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CHROMATOGRAPHIC DETERMINATION OF THE MOLECULAR WEIGHT OF DNA

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SUMMARY

Further evidence is presented that the chromatographic behaviour of DNA on DEAE-cellulose depends only upon macromolecular heterogeneity and the integrity of the double helical structure. This evidence was obtained by correlating the sedimentation coefficients and the chromatographic profiles of the samples from sucrose gradients.

Molecular weights of the eluted fractions were calculated by solving the system $\bar{M}_w = \Sigma \bar{M}_i F_i$, where the $\bar{M}_w$ values are determined by sedimentation and/or viscosimetry and F_i are the weight percentages of the chromatographic fractions.

With this calibration, the weight-average molecular weight of a DNA sample can be determined solely from the chromatographic data in the range $1 \cdot 10^6$–$25 \cdot 10^6$ daltons.

INTRODUCTION

A continuous molecular-weight distribution is characteristic of DNA preparations obtained by standard methods. This macromolecular heterogeneity can be neglected for most work in molecular biology, but when enzymatic, chemical or physical mechanisms of DNA degradation are being investigated this heterogeneity becomes an important parameter.

The evolution of the molecular-size distribution throughout the degradative process is particularly simple when an initially monodisperse preparation is employed and the observation is limited to the very early stages of the process. This special situation applies only when phage DNA, obtained by very careful methods, is used[1–3]. As soon as degradation has begun to any extent, or at any time if we used DNA from an origin other than phages, it is a great problem to establish the sample polydispersity.

The ratio between two average molecular weights depends upon the molecular-weight distribution. Consequently, some information on the polydispersity could be obtained even by simply taking average molecular weights, whenever it is possible to

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determine more than one (for instance the number-average and the weight-average molecular weights).

Unfortunately, this dependence is not defined and so its utility is very limited. The exponential character of the molecular-weight distribution of a DNA sample subjected to random cleavage of the internucleotide bonds has been established in this way[4].

On the other hand, it is possible to establish the actual distribution of molecular weights in a polymer preparation by determining the distribution of sedimentation coefficients[5–8]. Apart from fractionation and, in some instances, electron microscopy, this is the only means of determining polydispersity directly. This method has also been applied to the study of the radiolytic degradation of DNA[9].

We have already found[10] that the molecular-weight heterogeneity of DNA preparations can be estimated by chromatography on DEAE-cellulose. We employed this technique to follow enzymatic and other DNA degradations[11] and, more recently, in the study of degradations by single-break production mechanisms[12].

In this paper we present more direct proof of our previous assertion that the chromatographic behaviour of DNA on DEAE-cellulose depends on the molecular weight, and the calibration of this chromatographic technique by comparing it with conventional hydrodynamic methods for molecular-weight determination is described.

EXPERIMENTAL

Chemicals

All chemicals employed were of reagent grade. Whatman DE-81 DEAE-cellulose, treated as previously described[10], was used for chromatography. Labelled [Me-^{3}H]thymine was obtained from the Radiochemistry Section of the Junta de Energía Nuclear, Madrid, Spain.

DNA preparations

Calf thymus DNA preparations were obtained by the procedure of Kay *et al.*[13]. Bacterial DNA from *Escherichia coli* B, *E. coli* TAU-bar and *Pseudomonas savastanoi* were prepared by the Marmur method[14]. The thymine auxotroph *E. coli* TAU-bar was used in order to obtain [^{3}H]DNA.

In all the DNA preparations, the protein content, as determined by the method of Lowry *et al.*[15] was $<2\%$, and the RNA content, assayed by the Schmidt–Tanhausser procedure (see ref. 16), was less than 2%.

Centrifugal DEAE-cellulose chromatography

The chromatographic analysis of the DNA samples was carried out by the technique already described[10]. Minor modifications were introduced, such as a special centrifuge tube with an upper compartment for the DEAE-cellulose paper pulp and the eluent, and a lower compartment for collection of the eluate.

DNA samples must be fixed to the cellulose in solvents at pH 7 with a cation concentration equal to or lower than that of the second eluent. The sample and the eluents are placed in the upper part of the tube and their passage through the DEAE-cellulose is accelerated by centrifugation at 100 *g* in a bench top centrifuge. Each eluate is collected from the lower compartment before loading with the next eluent.

The compositions of the eight eluents used and their order are shown in Table I. This series was selected from the twelve eluents listed by Ledoux[17].

The fractions eluted were analyzed spectrophotometrically in a Unicam SP700 instrument or by liquid scintillation counting in a Nuclear Chicago 725 spectrometer.

TABLE I

SERIES OF ELUENTS USED

No. in series	*Composition*
1	10^{-2} *M* phosphate buffer, pH 7.0
2	10^{-2} *M* phosphate buffer, pH 7.0 + 0.14 *M* NaCl
3	10^{-2} *M* phosphate buffer, pH 7.0 + 0.5 *M* NaCl
4	10^{-2} *M* phosphate buffer, pH 7.0 + 1.0 *M* NaCl
5	0.2 *M* NH_4OH, pH 10.9 + 2.0 *M* NaCl
6	0.4 *M* NH_4OH, pH 11.1 + 2.0 *M* NaCl
7	1.0 *M* NH_4OH, pH 11.4 + 2.0 *M* NaCl
8	1.0 *M* NaOH

Viscosimetry

Intrinsic viscosities were determined in a low-shear viscosimeter (Beckman), similar to the model A described by Zimm and Crothers[18]. The concentration was usually lower than 20 μg/ml and its independence of shear rate was verified.

Concentration effects were corrected by the expression

$$\eta_{sp}/C = [\eta] + k'[\eta]^2 C$$

with a value of k' of 0.5 as proposed by Eigner *et al.*[19]. Molecular weights were calculated using the equation proposed by Crothers and Zimm[20]:

$$0.665 \log M_w = 2.863 + \log ([\eta] - 5)$$

Sedimentation in sucrose gradients

A Spinco density gradient former and a Spinco L2 centrifuge with a SW39 swinging bucket rotor were employed. The gradients ranged from 5 to 25% (w/v) sucrose in SSC (0.15 *M* sodium chloride + 0.015 *M* trisodium citrate) and the samples, containing about 10 μg of [^{3}H]DNA in 0.1 ml, were placed in the top of the pre-formed gradient.

In the case of alkaline gradients, the solvent for the sucrose solution and also for the DNA solution was SSCOH (0.15 *M* sodium hydroxide + 0.015 *M* trisodium citrate).

Centrifugation was carried out at 63,000 *g* at 25° for 14–17 h, depending on the sample. Sedimentation coefficients were estimated from the experimental data using the tables and equations of McEwen[21].

Analytical ultracentrifugation

Sedimentation rate experiments were carried out with a Beckman Model E

analytical ultracentrifuge by using the UV optics and the photoelectric scanner. Charcoal-filled Epon (Shell Chem. Co.) centre-pieces of 12 mm were used in an AnF rotor and results were recorded every 16 min. The solvent was SSC in all instances.

The measured sedimentation coefficients were corrected to standard conditions and extrapolated to zero concentration. Molecular-weight calculations were made using the equation proposed by Crothers and Zimm[20]:

$$0.445 \log M_w = 1.819 + \log (s^0_{20w} - 2.7)$$

DNA degradations

DNA preparations were degraded enzymatically with bovine pancreas deoxyribonuclease in 0.05 *M* Tris–hydrochloric acid, pH 7.5 + 0.005 *M* magnesium chloride. Several incubation times and enzyme/substrate ratios were used, according to the particular degradation desired.

Solutions of DNA in SSC were irradiated using a cobalt-60 source at different concentrations, dose rates and times. The absorbed doses were determined by dosimetry according to Fricke and Hart[22].

Mechanical degradations were carried out with a Virtis 45 homogenizer at several speeds and times.

RESULTS AND DISCUSSION

Further evidence that the fractionation is a function of molecular-weight distribution

Some years ago, we showed[10,11] that the chromatographic behaviour of DNA on DEAE-cellulose is a function of molecular-weight heterogeneity. The evidence was based on the fact that all of the degradation agents tested produced a qualitatively equivalent evolution from the higher chromatographic fractions (F-8, F-7, F-6) to the lower fractions (F-5, F-4, F-3). We have now obtained more direct proof by chromatography of DNA obtained from various zones of several sucrose gradients.

Preparations of DNA with different degrees of heterogeneity, produced during isolation or by a degradative treatment, were sedimented on sucrose gradients as described under Experimental. In order to determine the position of the DNA peak, only one out of ten drops was counted. Once the peak position had been established, we selected four groups (20 drops per group), each one being equivalent to a zone of the peak, and the sedimentation coefficient of DNA from every group was calculated. These groups were independently chromatographed on DEAE-cellulose.

Table II shows the results of these experiments. The results prove that high sedimentation coefficients are correlated with high percentages of the chromatographic fractions eluted by solvents of high ionic strength or pH (F-8, F-7). The opposite is true for samples from zones with low sedimentation coefficients.

We emphasize that the DNA found in any of these zones is chromatographically heterogeneous. This result is not due to artifacts or poor resolution in the chromatographic procedure. In fact, the polydispersity already exists in the zones obtained from the sucrose gradient. Fig. 1 shows the result of re-sedimentation in a second gradient of the DNA from the light, medium and heavy zones of the peak obtained in a first gradient sedimentation. It is evident that in each zone there is a significant proportion

TABLE II

s_{20w} OF DNA SAMPLES AND PERCENTAGE OPTICAL DENSITY OF THEIR CHROMATOGRAPHIC FRACTIONS

s_{20w}	*F-3*	*F-4*	*F-5*	*F-6*	*F-7 + F-8*
2.6	60.9	15.7	11.1	2.4	5.1
4.7	62.7	17.3	14.1	2.7	2.3
5.4	42.5	47.2	6.4	2.2	0.9
7.6	32.3	41.7	17.4	4.7	3.4
7.9	34.4	46.4	12.5	5.2	1.0
9.1	36.9	34.1	18.0	7.8	1.8
10.8	26.9	29.4	18.5	19.0	5.8
11.4	30.8	30.1	18.1	16.5	3.7
13.8	17.1	31.3	21.5	24.7	5.4
14.7	15.7	22.4	21.5	32.3	8.1
16.9	19.5	27.4	20.4	27.5	5.1
17.5	12.7	24.1	25.6	30.8	6.7
19.4	10.6	15.8	24.3	38.5	10.8
20.0	13.5	18.3	18.7	42.0	7.0
22.3	9.1	18.1	24.7	37.1	10.9
24.0	11.5	18.0	24.4	35.4	9.9
29.9	10.5	13.9	23.1	39.7	13.1
36.0	7.5	13.7	22.5	36.2	19.9

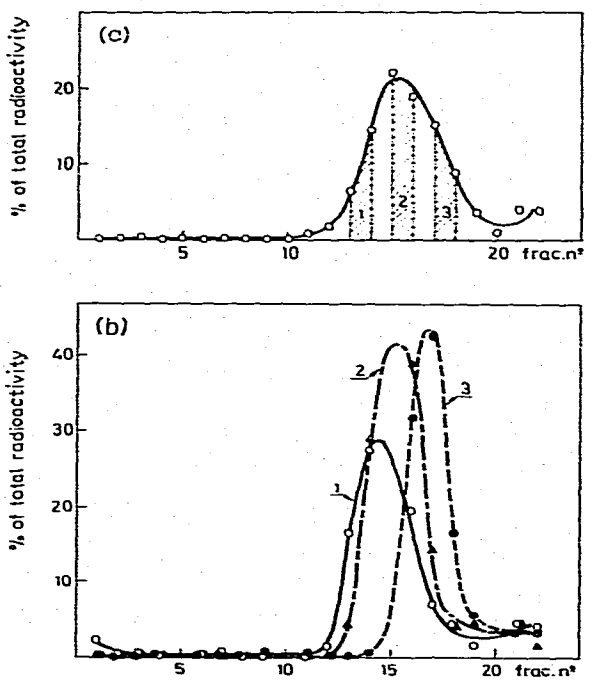

Fig. 1. Re-sedimentation (b) in 10–30% (w/v) sucrose gradient in 0.15 *M* sodium chloride + 0.015 *M* sodium citrate of the three (1, 2 and 3) zones obtained in a previous sucrose gradient sedimentation (a) of analogous characteristics. SW-65K rotor, 23,000 rpm, 16 h, 5°. [^{3}H]DNA from *E. coli* TAU-bar.

of molecules that should be in other zones, as demonstrated by the overlapping of the peaks in the second gradient.

Insensitivity to the single breaks

Fig. 2 shows the chromatographic profiles of two degraded DNAs, one degraded by radiolysis in solution (20 krad) and the other by mechanical shear. They

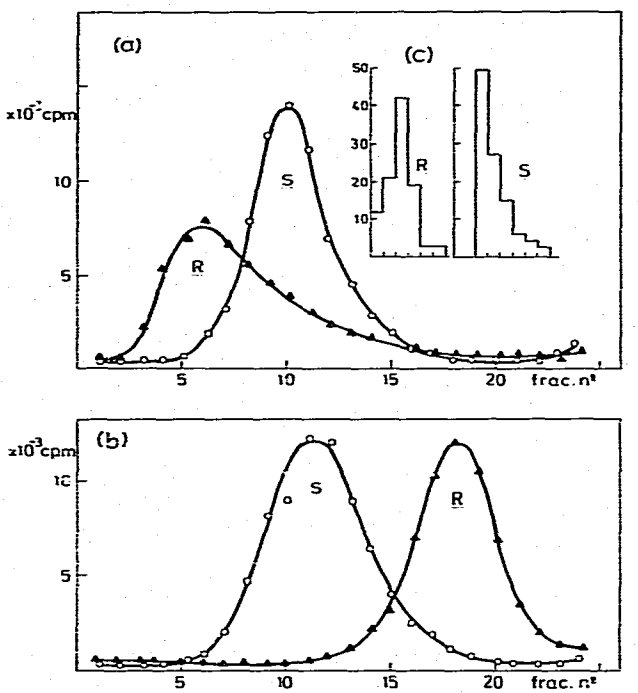

Fig. 2. (a) Sedimentation in neutral 5–25% (w/v) sucrose gradient in 0.15 *M* sodium chloride – 0.015 *M* sodium citrate of [^{3}H]DNA from *E. coli* TAU-bar degraded by: (R) gamma-radiation from a cobalt-60 source, 20 krad; (S) mechanical shear in high-speed homogenizer, SW-65K rotor, 25,000 rpm, 16 h, 20°. (b) Sedimentation in alkaline 5–25% (w/v) sucrose gradient in 0.15 *M* sodium hydroxide – 0.015 *M* sodium citrate of the same (R) and (S) [^{3}H]DNA samples. Same sedimentation conditions as before. (c) Chromatographic profiles of the (R) and (S) [^{3}H]DNA samples obtained by the centrifuged DEAE-cellulose technique[11,12].

indicate that the radiolytically degraded sample has a higher molecular weight, as suggested by the predominance of fractions of higher index than in the sheared sample. This is in agreement with the results of the sedimentation in a neutral sucrose gradient, also shown in Fig. 2.

However, in spite of its higher molecular weight, the radiolytically degraded sample presents a higher density of single breaks, as revealed by the inversion of the peak positions in the alkaline sucrose gradient. Consequently, we can say that the chromatographic behaviour, which is in accordance with the neutral gradient, is not affected by local discontinuities if the macromolecular structure is preserved.

Estimation of average molecular weight of chromatographic fractions

Once the qualitative correlation between hydrodynamic and chromatographic parameters has been verified, we have postulated that the average molecular weight of a DNA sample can be expressed by the equation

$$\bar{M}_w = \Sigma \bar{M}_i F_i$$

where $\bar{M}_w$ is the weight-average molecular weight of the sample, $\bar{M}_i$ is the weight-average molecular weight of each chromatographic fraction and F_i is the weight percentage that each fraction represents in the sample. Of course, the condition $\bar{M}_1 < \bar{M}_2 < \ldots < \bar{M}_8$ must be fulfilled according to the results previously presented.

One set of 76 determinations, made on 24 different preparations, has enabled us to fit this expression to give the best set of M_i values. The data were fitted by the least-squares method to a linear regression between $\bar{M}_{w(\text{chromatographic})}$ and $\bar{M}_{w(\text{hydrodynamic})}$. The best set of $\bar{M}_i$ values was searched for by trial in order that the slope should approach unity and also to minimize the ordinate at the origin. The approximation intervals in the $\bar{M}_i$ values were $0.5 \cdot 10^6$ daltons.

The best set of $\bar{M}_i$ values obtained is shown in Table III.

TABLE III

BEST SET OF AVERAGE MOLECULAR WEIGHTS ($\bar{M}_i$ VALUES)

Chromatographic fraction	*Average molecular weight ($\times 10^6$ daltons)*
F-3	1 (we use 0.5)
F-4	1 (we use 0.5)
F-5	2
F-6	6
F-7	17
F-8	50

Fig. 3 shows the regression line found between the $\bar{M}_w$ determined by hydrodynamic methods and the $\bar{M}_w$ estimated by our chromatographic procedure, applying the set of $\bar{M}_i$ values in Table III. It can be seen that dispersion of the chromatographic determinations is of the same order of magnitude as the standard confidence limits of the linear regression.

Another means of indicating the goodness of fit is to consider the standard deviation of the relation $\bar{M}_{w(\text{chromatographic})}/\bar{M}_{w(\text{hydrodynamic})}$, which was found experimentally to be 0.968 ± 0.081.

DISCUSSION

The results obtained confirm our previous conclusions that the chromatographic behaviour of DNA fixed in DEAE-cellulose depends upon the macromolecular heterogeneity and integrity of the double helical structure. In particular, the experiments on the chromatography of zones from sucrose gradients and on the resedimentation of these zones showed that the heterogeneity is not eliminated by sedimentation and so should be detected by chromatography.

The linear regression obtained for chromatographic and hydrodynamic molec-

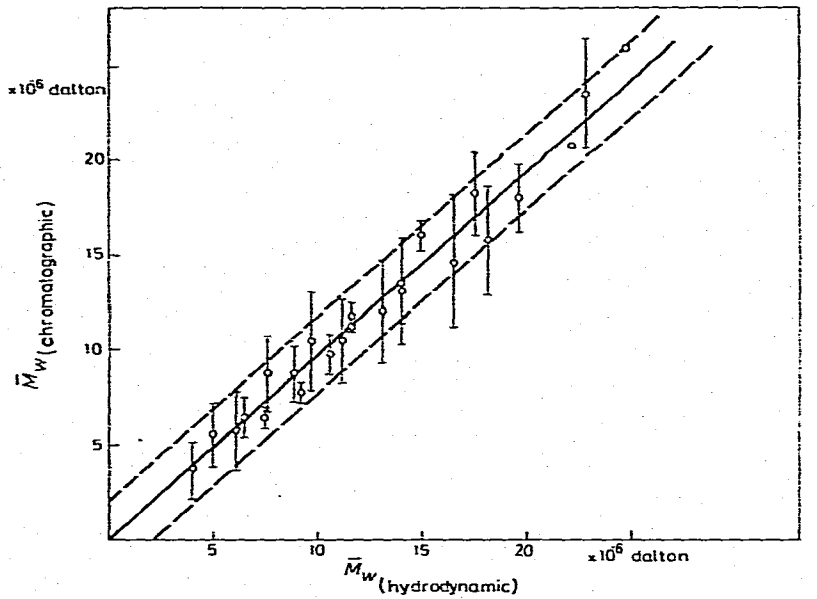

Fig. 3. Linear regression line of chromatographically and hydrodynamically estimated molecular weights of DNA samples from different origins. Dashed lines: standard confidence limits of the linear regression. $\bar{M}_{w\ (chromatographic)} = 0.9600\ \bar{M}_{w\ (hydrodynamic)} + 0.070$. Correlation, 0.9301.

ular-weight estimations also provides indirect proof of the dependence of the chromatographic elution of DNA on macromolecular size. The calibration presented here is applicable to the most useful $\bar{M}_w$ range of DNA preparations ($1 \cdot 10^6$–$25 \cdot 10^6$ daltons). This chromatography is one of the most simple, reliable and economic procedures for carrying out simultaneous molecular-weight estimations on several DNA samples.

It must be kept in mind, however, that the series of eluents employed does not provide sensitivity outside the limits of the calibration range: $0.5 \cdot 10^6$ daltons is the lower limit and $25 \cdot 10^6$ daltons is the upper limit. This corresponds to (F-3 – F-4) $\geq$ 75% and F-8 $\geq$ 50%.

This limitation is derived from the eluents employed in the calibration and not from the chromatography itself. It would be sufficient to develop a new series and to calibrate it again. Nevertheless, it is probable that an expanded series of eluents would be cumbersome in practice and would require a larger amount of DNA.

Finally, it must be pointed out that among the wide variety of DNA preparations from many different origins (phage, bacteria, mammals) studied during recent years in our laboratory[13] we have found anomalous behaviour only in the case of the T2 DNA. This phage DNA does not fulfil the correlation established here. We suspect that the reason for this anomaly lies in the glucosylated nature of this T2 DNA and not in its very high molecular weight.

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