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Fractionation of Native and Denatured Deoxyribonucleic Acid on Agarose Columns

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SUMMARY

Enzymatically prepared DNA fragments have been fractionated according to molecular weight on agarose columns. The upper limit of fractionation on the most porous gel used (Sepharose 2B) corresponds to a molecular weight of about $0.85 \cdot 10^6$ for double-stranded DNA. The relationships between k_{av} and $[\eta]$ or s as well as the $[\eta]$ *versus* s relationship were determined for the 2B fractions. Sepharose 6B columns have been used to fractionate both native and denatured DNA fragments having a molecular weight lower than about $3.5 \cdot 10^5$. Sedimentation equilibrium studies using the short column technique (VAN HOLDE, K. E., AND BALDWIN, R. L. (1958) *J. Phys. Chem.* 62, 734) at high speed (YPHANTIS, D. A. (1964) *Biochemistry* 3, 297), which only requires 0.5 μg of DNA, were done on the 6B chromatographic fractions. These were found to be practically monodisperse ($M_z/M_w \leq 1.1$). Relationships between the sedimentation coefficients and the molecular weights of the 6B chromatographic fractions of native and denatured DNA and their elution volumes were determined. At the same time, the relationships between s and M_w were established in a molecular weight range where no data were previously available.

Gel chromatography has been widely used in recent years as a technique for the determination of molecular weights of proteins and polysaccharides (see References 1 and 2 for reviews). Applications of this method in the area of nucleic acids have been essentially limited to the fractionation of synthetic, single-stranded oligo- and polynucleotides of molecular weight lower than 10^5 on cross-linked dextran, polyacrylamide, and agarose gels (3–6). Because of their very large hydrodynamic volumes, polynucleotides are excluded from agarose columns at a much lower molecular weight level than globular proteins (6, 7). However, the fractionation range of agarose columns is interesting in studies of DNA properties dependent upon molecular weight (such as melting, renaturation and hybridization kinetics, etc.) and secondary structure (such as flexibility), as well as in investigations on compositional heterogeneity of DNA in the "gene-size" range, the exclusion of native DNA taking place at a molecular weight of $0.85 \cdot 10^6$ on the most porous agarose columns used in this work.

In the present paper we report investigations on the fractionation on agarose columns of both native and denatured DNA fragments. We have shown that this separation procedure is very powerful in that it can yield precise information on the molecular weight distribution of DNA samples and provide practically monodisperse DNA fractions. We have been able, using these fractions, to establish relationships between elution volume on one hand and sedimentation coefficients and intrinsic viscosity on the other hand for native DNA ranging in molecular weight from $0.85 \cdot 10^6$ to $1.4 \cdot 10^5$. Furthermore, we could fractionate both native and denatured DNA fragments having a molecular weight lower than $3.5 \cdot 10^5$; in this case we could determine the molecular weights of the chromatographic fractions by sedimentation equilibrium using the short column technique (8) at high speed (9), and establish relationships between s and M_w in a molecular weight range where no data were available.

MATERIALS AND METHODS

DNA Preparations—Calf thymus DNA was obtained following the detergent procedure of Kay *et al.* (10) according to Bernardi and Sadron (11). *Escherichia coli* DNA was a preparation described elsewhere.[1] Mitochondrial DNA from wild type *Saccharomyces cerevisiae* strain A (12) was preparation I-II of Bernardi *et al.* (13). All DNA preparations were degraded with hog spleen acid DNase B (14). The sedimentation coefficients of the degraded calf thymus, *E. coli*, and yeast mitochondrial DNA samples were 7.7 S, 5.2 S, and 8.8 S, respectively. Sedimentation coefficients were measured as indicated below.

DNA Denaturation—DNAs from *E. coli* and yeast, degraded by spleen acid DNase, were dialyzed against 0.15 M NaCl-0.015 M sodium citrate (Solvent A), pH 7.7, adjusted to 1% formaldehyde with 37% formaldehyde Merck (Darmstadt, West Germany), heated 5 min in boiling water, and rapidly cooled in an ice bath. Heat denaturation was done immediately before the chromatographic experiments. The sedimentation coefficients in alkaline solvent (see below) of the denatured samples were 4.0 S and 5.2 S for two *E. coli* DNA samples and 6.4 S for yeast mitochondrial DNA.

Chromatography—Sepharose 2B, 4B, and 6B (Pharmacia, Uppsala, Sweden) were equilibrated with Solvent A in the case of native DNA, Solvent A containing 1% formaldehyde (Solvent B) in the case of denatured DNA, and loaded on columns (1.2 × 52 to 60 cm).

[1] J. P. Thiery, S. D. Ehrlich, A. Devillers-Thiery, G. Bernardi, manuscript submitted for publication.

One to 16 A_{260} units of native DNA in Solvent A volumes equal to 1 to 4% of the column bed were loaded on the columns. Denatured DNA loads were kept below 5 A_{260} units.

The flow rate of the solvent was maintained at 6 to 10 ml per hour using a Technicon (Chauncey, N. J.) peristaltic pump. Except where otherwise stated, 0.5- to 0.6-ml fractions were collected using an LKB UltroRac (LKB, Stockholm, Sweden) fraction collector. The transmission of the effluent at 253.7 nm was recorded using a Uvicord (LKB, Stockholm, Sweden) equipped with a 0.3-cm cell. The absorbance at 260 nm of the fractions was read on a Zeiss spectrophotometer PMQ II.

Collection was started when the load was put on the top of the previously drained column. Elution volumes were determined by multiplying the fraction number by the fraction volume; $k_{av(ailable)}$ values were calculated using the equation $k_{av} = V_e - V_0/V_t - V_0$, where V_e is the elution volume of the fraction, V_0 and V_t the void volume and the total volume of the column, respectively. V_0 was determined as the elution volume of the leading peak of dextran blue 2000 (Pharmacia, Uppsala, Sweden). With DNA samples partially excluded by the gel, the void volume was found to correspond to the half-height of the leading peak, the presence of some excluded DNA providing, therefore, an internal marker of the void volume.

Some experiments were performed, using native calf thymus and *E. coli* DNAs on Sepharose 2B and native *E. coli* DNA on Sepharose 6B columns, to check the influence of the load volume and its DNA concentration on the chromatogram. In the first set of experiments, the DNA load was kept constant and equal to 3 A_{260} units; the chromatograms obtained with volumes of the loaded solution ranging from 1 to 6% of the column volume were superimposable. In the second set of experiments, the volume of the DNA solution was kept constant and equal to 4% of the column volume; the chromatograms obtained with amounts of DNA ranging from 1 to 16 A_{260} units were homothetic. All chromatographic experiments were done at room temperature (about 25°). In all cases, recoveries of DNA from Sepharose columns were better than 95%.

Viscosity—Viscosity of chromatographic fractions obtained from native calf thymus DNA ($s_{20,w}$ = 7.7 S), having concentrations comprised between 8 and 24 μg per ml was measured at 26 ± 0.01° using a rotating cylinder viscometer (15) equipped with an automatic recording system (16), the solvent being Buffer A. The shear stress was $3.8 \cdot 10^{-3}$ dyne per cm^2; shear gradients were close to 0.4 s^{-1}. No concentration, or shear dependence corrections were made for the low molecular weight DNA samples used in the present work since they were unnecessary.

Sedimentation Velocities—These were carried out in a Spinco model E instrument equipped with a monochromator, a photoelectric scanner, and a multiplexer. Boundary sedimentation of calf thymus DNA fractions was performed in 30-mm single-sector cells, band sedimentation of *E. coli* DNA and mitochondrial DNA fractions in Vinograd 30-mm Kel-F single-sector cells. In this latter case, 0.6-ml DNA fractions were dialyzed against 0.05 M ammonium acetate, pH 5.5, (a solvent chosen because samples were later used for enzymatic digestions) and concentrated by rotary evaporation to an A_{260} = 0.6; 25-μl aliquots of the samples were then layered on 1 M NaCl-0.01 M Tris buffer, pH 7.6 (native DNA fractions), or 0.9 M NaCl-0.1 M NaOH, pH 12.6 (denatured DNA fractions), and centrifuged (17). Scannings were done at 265 nm; the horizontal enlargement of the scannings was equal to 23.4. Sedimentation coefficients were calculated using a Programma 102 (Olivetti, Ivrea, Italy) desk computer. For the correction to standard conditions see "Partial Specific Volume."

Sedimentation Equilibrium—Measurements were performed, using short columns (8) at high speed (9), on native and denatured DNA fractions from Sepharose 6B columns. Fractions of native and denatured DNA having molecular weights higher than $3.5 \cdot 10^5$, therefore above the fractionation range of Sepharose 6B were not usually studied by sedimentation equilibrium because of the increasingly longer times required to reach equilibrium (see below). Kel-F double-sector cells (12 mm) and a 6-holes titanium rotor, model An-G, were used. Solvent, 0.15 ml (Solvent A or Solvent B in experiments performed on native or denatured DNA, respectively), was introduced in the first sector; 0.10 ml of DNA solution, A_{260} = 0.1, plus 20 μl of fluorochemical FC 43 (Spinco, Palo Alto, Calif.) in the second sector (this corresponds to a liquid column height of about 0.28 cm in the cell). The angular velocities used ranged from 8,000 to 36,000 rpm and were checked by odometer readings at the beginning and at the end of the runs; since five DNA samples of different molecular weights were centrifuged at the same time, two or three different velocities were used in succession. Equilibrium was attained in all cases in less than 24 hours (see below). The temperature of the rotor during experiments was kept constant at values comprised between 22° and 26°. Scannings were routinely done at 265 nm with a slit width of 0.06 mm; wave lengths comprised between 265 nm and 280 nm were sometimes used, the choice being made so as to keep the absorbance at the bottom of the cell lower than 1.0. This procedure was useful in some of the experiments where the effects of concentration and rotor speed on the apparent molecular weights were studied (see below), and was allowed by the use of an achromatic mirror (Beckman, Munich, Germany) replacing the flat mirror and the lens of the usual set-up.

The molecular weights of the DNA solutions were calculated using the equation:

$$M_w = \frac{\bar{\sigma}_w \cdot RT}{\omega^2(1 - \bar{v}\rho)}$$

In this equation R is the gas constant, T the absolute temperature, ω the angular velocity, $\bar{v}$ the partial specific volume of the solute (see below), ρ the density of the solution; $\bar{\sigma}_w$, the weight average effective reduced molecular weight over the whole cell is defined (9) as:

$$\bar{\sigma}_w = \frac{c(b) - c(a)}{\int_a^b rc(r)\, dr}$$

where $c(a)$, $c(b)$, and $c(r)$ are the concentrations at the radii a, b, and r, corresponding, respectively, to the top and the bottom of the solution column, and to the radius r. $\bar{\sigma}_w$ was obtained by extrapolating to the bottom of the cell $\sigma_n(r)$, the number average effective reduced molecular weight (9) for the points centrifugal to the point of radius r', where the concentration is large enough to permit neglecting the concentration at the meniscus.

$$\sigma_n(r) = c(r) \Big/ \int_{r=a}^{r} rc(r)\, dr \qquad r > r'$$

The tracing was perfectly horizontal in at least the proximal third of the cell in all experiments used for calculations. $c(r)$ values were taken as the ultraviolet absorbances measured at about 50 equidistant points along the scanning whose horizontal

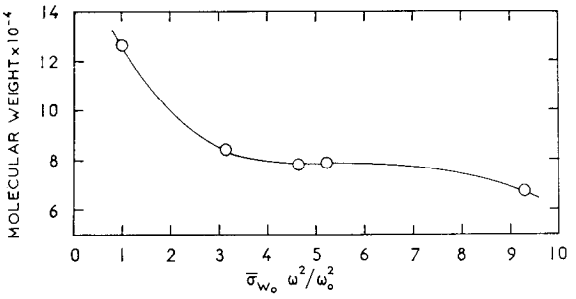

FIG. 1. Plot of molecular weight *versus* $\bar{\sigma}_{w0}\,\omega^2/\omega_0^2$ for five identical DNA samples. $\bar{\sigma}_{w0}$ and ω_0^2 are the values of $\bar{\sigma}_w$ and ω^2 for the particular speed where $\bar{\sigma}_w$ of the sample is close to 5. $\bar{\sigma}_{w0}\,\omega^2/\omega_0^2$ would be the real value of $\bar{\sigma}_w$, if no effect of speed existed.

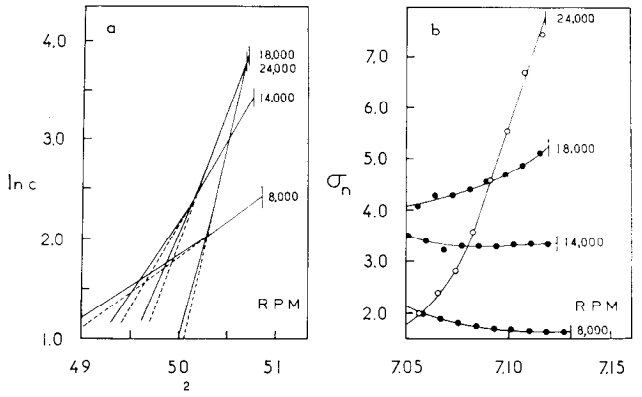

FIG. 2. *a*, plot of ln *c versus* r^2 (in square centimeters) for four identical DNA samples spun at different rates. The samples are those of Fig. 1. *Vertical lines* indicate the position of the bottom of the cell. *Broken lines* show the linear extrapolations of the bottom part of the plots. *b*, plot of σ_n *versus* r (in centimeters) for the same DNA samples.

enlargement was equal to 112; a base-line prolonging the tracing in its horizontal part was subtracted from the absorbance readings.[2] A FORTRAN program[3] was used (*a*) to smooth the curve given by the readings; (*b*) to correct them for the non-linearity of the scanner response; (*c*) to evaluate $\sigma_n(r)$; the integral $\int_a^r rc(r)\,dr$ was determined using the Simpson method; (*d*) to extrapolate $\sigma_n(r)$ to the bottom of the cell from its last two values; (*e*) to calculate M_w from the extrapolated value of $\sigma_n(r)$.

In order to establish the range of $\bar{\sigma}_w$ values in which valid molecular weight measurements could be obtained, the effect of angular velocity on the apparent molecular weight was investigated. The results obtained in an experiment in which five different velocities were used on the same DNA sample are shown in Fig. 1. It is clear from Fig. 1 that the optimal value of $\bar{\sigma}_w$ is close to 5 cm^{-2}, in agreement with Yphantis (9). In fact, the calculated M_w values decrease very slightly when the angular velocities increase above a value corresponding to $\bar{\sigma}_w$ = 5, whereas they show a stronger increase for lower angular velocities.

The decrease in the apparent molecular weights at high speed seems to be due to a small effect of polydispersity in the DNA samples, causing a piling up of the heavier molecules on the solution-fluorochemical meniscus. The polydispersity of the DNA fractions was found to be very small, M_z/M_w being lower than 1.1, all over the molecular weight range of fractionation on Sepharose 6B columns. M_z values were calculated according to Yphantis (9) from the slopes of the linear parts of the ln *c versus* r^2 plots (Fig. 2*a*).

The increase in the apparent molecular weight at low speed appears to be due to the presence of some material in the air-solution meniscus region, a fact which prevents a correct estimate of $c(r)$ and, therefore, of the molecular weight. It can be easily shown that M_w and M_z will be increasingly overestimated as the amount of material left at the meniscus increases and that $\sigma_n(r)$ will be increasingly overestimated as r decreases, a fact shown in Fig. 2*b*.

The time, t, required to attain equilibrium is related to the diffusion constant, D, and, therefore, to the molecular weight of the sample. For $\bar{\sigma}_w = 5.0\ cm^{-2}$, $(b - a) = 0.3$ cm, Dt is close to $1.4 \cdot 10^{-2}\ cm^2$ for $\epsilon = 10^{-2}$ (ϵ being the relative deviation of $c(b) - c(a)$ from its equilibrium value) and to $2.0 \cdot 10^{-2}\ cm^2$ for $\epsilon = 10^{-3}$ (9). On the basis of the empirical relationship between s and M, $s = kM^a$, and of Svedberg's equation $s = DM\,(1 - \bar{v}\rho)/RT$, one can see that t and M are related by an equation of the type $t = A \cdot M^B$, where $A = Dt\,(1 - \bar{v}\rho)\ 10^{13}/kRT$ and $B = 1 - a$, t being in seconds. It could be shown using this equation that, in our case, the routine time of 24 hours was sufficient to reach equilibrium within $\epsilon = 10^{-3}$ for the large majority of the samples (and was actually in excess for the samples of smallest molecular weight), and within $\epsilon = 10^{-2}$ for the largest ones.

Some experiments were done in order to check the effects of DNA concentration and solvent upon the apparent molecular weight. Using a native calf thymus DNA fraction (M_w = 127,000), no dependence of molecular weight was found upon DNA concentration (A_{260} values varied from 0.03 to 0.25), nor ionic strength (Solvent A or Solvent A + NaCl, final Na^+ = 0.6 M, were used). Similarly, molecular weights for *E. coli* denatured DNA samples did not show any difference whether DNA concentrations were 5 or 10 μg per ml.

Partial Specific Volume—The $\bar{v}$ value of DNA in water has been taken equal to 0.556 ml per g (18). In salt solutions, ϕ' values have been used instead (19), ϕ' being defined by the equation:

$$\left(\frac{\partial\rho}{\partial c_2}\right)_{\mu_1,\mu_3} = 1 - \phi'\rho$$

where $\partial\rho$ is the density increment caused by the concentration increment ∂C_2 of DNA, μ_1, and μ_3 being the chemical potentials of components 1 (water) and 3 (salt); ϕ' values in 0.2 M and 1 M Na^+ were taken as equal to 0.541 and 0.565 ml per g, respectively (19). These values differ from the 0.55 ml per g (20) used by Eigner (21) and 0.556 ml per g (22) used by Studier (17). As a consequence of using the Cohen and Eisenberg (19) ϕ' values: (*a*) the molecular weight by sedimentation equilibrium is decreased by 3.4%; (*b*) the sedimentation coefficient in 1 M Na^+, neutral or alkaline, is increased by 3.2%; the correction factors for solvent density and viscosity become 1.181 and 1.196

[2] Near the air-solution meniscus the absorbance tracing did not coincide, as a rule, with the zero absorbance level. In order for the $c(r)$ values to be correct, this small shift must not be due to the presence of absorbing material. The following observations showed that this was not the case. (*a*) When a DNA solution of known absorbance was introduced in the cell and spun at low speed for a short time, the recorded absorbance showed a higher value which was constant through the cell; when the DNA was removed by centrifugation from the air-solution meniscus region, the recording near this meniscus was equal in value to the difference between the recorded and the previously measured absorbance. (*b*) The shift was variable in magnitude from experiment to experiment and also for different cells in the same experiment, but it did not vary with time or with rate of centrifugation.

[3] A listing of this program is available upon request.

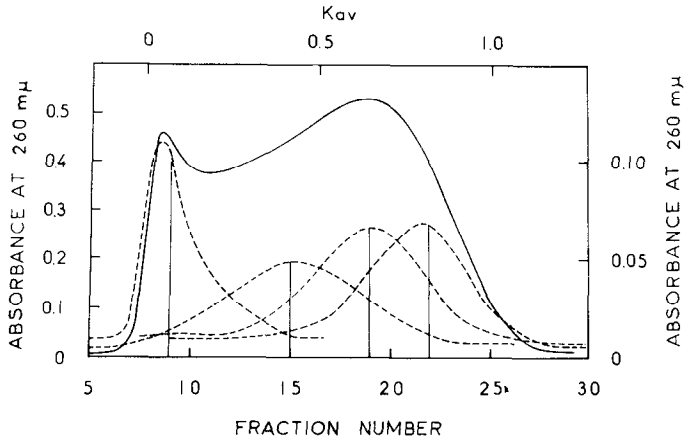

FIG. 3. The continuous line shows a chromatogram of 16 A_{260} units of 7.7 S calf thymus DNA. Two milliliters of DNA solution in Solvent A were loaded on a Sepharose 2B column (1.2 × 55.2 cm) (left-hand ordinate). Flow rate was 9.7 ml per hour; 2.42-ml fractions were collected. The *broken lines* show the chromatograms of fractions 9, 15, 19, and 22 (indicated by *vertical lines*) on the same column (right-hand ordinate).

instead of 1.144 and 1.160 (17) for 1.0 M NaCl and 0.9 M NaCl-0.1 M NaOH, respectively; (*c*) in contrast, the sedimentation coefficient in 0.15 to 0.20 M Na^+ is decreased by 3.3%.

It should be pointed out that we have also used the Cohen and Eisenberg (19) ϕ' values, which were determined on calf thymus DNA (44.5% GC[4]), for yeast mitochondrial DNA (18% GC (13)), neglecting the possible difference due to different base composition; and for denatured DNA in Solvent B, assuming, as other authors previously did (17, 21), that the $\bar{v}$ of denatured DNA is the same as that of native DNA; an additional uncertainty in our case derives from the fact that formaldehyde-reacted denatured DNA was used.

RESULTS

Fractionation of Native DNA on Sepharose 2B—Fig. 3 shows a chromatogram obtained by running the 7.7 S calf thymus DNA sample on a Sepharose 2B column (*continuous line*). The chromatogram is characterized by the presence of a minor component, which is excluded by the gel and a major component which is retarded. Four chromatographic fractions were re-run on the same column (*broken lines* of Fig. 3). The elution volume of each fraction, as determined at the top of each peak, is identical with that of the starting material, except for the first one which was, however, a fraction containing both excluded and retarded material. The experiments shown in Fig. 3 demonstrate that the k_{av} values of the retarded chromatographic fractions, as determined by rechromatography, coincide with the original ones. The peaks obtained upon re-chromatography are rather broad; this effect might be due not only to diffusion but also to the polydispersity of the fractions, which was not negligible in this case because of the large volume of the samples used (2.42 ml).

Fig. 4 shows a semi-logarithmic plot of $s_{20,w}$ and $[\eta]$ for different Sepharose 2B fractions of 7.7 S calf thymus DNA *versus* k_{av}. Sedimentation experiments on chromatographic fractions obtained by running the 8.8 S mitochondrial DNA from wild type yeast cells are also shown in Fig. 4. In the *s* plot there is a linear region comprised between k_{av} values of 0.15 and 0.7; the deviation from linearity at lower k_{av} values (also seen in the

[4] J. Filipski, J. P. Thiery, and G. Bernardi, manuscript in preparation.

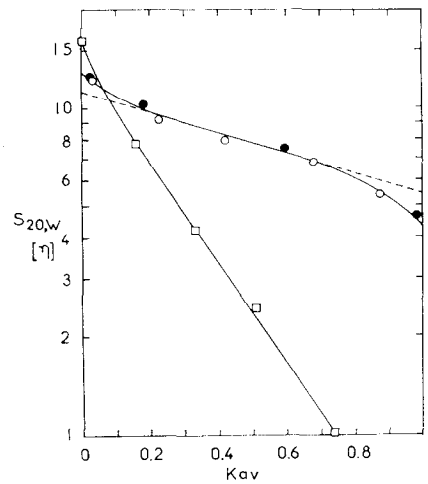

FIG. 4. $s_{20,w}$ (Svedberg units, *circles*) and $[\eta]$ (deciliters per g; *squares*) values as determined for Sepharose 2B chromatographic fractions of 7.7 S calf thymus DNA plotted *versus* k_{av} values. $[\eta]$ values were measured on the fractions from the chromatogram of Fig. 3, $s_{20,w}$ values on fractions obtained from another chromatogram run on the same DNA. ●, $s_{20,w}$ values of fractions obtained from a Sepharose 2B chromatogram of 8.8 S mitochondrial DNA from *Saccharomyces cerevisiae*.

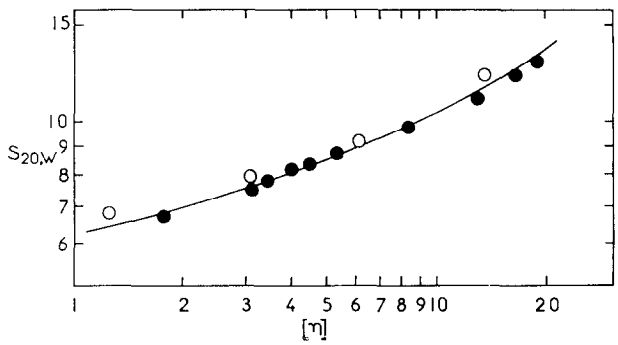

FIG. 5. $s_{20,w}$ (Svedberg units) and $[\eta]$ (deciliters per g) values of calf thymus DNA fractions as obtained from Fig. 4 are plotted against each other on a double logarithmic scale (*circles*). *Solid circles* show values taken from Tables 1 and 2 of Eigner and Doty (23); only the points lying on the curve of Fig. 2 of Eigner and Doty (23) were used. These were corrected according to Rinehart and Hearst (18).

$[\eta]$ plot) is due to the contamination of the early retarded fractions by excluded material. It is important to point out that this effect is only caused by the particular molecular weight distributions of the DNA samples used (Figs. 7 and 8 show that no such effect is seen when excluded material is absent in the DNA sample). The deviation at higher k_{av} values is a well known effect, which can be seen when material of molecular weight below the lower fractionation limit of the gel contaminates the late-eluting fractions (see, for instance, Fig. 6). By extrapolation of the linear region of the *s* plot it can be seen that the limits of fractionation of native DNA on Sepharose 2B are 11.2 S and 5.5 S (corresponding to molecular weights of $0.85 \cdot 10^6$ and $1.1 \cdot 10^5$, respectively; see below).

The relationship between log $s_{20,w}$ and log$[\eta]$, and k_{av} was calculated using the least square method from the points of Fig. 4, between k_{av} values of 0.15 and 0.7.

$$\log s_{20,w} = -0.309\, k_{av} + 1.047 \quad (1)$$

$$\log [\eta] = -1.514\, k_{av} + 1.135 \quad (2)$$

A double-logarithmic plot of $s_{20,w}$ *versus* $[\eta]$ is shown in Fig. 5,

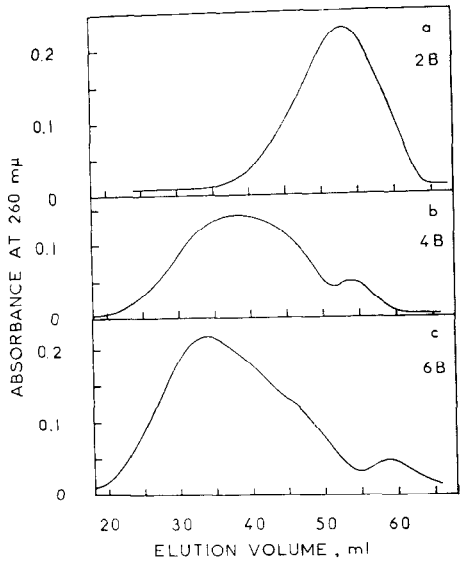

FIG. 6. *a*, chromatogram of 3 A_{260} units of 5.2 S *Escherichia coli* DNA in 1 ml on a Sepharose 2B column (1.2 × 50 cm). Flow rate was 9.8 ml per hour; 3.2-ml fractions were collected. *b*, chromatogram of 3 A_{260} units of 5.2 S *E. coli* DNA in 1 ml on a Sepharose 4B column (1.2 × 54 cm). Flow rate was 6.3 ml per hour; 2.11-ml fractions were collected. *c*, chromatogram of 4.5 A_{260} units of 5.2 S *E. coli* DNA in 3 ml on a Sepharose 6B column (1.2 × 56.6 cm). Flow rate was 10 ml per hour; 0.6-ml fractions were collected.

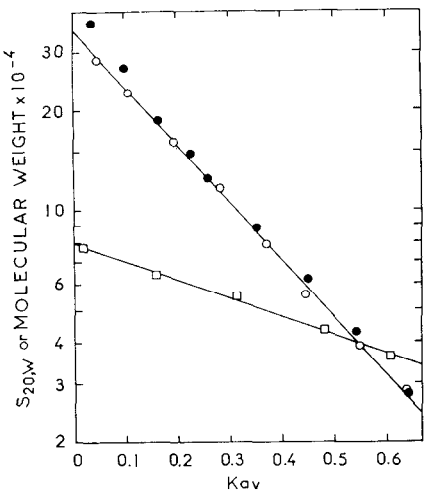

FIG. 7. $s_{20,w}$ values (*squares*) and molecular weights (*circles*), as determined by sedimentation equilibrium, of chromatographic fractions of the 5.2 S *Escherichia coli* DNA sample are plotted *versus* their k_{av} values. *Open symbols* refer to fractions from the experiment of Fig. 6*c*; *solid symbols* to data from another experiment.

which also indicates that this plot is practically coincident with the best-fit line of the data of Eigner and Doty (23).

Fractionation of Native DNA on Sepharose 6B—Fig. 6 shows chromatograms obtained by running the 5.2 S *E. coli* DNA sample on Sepharose 2B, 4B, and 6B columns. In the case of the 6B column, the chromatogram shows a main peak which exhibits a rather evident shoulder on its right side and a minor component which is strongly retarded. Interestingly, the shoulder becomes less apparent in the 4B chromatogram and disappears in the 2B chromatogram. Since the major peak is displaced to higher k_{av} values when the DNA is run on gels of increasing porosity, the minor peak is less well separated from the major one on the 4B compared to the 6B column and is included in the major peak on the 2B column. The minor peak seems to be due to the fact that the lowest molecular weight material present in the degraded DNA has undergone melting. In fact, samples obtained at earlier or later digestion times than that shown in Fig. 6 exhibited a smaller and a larger amount of this component, respectively.

Fig. 7 shows the relationship found between the sedimentation coefficients of the chromatographic fractions from the experiment of Fig. 6*c* and their k_{av} values. The following equation can be calculated from these data.

$$\log s_{20,w} = -0.554\ k_{av} + 0.902 \qquad (3)$$

It is interesting to remark that, in this case, in which no excluded material was present in the chromatogram (in contrast to the case of Fig. 3), the plot remained linear down to a k_{av} value equal to 0. The upper fractionation limit of native DNA on Sepharose 6B columns is 8.0 S (corresponding to a molecular weight of $3.5 \cdot 10^5$; see below); the lower limit as calculated by extrapolation to a $k_{av} = 1$ corresponds to a $s_{20,w}$ value of 2.2 S. This value is well below the lower size limit at which the native DNA structure can be preserved in the experimental conditions used. In fact, the lower stability limit for *E. coli* DNA fragments with no nicks is close to 3.6 S, equivalent to a molecular weight of $2.9 \cdot 10^4$ (see Footnote 5).

Fig. 7 also shows a plot of the sedimentation equilibrium molecular weights found for different fractions obtained from two *E. coli* DNA chromatograms on Sepharose 6B against their k_{av} values. The following equation can be calculated from these data.

$$\log M_w = -1.731\ k_{av} + 5.542 \qquad (4)$$

The relationship between s and M_w can be calculated from the two equations given above:

$$s_{20,w} = 0.1345 \cdot M^{0.320} \qquad (5)$$

and is shown in Fig. 9.

Fractionation of Denatured DNA on Sepharose 6B—Fig. 8 shows a plot of sedimentation coefficients of denatured DNA fractions *versus* k_{av} values. The DNA fractions used in these experiments were obtained from the chromatograms of two different denatured *E. coli* DNA samples (having alkaline $s_{20,w}$ values of 4.0 S and 5.2 S, respectively) and of a denatured yeast mitochondrial DNA sample ($s_{20,w}$ = 6.4 S in alkali). In this case, too, as in that shown in Fig. 4, the linearity of the plot is not preserved above a k_{av} value of 0.7. The reason of this deviation is, in all likelihood, the same as that already commented upon for native DNA.

The upper limit of fractionation of denatured DNA on 6B columns appears to correspond to an alkaline $s_{20,w}$ value of 9.6 S (equivalent to a molecular weight of $3.4 \cdot 10^5$; see below). This upper limit can be increased (*upper line* of Fig. 8) to an alkaline $s_{20,w}$ value of 12.5 S, if the DNA solvent and the column eluent is Solvent B + 5 M NaCl (4:1; v/v), therefore, by using experimental conditions causing a collapse of the highly extended DNA configuration existing in Solvent B (see "Discussion"). The lower limit of fractionation of denatured DNA on 6B columns appears to correspond to molecular weights lower than 10^4.

[5] A. Prunell and G. Bernardi, manuscript in preparation.

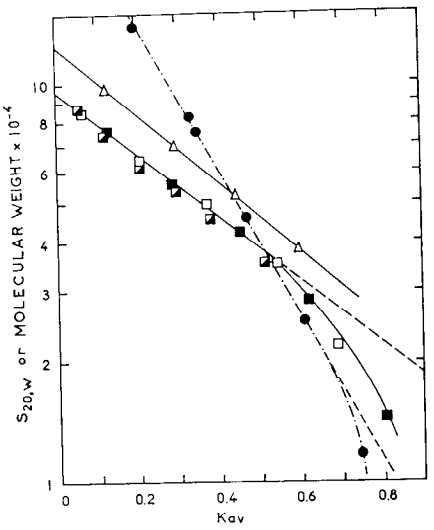

FIG. 8. $s_{20,w}$ values (*squares* and *triangles*), as determined in alkaline solvent, and molecular weights (*circles*), as determined by sedimentation equilibrium, of chromatographic fractions of denatured DNA are plotted *versus* their k_{av} values. *Solid squares* and *circles* and *open squares* refer to *Escherichia coli* DNA fractions obtained from two different experiments, *half-filled squares* to yeast mitochondrial DNA. These chromatographic experiments were performed on Sepharose 6B columns using Solvent B as the solvent. *Triangles* show the alkaline $s_{20,w}$ values as determined for *E. coli* DNA fractions obtained from a Sepharose 6B column using Solvent B + 5 M NaCl (4:1; v/v) as the DNA solvent and the eluent.

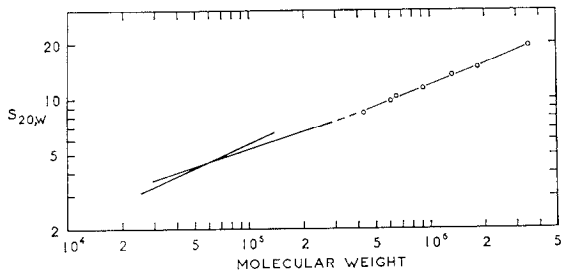

FIG. 9. Double-logarithmic plot of sedimentation coefficients of DNA samples *versus* their molecular weights. The line covering the $2.9 \cdot 10^4$ to $2.9 \cdot 10^5$ range refers to native DNA (Equation 5); *circles* are the data of Richards and Bernardi. The line covering the $2.5 \cdot 10^4$ to $1.4 \cdot 10^5$ range refers to denatured DNA (Equation 8).

Fig. 8 also shows the semi-logarithmic plot of sedimentation equilibrium molecular weight *versus* k_{av}.

Equations corresponding to equations 3 and 4 were calculated for denatured DNA samples.

$$\log s_{20,w} = -0.790\, k_{av} + 0.983 \quad (6)$$

$$\log M_w = -1.820\, k_{av} + 5.514 \quad (7)$$

Only k_{av} values lower than 0.6 were used to calculate these equations. The relationship between s and M_w was calculated from the above equations:

$$s_{20,w} = 0.0388\, M^{0.434} \quad (8)$$

and is shown in Fig. 9.

DISCUSSION

s-M Relationship for Native DNA—The *s-M* relationship established here is the first one determined in this low molecular weight range ($2.9 \cdot 10^4$ to $2.9 \cdot 10^5$), and also the first one in which molecular weights were determined by sedimentation equilibrium and practically monodisperse samples were used. The presence of some nicks in the fragments seems not to affect their chromatographic behavior on agarose columns or their sedimentation coefficients[5]; this latter point is in agreement with the findings of Hays and Zimm (24).

The present *s-M* curve shows a continuity (Fig. 9) with that established by Richards and Bernardi (25, 26) on DNA samples ranging in molecular weight from $4.3 \cdot 10^5$ to $3.5 \cdot 10^6$; these samples were also obtained by acid DNase degradation from calf thymus DNA.[6]

In contrast, slight discontinuities, which cannot be only explained by the different $\bar{v}$ values used, are found with the relationships proposed by other authors (17, 23). Their significance is not clear, however, because of uncertainties in the *dn/dc* value used or for other reasons (see criticisms presented in References 19, 29, 30), or both. Interestingly, however, the slopes of the corresponding curves, which are less sensitive than their intercepts to a number of variables, are in reasonable agreement with that of Richards and Bernardi.

Finally, it should be mentioned that the a constant in the equation $s = kM^a$ found here is lower than in the immediately higher molecular weight range. This effect, which appeared surprisingly small to us, is expected from the Hearst and Stockmayer (31) and Crothers and Zimm (32) analysis of the *s-M* relationship of wormlike chains (33).

Fractionation of Denatured DNA—Our choice of denaturing DNA in the presence of 1% formaldehyde and of using formaldehyde-containing Solvent A (Solvent B) as the eluent is due to three reasons. (*a*) The presence of formaldehyde during DNA heating was required in order to avoid renaturation of fast-renaturing DNA fractions during cooling down; renaturation would obviously prevent formaldehyde from reacting with these fragments if reaction was carried out after cooling down. (*b*) Experimental conditions involving either chromatography at high temperature or the use of formamide, alkali, or concentrated urea solutions are impractical or impossible with agarose columns. (*c*) Under the conditions chosen, the secondary structure and the hydrodynamic volume of denatured DNA fragments are independent of their base composition (see Fig. 8; the same applies to native DNA fragments, Fig. 4). It should be stressed, however, that so far we have not examined in detail other possible solvents for the fractionation of denatured DNA.

The configuration of single-stranded fragments in our experimental conditions is very highly extended as shown by the fact that their $s_{20,w}$ values, as measured in Solvent B are even lower than those obtained in alkaline M NaCl and reach the latter only when the ionic strength of the solvent is raised to 1. Now it is well known (17, 34) that sedimentation coefficients of DNA in alkaline M NaCl already correspond to greatly extended configurations. A consequence of the remarkably stiff configuration of formaldehyde-reacted denatured DNA is that its fractionation range on Sepharose columns is very close to that of native DNA as it can be seen by comparing Fig. 8 with Fig. 7, a fact

[6] In this case, molecular weights were determined by light scattering, well within the validity range of this method (27), using the experimentally determined dn/dc = 0.174 ml per g at 546 nm (28); since s values had been determined in 1 M NaCl and calculated using the $\bar{v}$ value of Tennent and Vilbrandt (20), the original relationship $s_{20,w} = 0.057\ M^{0.382}$ was corrected to $s_{20,w} = 0.0594\ M^{0.382}$.

which indicates that the hydrodynamic volume of the denatured DNA fragments, in our experimental conditions, is even slightly higher than that of native DNA fragments having half their length. Besides, the slopes of the M_w *versus* k_{av} plots for native and denatured DNA in Solvent B (Equations 4 and 7) are the same within 5%, indicating that the hydrodynamic volumes show practically the same dependence upon molecular weight in both cases. An obvious way to increase the upper limit of fractionation of denatured DNA is to use solvents of high ionic strength in order to cause a relative folding of the molecules. As shown in Fig. 8, increasing the ionic strength of Solvent B to about 1 almost doubles the molecular weight corresponding to the upper limit of fractionation on Sepharose 6B. Similar effects of ionic strength on the chromatographic behavior of RNA on agarose columns were observed by Öberg and Philipson (35).

A comparison of the *s-M* relationship established here for denatured DNA fragments of $2.5 \cdot 10^4$ to $1.4 \cdot 10^5$ daltons (Fig. 9), $s_{20,w} = 0.0388\ M^{0.434}$ and that determined by Studier (17) in the 1.7 to $67 \cdot 10^6$ daltons range, $s_{20,w} = 0.0528\ M^{0.400}$ shows a significant difference. In view of the many uncertainties existing in the available data ($\bar{v}$ of denatured DNA in alkali; $\bar{v}$ of formaldehyde-reacted denatured DNA; molecular weight estimates used by Studier) it is not very useful to comment on this difference. Obviously, this uncertainty in the *s-M* relationship does not detract from the validity of agarose chromatography as a method for studying the molecular weight distribution of denatured DNA fragments.

DNA Molecular Weight by Sedimentation Equilibrium—The possibility of using the sedimentation equilibrium method for the molecular weight determination of DNA fractions is due to the extremely narrow size distribution of the fractions, a point already commented upon earlier. It should be pointed out that the short column technique of Van Holde and Baldwin (8) was already successfully used by Scheffler *et al.* (36, 37) and by Pohl and Jovin (38) for determining the molecular weight of fractionated dAT oligomers and of poly(dG-dC), respectively.

While this work was in progress, a report concerning the fractionation of poly(dAT:dAT) on agarose columns (Bio Gel A-5m, BioRad Laboratories, Richmond, Calif.) appeared (39). In this case, sedimentation equilibrium experiments were also performed using the classical technique of Van Holde and Baldwin (8), but molecular weights could not be calculated, essentially because of the polydispersity of the fractions. According to our experience, the polydispersity found by the authors is due to the very large loads (100 A_{260} units) used on columns (1 × 100 cm) and the rather large volumes (1 ml) of the fractions. As mentioned under "Materials and Methods," we could not confirm the conclusion of Jang and Bartl (39) that the efficiency of the gel separation decreases with increasing polymer size.

From a practical point of view, two additional comments should be made on the sedimentation equilibrium method. The first one is that higher molecular weights than those in the range investigated here can be determined. Keeping the solution depth in the cell equal to that used here (about 0.28 cm), 48, 72, and 96 hours would be required to reach equilibrium, with an $\epsilon = 10^{-2}$, for DNA fractions having molecular weights of $6 \cdot 10^5$, $1 \cdot 10^6$, and $1.6 \cdot 10^6$, respectively. Even in the latter case, angular velocities do not need to be lower than 4000 r.p.m. in order to keep the effective reduced molecular weight equal to the value of 5 cm^{-2} recommended by Yphantis (9). The second point is that the already very low amount of DNA (0.5 μg) required for an experiment, could be decreased by a factor of about 10 with improved scanners already in use in some laboratories.

In conclusion, chromatography of DNA on agarose columns, as developed in the present work, appears to be a new technique for dealing with DNA fragments in the "gene-size" range. The main interest of this technique is that it practically closes the previously existing gap between the macromolecular range, usually studied by physical techniques, and the oligonucleotide range, normally investigated by enzymological and chemical methods. Several applications of the fractionation of DNA fragments on agarose columns will be published elsewhere in due time.

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