the denatured material is rather susceptible to degradation on rapid changes from the dissolved to the solid state.

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DEOXYPENTOSE NUCLEIC ACIDS

XV. THE SEDIMENTATION OF CALF THYMUS DEOXYRIBONUCLEIC ACID IN 95% ETHANOL

J. H. COATES AND D. O. JORDAN

Department of Physical and Inorganic Chemistry, The Johnson Laboratories, University of Adelaide, South Australia (Australia)

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SUMMARY

The sedimentation properties of calf thymus DNA dissolved in ethanol have been studied and sedimentation coefficient distributions have been obtained. Evidence is presented for the collapsed state of the DNA molecule in ethanolic solution compared with aqueous solutions.

Abbreviation: DNA, deoxyribonucleic acid.

INTRODUCTION

The sedimentation coefficient of calf thymus DNA when measured in dilute sodium chloride solution depends only to a limited extent on the molecular weight of the sample[1,2]; this is exemplified by the observation of DOTY, MCGILL AND RICE[3] that for a series of samples of DNA of different molecular weights, the sedimentation coefficient ($S°$) for solutions in 0.2 *M* NaCl is proportional to $M^{0.37}$, where M is the molecular weight of the sample. Consequently such sedimentation experiments are of limited use for characterising the heterogeneity of DNA preparations. In principle sedimentation experiments would be more sensitive to molecular weight heterogeneity if they could be carried out under conditions such that the sedimentation coefficient depended more directly on the molecular weight. In this communication experiments will be described in which DNA was sedimented when dissolved in 95 % ethanol. For a variety of reasons (see below) it is believed that under these conditions the sedimentation coefficient depends to a much greater extent on the molecular weight of the DNA.

Consequently it is possible, by means of an analysis of the boundary spreading during sedimentation, to obtain estimates of the heterogeneity of DNA preparations which are more directly related to the molecular weight heterogeneity of the preparation, than are the results obtained on aqueous solutions.

The insensitivity of the sedimentation coefficient of DNA to the molecular weight of the sample when it is dissolved in 0.2 *M* NaCl is a consequence of the extended free-draining nature of the molecule. Theoretically the sedimentation coefficient of a free-draining random coil should be independent of the molecular weight[4]. The limited molecular weight dependence of the sedimentation coefficient of the DNA molecule in 0.2 *M* NaCl must therefore indicate that DNA is not behaving as a completely free-draining random coil under these conditions. The configuration of the DNA molecule in solution depends on the following factors: (a) the inter-nucleotide hydrogen bonds which confer considerable stiffness on the molecule, (b) the repulsions between the negative charges situated on the ionised phosphate groups of each nucleotide, and (c) the solvation of the molecule. In a "good" solvent a flexible molecule is extended allowing many solute–solvent interactions, and the free energy of the molecule is consequently reduced.

When dissolved in 0.2 *M* NaCl the above factors collectively lead to an extended free-draining structure for the DNA molecule. In order to carry out sedimentation measurements in a manner such that the sedimentation coefficient depends more directly on the molecular weight, this extended molecule must be collapsed giving rise to a non or partial free-draining molecule. GEIDUSCHEK AND GRAY[5] showed that considerable contraction occurred in the DNA molecule if it was dissolved in 95 % ethanol in the absence of traces of sodium chloride. Using light scattering measurements they observed that the radius of gyration of the DNA molecule and its molecular weight were 980 Å and $6.6 \cdot 10^6$ respectively when dissolved in 95 % ethanol, compared with 3000 Å and $7.7 \cdot 10^6$ when dissolved in 0.2 *M* NaCl. This contraction probably comes about by modification of the second and third factors mentioned above. There is no reason to suspect *a priori* that dissolution in ethanol will lead to the scission of internucleotide hydrogen bonds. However, in view of the conclusions of THOMAS[6], the prolonged dialysis of DNA against distilled water could

conceivably lead to hydrogen bond scission, since he found that DNA was denatured in solutions of low ionic strength. Such denaturation is not likely to have occurred in the experiments described here because the dialyses were carried out at a concentration of DNA of 0.1 %, and INMAN AND JORDAN[7] have found that denaturation does not occur if the DNA is kept at a concentration above 0.007 % even in the total absence of added electrolyte. The results of GEIDUSCHEK AND GRAY support our contention on this point since they found that there was no increase in the u.v. absorption of ethanolic solutions of DNA compared with saline solutions such as would have occurred had the DNA been denatured.

Conductivity measurements show that the charge on the DNA molecule is much reduced when dissolved in 95 % ethanol as compared with water. This indicates that the effective number of charged phosphate groups is reduced. The equivalent conductivity of DNA in 95 % ethanol is of the order of one percent of the equivalent conductivity of DNA in water. Presumably this is a consequence of the lower dielectric constant of ethanol (24 as opposed to 82 for water) leading to greater ion pair formation between sodium ions and ionised phosphate groups. It is also reasonable to suppose that the solvation of the DNA molecule will be less in ethanol than in water because of the lower hydrogen bonding capacity of ethanol. Furthermore, if the hydration of DNA is closely connected with an ice-like arrangement of water molecules on its surface as suggested by JACOBSEN[8], it is unlikely that ethanol will solvate to the same extent as water.

EXPERIMENTAL

The calf thymus DNA was prepared by the method of KAY, SIMMONS AND DOUNCE[9] and its properties have been described previously[10].

Since the 95 % ethanol DNA solutions contained no involatile substances other than DNA, DNA concentrations were determined by weighing the amount of solid material in a weighed amount of solution. A weighed quantity of ethanolic DNA solution was placed in a desiccator over KOH pellets at atmospheric pressure. The DNA residue reached constant weight in about three weeks; this procedure was found to give reproducible results.

A pure azeotropic 95 % ethanol solution was prepared by refluxing redistilled spirits of wine over caustic potash for several hours, and distilling the product through a fractionating column. The middle third only of the distillate was subsequently used. Spirits of wine was used as a starting material because of the difficulties of removing traces of benzene from commercial absolute alcohol.

The dissolution of DNA in ethanol was carried out by the method of GEIDUSCHEK AND GRAY[5] at 0–4°. A sample of DNA dissolved in distilled water was freed from salt by dialysis against distilled water. The salt free aqueous DNA solution was then dialysed against a 25 % ethanol/water solution which was replaced with increasingly concentrated ethanol solutions up to 95 % azeotropic ethanol. The solution was finally dialysed twice against 95 % ethanol. All dialyses were carried out at 0–4° dynamically, by slowly oscillating about its vertical axis a sealed jar containing the dialysis bag and the outside solution.

Sedimentation experiments were carried out using a Spinco model E ultracentrifuge equipped with schlieren optical system. The schlieren pattern was produced

by means of an inclined wire of bright 33 gauge Nichrome. Runs were carried out at 39,460 rev./min using a standard A analytical rotor and standard 4° sector 12 mm cells. Solution temperatures were determined by measuring the rotor temperature with a thermocouple before and after the run and taking a mean value. No correction was applied for the adiabatic cooling of the rotor. The photographic plates were measured in a Pye two-dimensional comparator reading to 0.01 mm. Sedimentation coefficients were corrected to a medium of the same viscosity as water at 20°; however, no correction was applied for the difference in density between water at 20° and 95 % ethanol. Graphs of refractive index gradient in the cell for boundary analysis, were obtained by tracing an enlarged projected image of the photographic plate onto graph paper. Areas were determined by planimetry of the tracings.

The partial specific volume of DNA in ethanol was determined by pycnometry. A graph of weight of solution against weight concentration of DNA was plotted, from which the partial specific volume term $(1 - \bar{V}\rho)$ was determined[11]. A value of 0.38_1 ml/g was obtained for the partial specific volume, $\bar{V}$.

RESULTS AND DISCUSSION

A typical ultracentrifuge photograph of the sedimentation of DNA in alcohol is shown in Fig. 1. It can be seen that a single rapidly sedimenting peak is obtained, which spreads very rapidly with time. In earlier experiments using shorter dialysis times it was often found that two peaks were observed in the ultracentrifuge (Fig. 2).

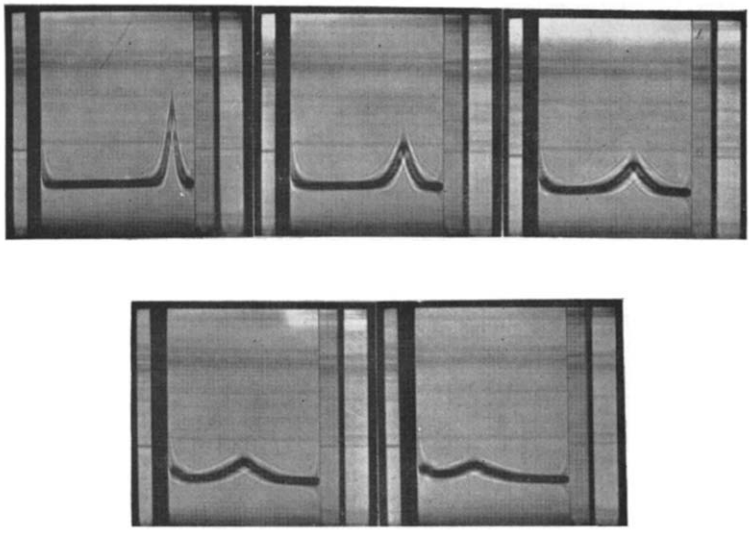

Fig. 1. Sedimentation of DNA in ethanol showing one peak (direction of sedimentation from right to left).

When the time of dialysis against distilled water was increased the smaller peak disappeared and symmetrical peaks of which Fig. 1 is typical were always obtained. The second smaller peak probably represents some form of DNA aggregate which arose because of a residual trace of sodium chloride in the solution. This conclusion is supported by the observation that the addition of 0.1 ml of ethanol saturated with

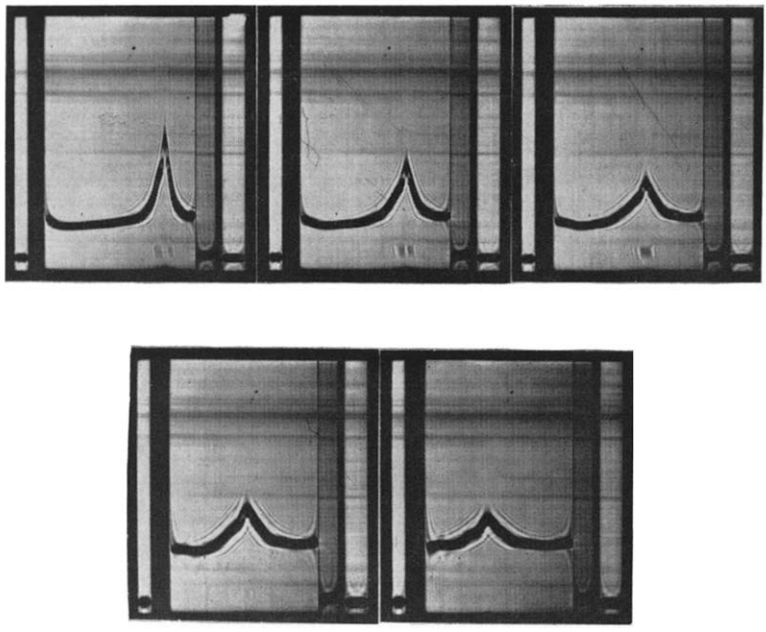

Fig. 2. Sedimentation of DNA in ethanol showing two peaks (direction of sedimentation from right to left).

sodium chloride to 1 ml of an ethanolic DNA solution causes immediate gelation of the solution.

Sedimentation experiments were carried out over a range of concentrations with both the single component and the two component ethanolic DNA solutions. The mean sedimentation coefficients for the various components were computed by measuring the rate of movement of the maxima of the refractive index gradient photographs. The results obtained are shown in Table I and Fig. 3.

TABLE I

SEDIMENTATION BEHAVIOUR OF DNA IN 95% ETHANOL WHEN TWO COMPONENTS ARE PRESENT

Total DNA concentration %	S_{20} *large peak* *S units*	S_{20} *small peak* *S units*
0.86	51.9	71.6
0.86	53.7	66.5
0.41	87.0	63.1
0.18	89.0	Only one peak detected
0.09	111.9	

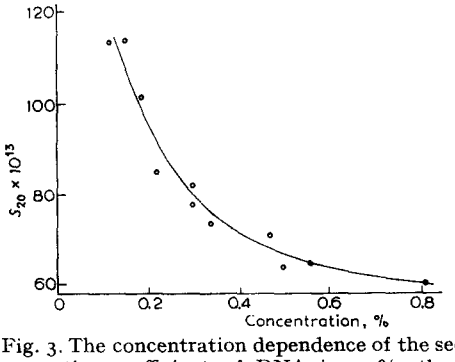

Fig. 3. The concentration dependence of the sedimentation coefficient of DNA in 95% ethanol when only one component is present.

From these data it can be seen that the larger of the two sedimentation peaks probably corresponds to the single peak observed when more prolonged dialysis was used to remove traces of sodium chloride. Restricting attention to the data for the

single component solutions, (Fig. 3), it can be seen that there is very marked concentration dependence of the sedimentation coefficients and the individual values are very much greater than those observed for DNA dissolved in 0.2 *M* NaCl[11].

It would be of interest to obtain a value for the sedimentation coefficient at infinite dilution. Extrapolation of the graph in Fig. 3 is clearly not possible with any precision. The theories of FESSLER AND OGSTON[12] and of BURGERS[13] for the concentration dependence of sedimentation coefficient predict a linear relation between $1/S$ and concentration. Accordingly the data of Fig. 3 have been plotted in this manner in Fig. 4. It is apparent that the linear relationship does not hold at high concentrations; but at low concentrations extrapolation, assuming the linear relation to hold, yields the value for S°_{20} of 182 S.

If we make two assumptions, namely, that the molecular weights of DNA in ethanol and in 0.2 *M* NaCl are identical and that a linear extrapolation of the low concentration part of the curve shown in Fig. 4 is valid, we may calculate the approximate ratio of the frictional coefficients of DNA in the two solvents. The first assumption rests on the work of GEIDUSCHEK AND GRAY[5] discussed earlier. The second assumption appears from the results to be justifiable, but if it is in error, it is apparent from the general shape of the curve that the extrapolation value of S_{20} will most probably be an under-estimate of the true value.

From a study of DNA in 0.2 *M* NaCl, DOTY[1] gives values for S°_{20} and $\bar{V}$ of 22.5 S and 0.55 respectively. The value of the frictional coefficient, f, in terms of the molecular weight M of the DNA molecule is then given by

$$f_{0.2\ M\ \mathrm{NaCl}} = \frac{\mathrm{M}\,(1 - \bar{V}\rho)}{S^0_{20}} = \frac{\mathrm{M} \times 0.45}{22.5}$$

Similarly for DNA in ethanol using our approximate extrapolated value for S°_{20} of 182 S and the value of 0.38 for the partial specific volume (which was determined on the solutions used in the sedimentation experiments), we obtain, assuming an identical value for the molecular weight

$$f_{\mathrm{ethanol}} = \frac{\mathrm{M}\,(1 - \bar{V}\rho)}{S^0_{20}} = \frac{\mathrm{M} \times 0.69}{182}$$

which gives

$$\frac{f_{\mathrm{ethanol}}}{f_{0.2\ M\ \mathrm{NaCl}}} = 0.19$$

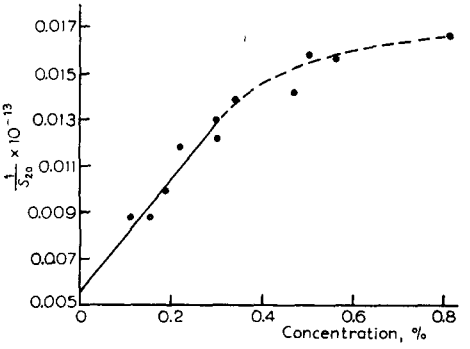

Fig. 4. The concentration dependence of the reciprocal of the sedimentation coefficient of DNA in 95% ethanol.

It is thus apparent that the molar frictional coefficient of DNA in ethanol has a value approximately one-fifth of that in 0.2 *M* NaCl.

The theories of BRINKMAN[14], DEBYE AND BUECHE[15] and KIRKWOOD AND RISEMAN[16], which attempt to describe the hydrodynamic behaviour of flexible chain macromolecules, all give rise to equations for the frictional coefficient that may be approximated by the expression

$$f = A\mathrm{M}^\varepsilon \qquad \tfrac{1}{2} \leqslant \varepsilon \leqslant 1$$

where the values of the "adjustable constants" A and ε depend on how much the flow of solvent through the polymer coil is restricted. For an open, very free-draining

coil, ε approaches 1, whilst for a collapsed, non free-draining coil, ε approaches $\frac{1}{2}$. It can thus be seen that if our assumptions are correct, and in particular if the molecular weight is constant in the two different media, the smaller value of f for an ethanolic solution must correspond to a decrease in the exponent; *i.e.*, the configuration of the molecular coil has changed from open free-draining to collapsed non free-draining. This conclusion is in agreement with the conclusions of GEIDUSCHEK AND GRAY. A consequence of the compact configuration of the DNA molecule in ethanol should be that, compared with aqueous solutions, if the DNA is polydisperse, a wider distribution of sedimentation coefficients will be observed.

It can be seen in Fig. 1 that the sedimentation boundaries observed for ethanolic DNA solutions spread rapidly, so it is of interest to examine the distribution of sedimentation coefficients, preferably at infinite dilution. Four graphs of refractive index gradient against distance from the centre of rotation of the cell were obtained at regular intervals during each sedimentation experiment. These graphs were then converted into apparent normalised sedimentation coefficient distributions $[g^*(S)]$ by the method of WILLIAMS, BALDWIN, SAUNDERS AND SQUIRE[17]. A single apparent normalised distribution showing the apparent distribution of the sedimentation coefficients of the sedimenting species on a refractive index concentration scale was obtained from each photograph; the effects of the radial increase in centrifugal field and dilution due to the sector shape of the cell having been eliminated. The only difference between these apparent distributions obtained at different times in a given centrifuge run is the time for which diffusion has been taking place across the initial sharp boundary. It is of interest that in the case of DNA dissolved in ethanol no significant difference was found between apparent distributions derived from the second and last photographs of the centrifuge run. The similarity between the normalised distributions is presumably due to the low diffusion coefficient of DNA in ethanol and the short duration of the ultracentrifuge runs. Consequently the mean of the apparent distributions obtained at a given concentration may be taken as the true sedimentation coefficient distribution $[g(S)]$ free from the effects of diffusion. The normalised sedimentation coefficient distributions obtained from typical ultracentrifuge experiments are shown in Figs. 5–7. It can be seen that the distributions become broader in the lower concentration experiments, this being a consequence of the concentration dependence of the sedimentation coefficient which leads to a sharpening of the boundary at higher concentrations. The most desirable way of presenting the results of such a boundary spreading analysis is in terms of $g(S^\circ)$, the distribution of sedimentation coefficients extrapolated to infinite dilution and thus

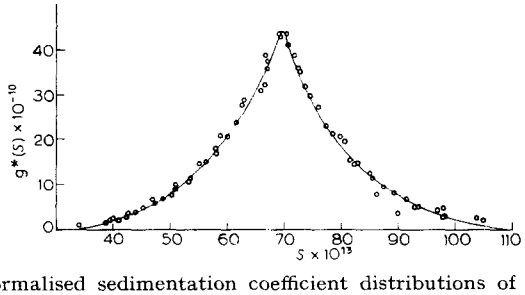

Fig. 5. Apparent normalised sedimentation coefficient distributions of DNA in 95% ethanol. Concentration 0.47%. Points derived from all five sedimentation photographs.

free from the effects of concentration dependence. This can only be done satisfactorily in a system where the dependence of S on concentration is linear, either by direct extrapolation or by application of the theory of BALDWIN[18]. The decidedly non-linear behaviour described here for DNA in ethanol precludes such a course in this case;

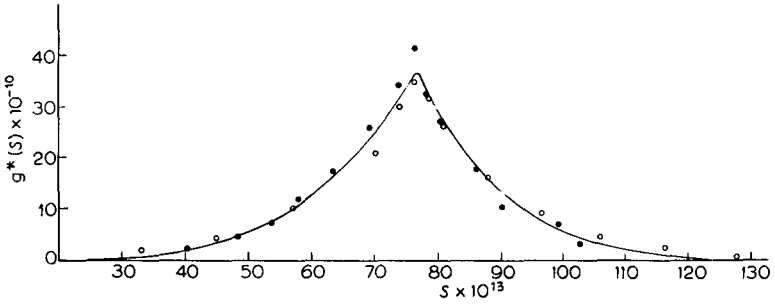

Fig. 6. Apparent normalised sedimentation coefficient distributions of DNA in 95% ethanol. Concentration 0.30%. O, second photograph; ●, fifth photograph.

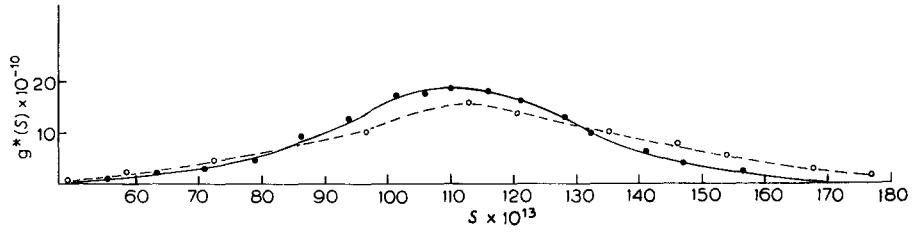

Fig. 7. Apparent normalised sedimentation coefficient distributions of DNA in 95% ethanol. Concentration 0.12%. O, second photograph; ●, fifth photograph.

the best that can be achieved is to consider the distribution obtained at the lowest concentration used, whilst acknowledging the probability that the true distribution is rather wider. On examination of Figs 5–7 it is seen that at the lowest concentration at which experiments were made there was a spread of sedimentation coefficients from 50 to 170 S with a maximum at 110 S. This contrasts with the distribution obtained by SCHUMAKER AND SCHACHMAN[19] for DNA in 0.2 M aqueous NaCl at a concentration of 0.001 %, where they found a distribution of sedimentation coefficients from 10 to 60 S of asymmetric shape with a maximum at about 20 S. However, the two distributions are not strictly comparable since the distribution in aqueous solution is effectively at zero concentration whereas the distribution in ethanol is not. Bearing in mind the much higher concentration of the distribution for DNA in ethanol it is probable that the distribution at zero concentration would be rather wider than the distribution for aqueous solutions, in terms of the ratio of the S values of the fastest to the slowest sedimenting species. In spite of this incompatibility it is possible to assert that calf thymus DNA shows a broad distribution of sedimentation coefficients which, in view of the collapsed state of the molecule compared with aqueous solutions, reflects heterogeneity of molecular weight in confirmation of the results of SCHUMAKER AND SCHACHMAN[19].

Two other possible reasons for the wide spread of sedimentation coefficients in ethanolic solution may be advanced. In ethanolic solution, random aggregates of

DNA molecules might be formed; in this connection is it significant that GEIDUSCHEK AND GRAY[5] determined the molecular weight of DNA in ethanol by light scattering, and found it to be within 15 % of the value obtained in dilute saline solution. As light scattering yields a weight-average molecular weight, the presence of aggregates seems unlikely. It might be that the sedimentation coefficient distribution represents different degrees of solvation and coiling in an assembly of uniform molecular weight molecules. The possibility of large variations in these properties, within the limitations of the known overall composition of DNA, and the WATSON AND CRICK structure seem quite small. Thus heterogeneity of molecular weight seems to be the most likely explanation. It has been shown[20,21] by means of gradient elution of DNA from weakly basic ion exchange columns, that a number of DNA samples are remarkably heterogeneous with respect to the relative amounts of the two possible base pairs (adenine-thymine and guanine-cytosine) in a given molecule. The work described here indicates that in addition to this heterogeneity of composition, there is almost certainly great heterogeneity of molecular weight.

It may thus be concluded that the ultracentrifugal analysis of DNA in ethanolic solution is a method that may be used to examine the heterogeneity of DNA preparations.

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