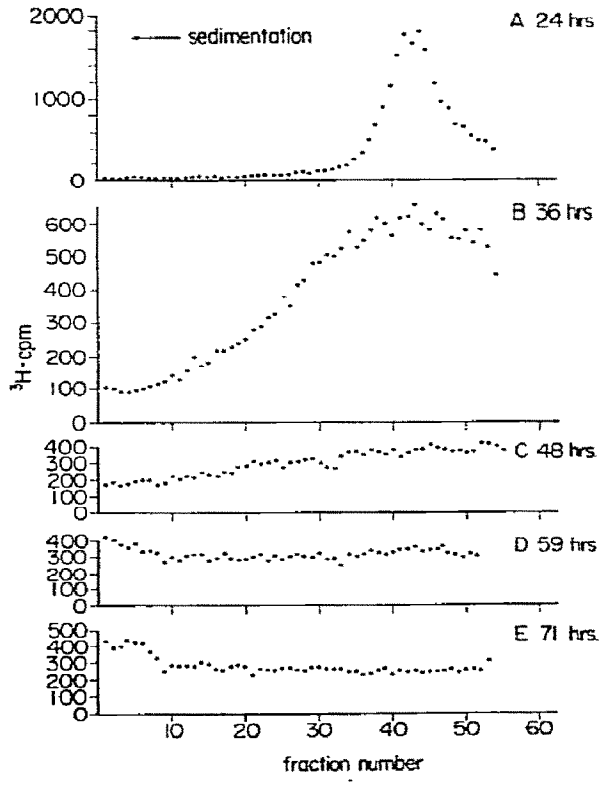

Fig. 3. Time dependence of *E. coli* DNA sedimentation using standard acceleration and coasting to take sample. The total elapsed time for each sample is shown.

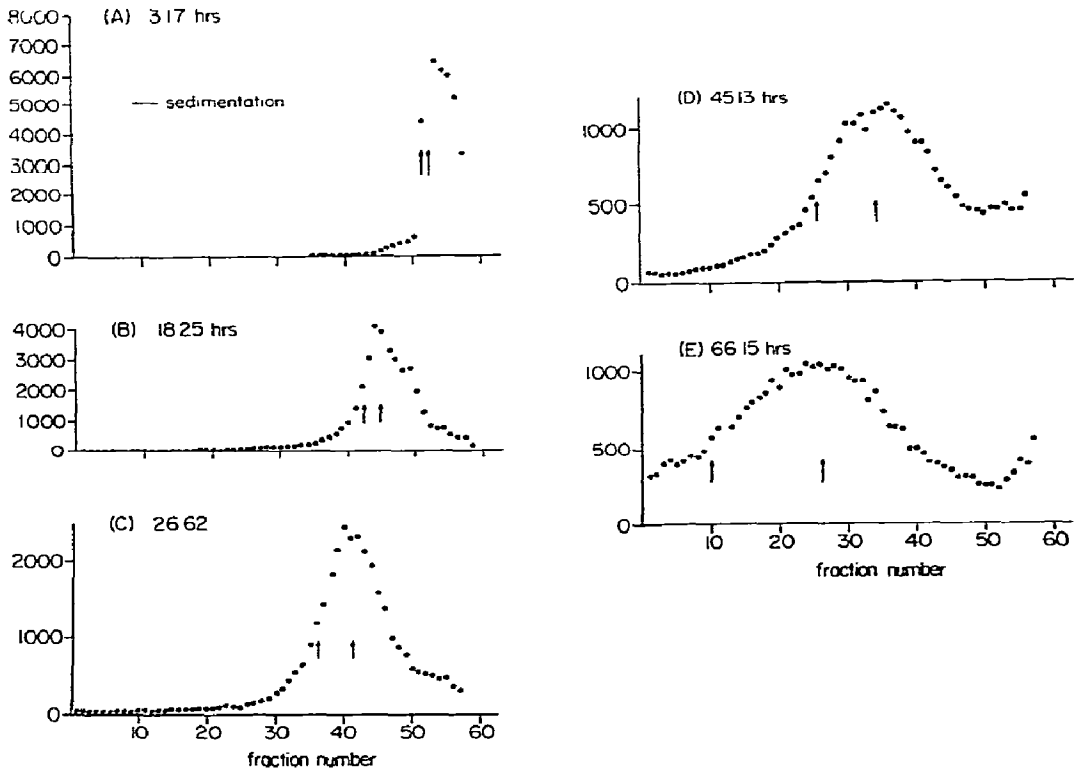

Fig. 4. Time dependence of stationary phase *E. coli* DNA sedimentation using the special procedures described in Methods. The rotor was decelerated according to the schedule shown in fig. 2. A tube was removed and an identical blank was replaced in the rotor. The rotor was then accelerated according to fig. 2. The elasped time for each tube is shown as well as the two positions whose time dependence was determined (2 arrows $r_{i/2}$ and r_{peak}). The vertical axis in each case is ^3H cpm. The concentration of DNA at the peak changes from 0.085 in (B) to 0.021 μg/ml in (E).

3. Results

3.1. Time dependence of the sedimentation of DNA from lysates of stationary phase and log phase cells

The results of ignoring the special procedures outlined in section 2 is shown in fig. 3. Here six identical gradient-lysate samples were prepared and time points were taken. The rotor was stopped by turning the timer to zero and allowing the rotor to coast to a stop without the brake. After removing a tube, the remaining gradients were accelerated to 6000 rpm at full current (~20 amp). Significant mixing progresses as each successive point is taken until the counts are uniformly distributed in the 4th gradient. (Tube 6 is not shown since it is identical to tube 5.) However if one utilizes the controlled acceleration and deceleration device discussed in the previous section and follows the accelera-

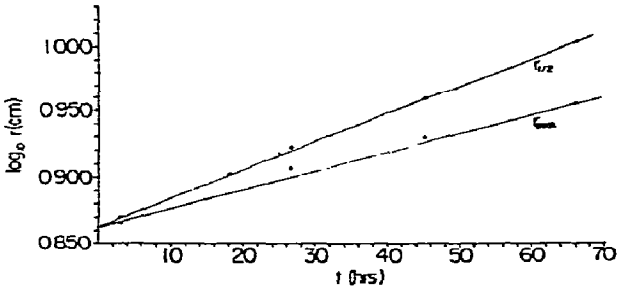

Fig. 5. Svedberg plot of data from fig. 4. The value of $\log_{10} r$ for the meniscus is 0.832.

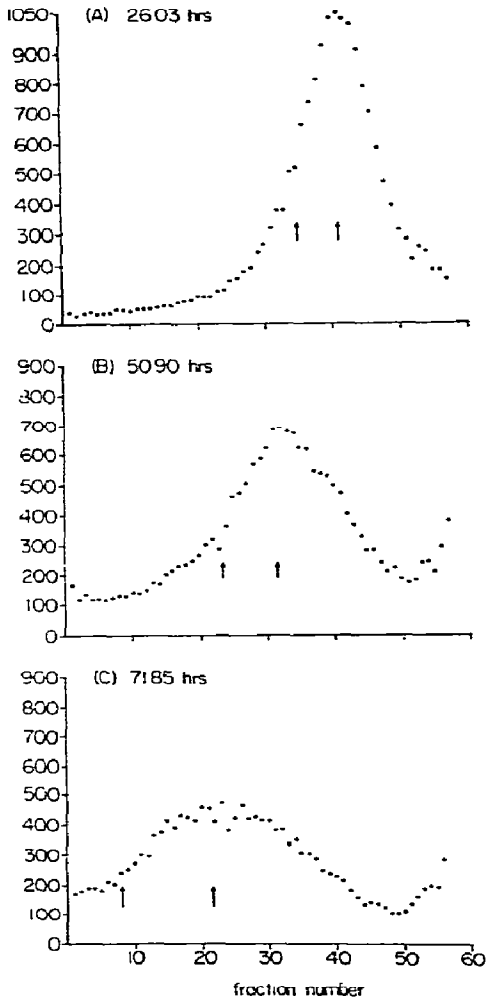

Fig. 6. Time dependence of log phase *E. coli* DNA sedimentation. Procedure used described in legend to fig. 4 and in Methods. The vertical axes are ^{3}H cpm.

tion and deceleration schedule as shown in fig. 2 then the results of taking time points on five samples is quite different. Fig. 4 shows the five gradients. The distribution of counts in each gradient is obviously stable as successive points are taken. In addition the peak and leading edge half height, $r_{1/2}$, move according to the Svedberg equation as shown in fig. 5. $\mathrm{Log}_{10} r_{1/2}(t)$ shows excellent linearity whereas there is some random error associated with the position of the peak. This error is probably due to the rather broad distribution of sedimentation coefficients obtained from this lysate. Note that the curves do not extrapolate to the meniscus at zero time. The sedimentation coefficient of the DNA corresponding to $r_{1/2}$ is 220 S; and that corresponding to the peak is 146 S. The value for $r_{1/2}$ may be the value for the undegraded *E. coli* as we will discuss later.

We have also carried out the same measurement on actively growing cells. The results of this sedimentation experiment are shown in fig. 6. Here, three identical gradient-lysate samples were centrifuged. The Svedberg plot is shown in fig. 7 for the two points followed. The data is linear and does not extrapolate to the meniscus at zero time. The sedimentation data for log phase cells and stationary phase cells are summarized in table 1.

3.2. Distribution of sedimentation coefficients of DNA

We determined the distribution of sedimentation coefficients in the stationary phase cell lysates by evaluating eq. (9) as described in Methods for the third, fourth and fifth time points of fig. 4. The resulting distributions are plotted in fig. 8. Notice that all three curves superimpose in their general features. This result argues very strongly against even slight convective mixing of any kind. The 45.13 hour time point (filled circles) fails to coincide with the other two at very low S values because of the detergent problem at the meniscus discussed in Methods. The distribution is very broad and is fairly

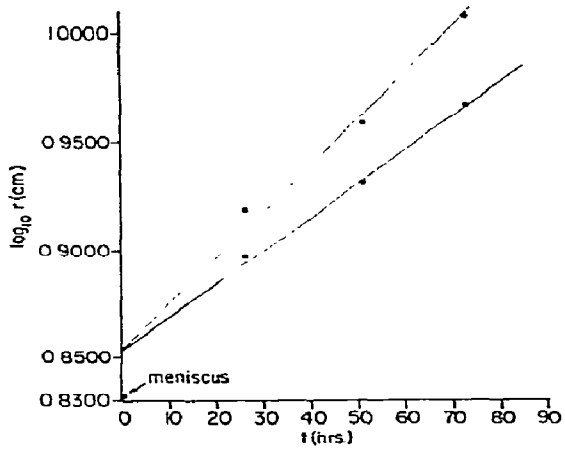

Fig. 7. Svedberg plot of data from fig. 6. The value of $\log_{10} r$ for the meniscus is 0.832.

Table 1
Observed and corrected sedimentation coefficients for *E. coli* from the analyses in figs. 5 and 7

S°_{obs}	Stationary phase	Log phase
r_{peak}	23.4 S	25.6 S
$r_{1/2}$	35.2 S	35.2 S
$S^{\circ}_{20,w}$		
r_{peak}	146 S	160 S
$r_{1/2}$	220 S	220 S

symmetrical. The broad peak is centered about a sedimentation coefficient of 155 S. The peak breadth at 0.606 times peak maximum is 118 S. The high side has a half width of 54 S and the low side has a half width of 64 S. 99% of the material present exhibits sedimentation coefficients from less than 10 S to 450 S. Also if one examines the three individual distributions carefully one can see minor components at 280–290 S, 245–250 S, 210–220 S, 165–175 S, and 130–150 S. While these components are certainly present, they do not represent a substantial portion of the DNA and also are not well resolved from the remainder of the heterogeneous material.

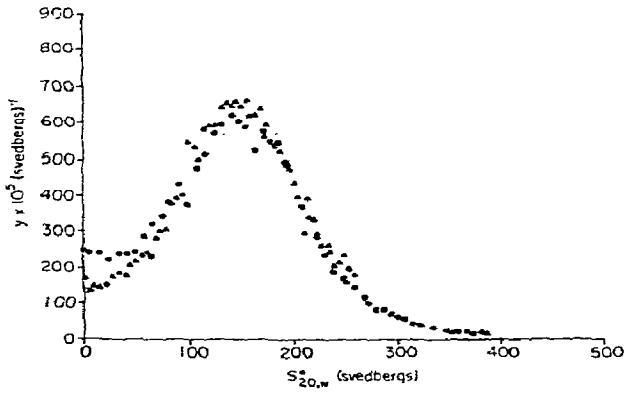

Fig. 8. Distribution of sedimentation coefficients. The function y was calculated from eq. (9) for three times of centrifugation: (○) 26.62 hours; (●) 45.13 hours; and (▲) 66.15 hours. This figure shows the fraction of molecules having a given $S^{\circ}_{20,w}$.

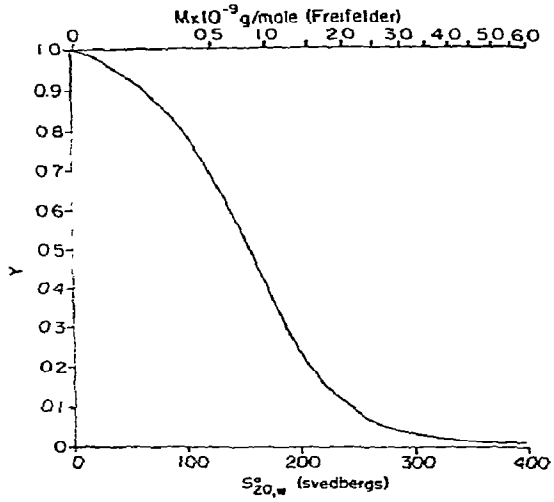

Fig. 9. Integral distribution of sedimentation coefficients. The function y was calculated from eq. (10) for the second and third times of centrifugation. The smooth curve through the data is plotted. The figure shows the fraction of molecules having a given $S^{\circ}_{20,w}$ or greater.

The integral distribution function, eq. (10), is plotted in fig. 9 as a continuous function. For a symmetrical distribution, the integral distribution function will equal 0.5 at the peak maximum which simply means that half of the material moves faster than the peak and half moves slower than the peak. Thus the weight average sedimentation coefficient will be the same as the sedimentation coefficient of the peak position for the symmetrical distribution. For our system, the integral distribution yields a weight average sedimentation coefficient of 155 S ($Y = 0.5$ at $S^{\circ}_{20,w} = 155$ S, fig. 9). From the slope of the r_{peak} curve in fig. 5 we obtained a value of 146 S for the peak. This discrepancy is more a reflection of the difficulty in locating the peak position accurately than it is an indication of an unsymmetrical distribution. We emphasize that the value of 155 S is the true weight average sedimentation coefficient.

Also from the Svedberg plot of $r_{1/2}$ in fig. 5 we have determined the rather precise value of 220 S for the leading edge half height. One can see from fig. 9 that 15% of the DNA has this sedimentation coefficient or greater. This means that the weight average sedimentation coefficient of the fastest 30% of the DNA is 220 S. We will return to this fact in the discussion of molecular weights in the next section.

4. Discussion

4.1. The molecular weight of the DNA sedimenting at the position $r_{1/2}$

We have measured a value of 220 S for the $S^{\circ}_{20,w}$ of the fastest moving 30% of the DNA in the Klotz and Zimm [6] lysate of *E. coli*. This sedimentation constant is probably good to within 5% as we will show later. Klotz and Zimm [6] have measured the molecular weight of the largest molecules of DNA in this lysate and have arrived at a value of 2.7×10^9 g/mole, the size of the whole *E. coli* chromosome, They also estimate that about 40% of the molecules in the lysate have this molecular weight. We have taken care to reproduce their growth and lysis conditions exactly and qualitative observations on our lysate agree with corresponding observations on theirs: (1) We do not observe a substantial fraction of circular molecules as these should sediment as a broad peak well ahead (280–320 S) of the remainder of the degraded DNA. (2) The breadth of the distribution (fig. 8) shows that there is some random degradation occurring in the lysate. (3) Lysis for times shorter than 22–25 hrs yields sedimentation coefficients in the range 500 S to several thousand S for about 10–20% of the counts. We take these molecules to be the same linear aggregates observed by Klotz and Kimm at short lysis times which give rise to very long relaxation times. In fact it appears (fig. 8) that a small amount of very fast material (> 350 S) is still present after 22–25 hrs of lysis. The fraction of DNA in this high-S form is less than 3% (fig. 9).

The Klotz and Zimm value of 2.7×10^9 g/mole is obtained from a combination of the theory of viscoelastic relaxation and the empirical formula relating intrinsic viscosity to molecular weight reported by Crothers and Zimm [22]. These authors also give an equivalent formula relating $S^{\circ}_{20,w}$ to molecular weight. There are however two other formulae which bear consideration. These are the ones reported by Rinehart and Hearst [23] and by Freifelder [7]. These three equations are plotted on a log–log scale in fig. 10. It is important to note that whereas the DNA molecular weights on which the Crothers–Zimm equation is based are very likely incorrect [24], their equation is similar to the Rinehart–Hearst equation which is based on reliable bacteriophage molecular weights. (The difference in the two equations as presented here shows up because they

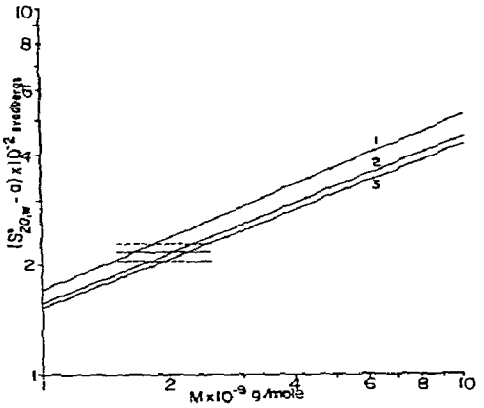

Fig. 10. Dependce of $S^{\circ}_{20,w}$ on molecular weight according to three empirical equations. The equation takes the general form $S^{\circ}_{20,w} = a + bM^c$. a, b and c for the three equations are:

Reference	a	b	c	Curve number
Freifelder [7]	2.8	8.34×10^{-3}	0.479	1
Rinehart and Hearst [23]	2.68	1.263×10^{-2}	0.455	2
Crothers and Zimm [22]	2.7	1.517×10^{-2}	0.445	3

The solid horizontal line is the value of 220 S measured for the $r_{1/2}$ position. The dashed lines show the limits of uncertainty in this number (see section 4.2).

are plotted in a molecular weight range far removed from that for which they were intended.) The horizontal bar on the figure corresponds to 220 S (minus 2.7). The dashed lines above and below are the estimated limits on the uncertainty of the value 220 S. (See section 4.2.) The three curves yield values of 2.3×10^9 g/mole (Crothers–Zimm [22]), 2.1×10^9 g/mole (Rinehart–Hearst [23]), and 1.7×10^9 g/mole /Freifelder [7]) for the molecular weight of DNA at 220 S. Of these only the Crothers–Zimm number is within the range of values accepted for the size of the whole *E. coli* chromosome. However, since the Klotz and Zimm calculation depends on the Crothers and Zimm molecular weight dependence, the above result should perhaps be viewed as a confirmation that we have reproduced their molecular weight distribution, not necessarily that the molecular weight is 2.3×10^9 g/mole.

The value of 1.7×10^9 g/mole from the Freifelder equation is interesting because it is close to the value expected for half size molecules (1.3×10^9 g/mole). A case can be made for the assertion that the largest molecules present are average half molecules. We observe that random degradation is certainly occurring in the lysate. In addition, the actual state of the genome is not known for this system. That is, unless one knows the cause of the onset of stationary phase and the result this cause would have on the replicative system of the cell, then one does not know whether the cells are arrested at the beginning of a replication cycle or whether their replicative mechanism was shut off in the middle of a cycle. Thus it may be that these cells are randomly distributed through the division cycle in stationary phase. If this were true there would be at least two replication forks per molecule and if random degradation cuts the chromosome apart at these points preferentially one expects from this model that the average of the largest molecules present would be approximately half of the full size genome.

There are two pieces of data which are in fact difficult to explain in any other way. First is the absence of circular forms. If completed circular genomes were present at the beginning of lysis, if the degradation of double stranded DNA had been random, and if the distribution of hits were Poisson, a non-negligible fraction of circles would have to be present at a time equal to that required to give a 30%–40% yield of whole linear molecules. No circles were observed at all indicating that there may be sites in the genome preferentially attacked by nucleases such as the single stranded regions in the replicating forks. Second, the sedimentation profiles of log phase and stationary phase cell lysates are very similar. They are coincident at $r_{1/2}$, both having a value of 220 S for the largest DNA present. The log phase cells are randomly distributed through the division cycle and by comparison we must conclude that the stationary phase cells are likewise randomly distributed in the cycle. These observations and arguments tend to support the use of the Freifelder relation for the determination of molecular weights from $S^{\circ}_{20,w}$ in the range 10^9 to 10^{10} g/mole.

The question of which $S^{\circ}_{20,w}$ function to use for molecular weight determination in the range of 10^9 to 10^{11} g/mole cannot be answered unequivocally at this time. The Freifelder relation is based on a choice of sedimentation coefficients and molecular weights after a very careful consideration of the best literature values for DNA from bacteriophages T4, T5, T7 and ϕX-174 replicative form. His equation represents an empirical fit to the data for these viral DNA's. However, the equation prepared by Rinehart and Hearst [23] is based on a no less careful choice of electron microscope data and they arrive at an equation closer to the Crothers and Zimm relation [22]. We view the Freifelder equation and the Crothers–Zimm equation as limiting cases.

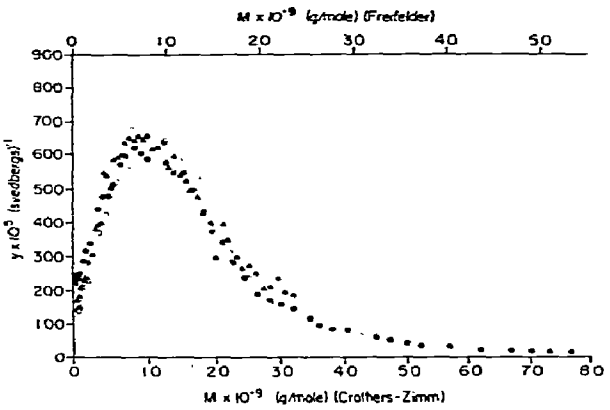

Fig. 11. The distribution of molecular weights according to two of the empirical curves in fig. 10. The function y is the same function as is plotted in fig. 8. See section 4.1 for explanation.

In view of these considerations we have placed two molecular weight scales on the distributions of fig. 11. The lower scale shows the molecular weights present in the lysate based on the Crothers–Zimm equation and the upper scale shows those calculated from the Freifelder equation. The Crothers–Zimm equation shows that the weight average molecular weight is 1×10^9 g/mole (peak) and half of the molecules in the lysate are larger than this value. The Freifelder equation yields a weight average molecular weight of 0.8×10^9 g/mole. We tentatively take the latter result to be closer to the true state because of the arguments above.

Extending either scale of fig. 11 into the range of 4 to 8×10^9 g/mole is certainly invalid since the presumed end to end aggregates [6] corresponding to the data in this range are probably not members of the same homologous series as the lower molecular weight DNA. However we do not have any way of choosing where to terminate the scales on the high side so we simply include all the data and recognize the fact that molecular weights

higher than about 3.5×10^9 g/mole may be incorrect by a large factor.

4.2. Expected uncertainty of the $S^{\circ}_{20,w}$ values

Examination of the data for $r_{1/2}$ of fig. 5 shows that the value of S_{obs} obtained from the slope of that line is good to better than 1%. There is wider latitude available from the slope of the line for r_{peak}, but as we have already pointed out, the point where the integral distribution equals 0.5 (fig. 9) is a more accurate value of the weight average sedimentation coefficient than is available from the data of r_{peak}. Because of the dripping artifact due to detergent at the top of each tube (discussed in Methods), the values of $S^{\circ}_{20,w}$ shown in the distribution of fig. 8 in the 0 to 50 S range are probably rather inaccurate. Thus, the values of S°_{obs} from our data are good to approximately 1% in the range 50 S to 350 S. A larger uncertainty arises from the factor 6.26 used to correct S°_{obs} to $S^{\circ}_{20,w}$. As we have discussed, the uncertainty in this number (arising from the uncertainty in the slope of the $(\partial\rho/\partial c)_{\mu}$ versus ρ curve at buoyancy) is about 4%. Thus the total uncertainty in the values of $S^{\circ}_{20,w}$ in the range 50 S to 350 S is about 5%. The uncertainty in the $S^{\circ}_{20,w}$ of the $r_{1/2}$ position for example is about 220 ± 11 S. This uncertainty is shown by the horizontal dashed lines of fig. 10.

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