A Defined Molecular-Weight Distribution of Deoxyribonucleic Acid after Extensive Sonication

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Concentrated solutions of low-molecular-weight DNA ($\bar{M}_w = 35000$) with a known molecular-weight distribution can be prepared in several hours, and require no additional fractionation procedures. This is achieved by sonication of the DNA in 1.0M-NaCl at high power at 0–2°C. No denaturation of the DNA is detectable, even after 8 h of continuous sonication. After 2 h, the molecular-weight distribution of the total DNA sample is that of the most probable Schulz distribution, described by $\bar{M}_n:\bar{M}_w:\bar{M}_z \dots = 1:2:3 \dots$ etc. Such a molecular-weight distribution is expected for a random break-up of indefinitely long macromolecules and indicates that the sonication process is essentially by random double-strand scission. DNA was also sonicated in the presence of ligands capable of modifying the DNA tertiary structure. The results support the idea that inflexibility of the DNA is required for efficient sonic degradation.

In the last two decades, many methods have been used to degrade DNA into low-molecular-weight fragments. The relative success of acid and alkaline degradation, enzymic hydrolysis, shearing through a syringe needle, French press and sonic-irradiation treatments have been discussed by Richards & Boyer (1965), who concluded that the most effective means of obtaining low-molecular-weight double-strand DNA is by a sonication procedure. Doty *et al.* (1958) have shown that sonication for 60 min could cause a 25-fold decrease of the molecular weight of the DNA to a weight-average molecular weight ($\bar{M}_w$) of 300 000. Although it is known that prolonged sonic irradiation does not significantly increase the concentration of lower-molecular-weight species present (Cohen & Eisenberg, 1966; Peacocke & Pritchard, 1968; Godfrey, 1976), there is no clear understanding of the optimum sonication conditions necessary to obtain the lowest possible molecular weight of DNA in a defined period of time. The most successful method to date has been to sonicate DNA for 30 min in a viscous solvent containing 75% (v/v) glycerol (Record *et al.*, 1975). This method has yielded a species of $\bar{M}_w$ 243 000 and, after fractionation on an agarose column, a trailing fraction of $\bar{M}_w$ 30 000.

It has become apparent that there is a need to be able to rapidly prepare high concentrations of low-molecular-weight DNA to facilitate n.m.r. studies of the exchangeable protons of DNA and for n.m.r. investigations of DNA–ligand interactions (Kearns, 1977). Such low-molecular-weight DNA yields measurable n.m.r. signals (Kearns, 1977). However, it is highly desirable that both the molecular weight

and the molecular-weight distribution be reproducible. The present paper reports a procedure that yields a $\bar{M}_w$ of 35 000 for the total solution within several hours, and not only ensures that the molecular-weight distribution is similar from one laboratory to another, but ensures that the molecular-weight distribution is defined and can be readily confirmed. The molecular-weight distribution that can be achieved is that of the most probable Schulz distribution (Fujita, 1962), which has ratios of successive molecular-weight averages in an arithmetic progression, i.e. $\bar{M}_n:\bar{M}_w:\bar{M}_z:\bar{M}_{z+1} \dots$ is as 1:2:3:4 ... etc. (Gibbons *et al.*, 1973). This distribution is expected for a random break-up of a linear polymer and can be confirmed by a linear plot of c^{-1} (c is the concentration) against the reduced radius ξ (Gibbons *et al.*, 1973). The validity of this procedure for the study of polydisperse samples has been demonstrated by Gibbons *et al.* (1973), who compared polydisperse preparations of intestinal epithelial glycoproteins and dextrans with a monodisperse glycoprotein.

Experimental

Materials

Calf thymus DNA (Worthington, Freehold, NJ, U.S.A.) was dissolved in 1.0M-NaCl for several days and undissolved DNA removed with a 3 μ m Millipore filter. A few drops of chloroform were added and the solution was stored at 4°C. The final DNA nucleotide concentration was 4 mM. Concentrations were determined spectrophotometrically by using a value of $6600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 259 nm. The G+C content was

41% and was determined spectrophotometrically (Hirschman & Felsenfeld, 1966) and from its buoyant density with respect to *Micrococcus lysodeikticus* DNA (Szybalski & Szybalski, 1971).

9-Aminoacridine was purchased from Sigma (London) Chemical Co., London S.W.6, U.K., distamycin A hydrochloride was from Boehringer Corp. (London), London W.5, U.K., and copper nitrate was from BDH Chemicals, Poole, Dorset, U.K. All were prepared in 1.0M-NaCl.

Methods

A 20 kHz Rapidis 600 sonicator (Ultrasonics) was used with a 32mm-diameter probe. A sample (20ml) of the DNA solution was routinely bubbled with N₂ for 15min before sonication and the N₂ was applied continuously during sonication. The sample was sonicated continuously for up to 8h at near maximum power (i.e. 45W of effective power). The temperature of the DNA solution was maintained at 0–2°C by a 'cold-finger' probe in 2 litres of methanol in which a 100ml Teflon beaker containing the DNA solution was immersed. The temperature was monitored continuously by using a platinum resistance thermometer (in the DNA solution) coupled to a Rosemount model 414L linearizer bridge and digital voltmeter, this system having been calibrated to indicate solution temperatures to $\pm 0.05^\circ\text{C}$.

A Chromatronix LC-9M chromatography column (2.6cm $\times$ 100cm) with only glass and Teflon components, was used for DNA fractionations. Sepharose 6B (Pharmacia) was used in the column at all times, 1.0M-NaCl was used as eluent and the flow rate maintained at 0.33ml/min (3.75ml/h per cm²). Samples (5ml) of DNA were applied to the column. Fractions (5ml) were collected (the exact volume was calculated from the weight of each fraction), and the absorbance was recorded at 259nm with a Zeiss DMR 10 spectrophotometer.

A Beckman model E analytical ultracentrifuge equipped with a photoelectric scanner and multiplexor was used for velocity-sedimentation and equilibrium-sedimentation studies (Yphantis, 1964) of the sonicated and fractionated DNA samples. Solvent (0.28 ml) and DNA sample (0.25ml) were used to provide a solution column height of 6mm. The rotor was run at 15000 rev./min and at 20°C, 72h being required to ensure an equilibrium-concentration distribution. The initial nucleotide concentration of DNA used was normally 60 μM and the equilibrium distribution monitored at 265nm.

When plotting the equilibrium-sedimentation data in terms of the most probable Schulz distribution (Gibbons *et al.*, 1973), the reduced radius, ξ , is used, where

$$\xi = \frac{r_b^2 - r^2}{r_b^2 - r_m^2}$$

r_b and r_m represent the radius to the bottom of the cell and to the meniscus respectively, and r represents the radius to any position in the cell.

The hyperchromicity of sonicated DNA was determined from thermal-denaturation profiles. These were carried out by using a Gilford 240 spectrophotometer equipped with a model 2527 Thermoprogrammer, with a heating rate of 0.5°C/min.

Results

Effective sonication power

The power dissipated in 50ml of water in a well-insulated Dewar flask was measured as a function of the power settings on the sonicator. The results are shown in Fig. 1 and illustrate that the effective power output is non-linear with respect to the power applied, and that the efficiency of the transducer is, as expected, quite poor (8% in this instance, the maximum power setting corresponding to 600W applied to the transducer). The same curves were obtained irrespective of the diameter of the probe used. As the efficiency of different transducers would be expected to differ, a more informative means of indicating the sonication conditions used would be in terms of the power actually dissipated in the solution, and these values can be readily determined in the manner indicated.

Effect of extensive sonication time

The rapid decrease of the sedimentation coefficient of DNA with sonication time is shown in Fig. 2. A value of 3.6S is obtained after 3h of sonication and does not decrease appreciably even after 8h of

Fig. 1. *Effective sonication power*
Sonicator setting of 12 corresponds to 600W applied to the transducer. See the Results section for experimental details.

Fig. 2. Effect of extensive sonication time

Samples (0.5ml) were withdrawn from the DNA solution (initially 20ml) after being subjected to sonication for the times shown. The sedimentation coefficient ($s_{20,w}$) ($\circ$), and hyperchromicity (expressed as a percentage of the initial DNA absorbance at 259nm) (Δ), were measured from the same sample after appropriate dilution with 1.0M-NaCl.

Fig. 3. Approach of the molecular-weight distribution of

DNA to that of the most probable Schulz distribution. The curves represent the equilibrium distribution of DNA after being sonicated continuously for 30min (a), 60min (b) and 120min (c), and were run under meniscus-depletion conditions. The A_{265} of the DNA was read directly from the equilibrium-sedimentation scanner charts and used as a measure of concentration for the purposes of calculating c^{-1} .

sonication. As the concentration used for all velocity-sedimentation runs was $33\mu\text{g/ml}$, there is a small difference between the $s_{20,w}$ and $s_{20,w}^0$ values. From the viscosity data of Godfrey (1976) and the equations of Eigner & Doty (1965) it is possible to show that the expected difference would be less than 1% of the $s_{20,w}$ values observed after several hours of sonication, consistent with similar calculations by Record *et al.* (1975). However, as a precaution, the $s_{20,w}^0$ value of the preparation sonicated for 4h was determined and also found to be 3.6S. This value therefore validates the use of $s_{20,w}$ values as a means

of monitoring relative molecular-weight changes. The $s_{20,w}$ value (after 3h of sonication) corresponds to a $\bar{M}_w$ of 35000, by using equation (2) of Record *et al.* (1975). Although this equation is strictly only applicable in the mol.wt. range of 40000–300000, there is a linear correlation of $\ln \bar{M}_w$ with $s_{20,w}$ for $s_{20,w}$ values between 4 and 5S, and this enables a simple extrapolation to our value of 3.6S. The value of 35000 is also consistent with the more recent equation derived for DNA of mol.wt. down to 32000 (Kovacic & van Holde, 1977).

The approach of the DNA molecular-weight distribution towards that of the most probable Schulz distribution is shown in Fig. 3 at increasing sonication times. Even after 30min of vigorous sonication, considerable non-linearity of the plot of c^{-1} against ξ is evident. However, after 2h of sonication, linearity is evident. Similar linear plots were also obtained after 4h and 8h of continuous sonication.

The hyperchromicity of the DNA is shown in Fig. 2 as a function of sonication time. The hyperchromicity is not detectably altered from that of non-sonicated DNA, even after 4h of continuous sonication. Moreover, sonication periods of up to 8h have been used, and the hyperchromicity of the DNA was found to be unaltered during the course of the sonication.

Fig. 4. Equilibrium distribution of DNA under meniscus-depletion conditions

DNA was sonicated for 2h in the presence of (a) no added ligands, (b) distamycin A (0.2mol/nucleotide), (c) 9-aminoacridine (0.2mol/nucleotide), (d) Cu^{2+} ions (0.5mol/nucleotide). Only in the presence of Cu^{2+} ions is the distribution significantly different from that of DNA alone (see the Results and Discussion sections).

Fig. 5. Gel-exclusion-chromatography elution profiles of extensively sonicated DNA

DNA was sonicated for 2 h in the presence of (a) no added ligands or of ligands that modify the DNA tertiary structure, (b) distamycin A (0.2 mol/nucleotide), (c) 9-aminoacridine (0.2 mol/nucleotide), (d) Cu^{2+} ions (0.5 mol/nucleotide). Only in the presence of Cu^{2+} ions is the elution profile significantly different from that of DNA sonicated in the absence of ligands (see the Results and Discussion sections).

Effect of ligands

Several ligands that are known to interact with DNA and to cause changes of the DNA tertiary structure were used to ascertain the role of DNA tertiary structure under sonicating conditions, so that optimal degradation conditions could be established. The effect of these ligands is shown in Figs. 4 and 5. Fig. 4 shows the extent of randomness of the molecular-weight distribution of DNA achieved after 2 h of sonication in each case. The most probable Schulz distribution is apparent for DNA in the absence of ligands, or in the presence of 9-aminoacridine or distamycin A. However, the molecular-weight distribution is considerably broader (as indicated by the non-linearity) for DNA in the presence of Cu^{2+} ions. When the same sonicated samples have been subjected to a gel-exclusion fractionation on Sepharose 6B, it is apparent from Fig. 5 that the DNA- Cu^{2+} system has not been degraded to the same extent as DNA, DNA-9-aminoacridine or DNA-distamycin A.

Discussion

The sonication conditions of 1.0M-NaCl and 0–2°C were selected for several reasons: 1.0M-NaCl was selected because DNA is known to exhibit its greatest thermal stability at this value (Schildkraut & Lifson, 1965). Any denaturation of DNA during the sonication process resulting from the high local temperatures experienced (Hughes & Nyborg, 1961) would therefore be minimized. A temperature of

near 0°C was also chosen for the same reason and because it was thought that the increased viscosity of the solution (relative to ambient temperatures) would tend to make the DNA more 'brittle' in much the same way as the 75% glycerol solvent would (Record *et al.*, 1975). The effect of the low temperature would therefore be to enhance the formation of lower-molecular-weight fragments, compared with higher temperatures, while simultaneously lowering the efficiency of the sonication process.

The observation that the power output of a sonicator bears little relationship to the power actually dissipated into solution suggests that some standardized means of indicating the effective power used should be adopted. We suggest the use of a simple calorimetric approach, as used in this work.

As the hyperchromicity of DNA is a function of helix length only for short sequences less than 20–50 base pairs (Pohl, 1974), any variation of hyperchromicity arising from similar small segments in the sonicated DNA is considered to be negligible. The constancy of the hyperchromicity (Fig. 2) therefore implies little disruption of the secondary structure of the DNA after 4 h of high-power sonication under these conditions. Indeed, there was no detectable hyperchromicity change after 8 h of sonication.

The upward curve of the plot of c^{-1} against ξ evident in Fig. 3 for 30 min and 60 min sonication periods indicates a broader molecular-weight distribution than that expected for a randomly degraded polymer (Gibbons *et al.*, 1973). The linearity of such plots after 2 h of sonication indicates that the molecular-weight distribution conforms to that of the most probable Schulz distribution (Gibbons *et al.*, 1973). It should be noted from Fig. 2 that although the sedimentation coefficient, and hence the $\bar{M}_w$, is largely unaltered in the sonication period from 30 min to 2 h, the molecular-weight distribution alters considerably in the same period (Fig. 3). These results are therefore entirely consistent with previous observations that prolonged sonication does not significantly increase the concentration of lower-molecular-weight species, but does sharpen the distribution (Cohen & Eisenberg, 1966; Peacocke & Pritchard, 1968; Godfrey, 1976).

9-Aminoacridine is known to interact with DNA by a non-specific intercalation process (Armstrong *et al.*, 1970), and this results in elongation of the DNA. Saturating amounts of the 9-aminoacridine were therefore used in an endeavour to make the DNA more rod-like. It was thought that the DNA would be held more rigid and 'brittle' than DNA in the absence of an intercalating agent, and that this might yield DNA of extremely low molecular weight after extensive sonication. Little difference of the final molecular weight was detected (Fig. 5) and the molecular-weight distribution was identical with that of DNA sonicated in the absence of intercalating

agent (Fig. 4). In contrast, when DNA is in the presence of saturating amounts of Cu^{2+} ions, known to decrease the asymmetry of DNA (Bryan & Frieden, 1967), the molecular weight obtained after the same sonication period is larger than that of DNA in the absence of Cu^{2+} ions (Fig. 5) and the molecular-weight distribution is broader (Fig. 4) and not described by the most probable Schulz distribution. The effects of these two ligands is thought to be due entirely to their effects on the DNA tertiary structure, and this in turn affects the efficiency of the sonication process. These results are also consistent with the fact that a lower $\bar{M}_w$ is detected at the low temperatures used in the present work compared with the higher temperatures used by others. This also seems to be due only to the flexibility of the DNA in solution and indicates that this low-temperature sonication procedure is a more effective manner in which to produce low-molecular-weight DNA than the 75%-glycerol method of Record *et al.* (1975). The latter method yields only a small fraction of the DNA with comparable low molecular weights after 30 min sonication times.

As there is a small deviation of the amount of 3'-termini after sonication from that of the base composition of calf thymus DNA (Bertazzoni, 1975), it was thought that this effect might be accentuated in the presence of a ligand, such as distamycin A, which binds preferentially to A·T sites (Luck *et al.*, 1974). However, there was no significant deviation of either the molecular weight or the molecular-weight distribution from that of DNA sonicated in the absence of distamycin A (Figs. 4 and 5).

Conclusion

After sonicating DNA solutions continuously at high power settings and at low temperatures for extremely long periods of time, the DNA is degraded in a random manner to a molecular-weight distribution that is described by the most probable Schulz distribution i.e. $\bar{M}_n:\bar{M}_w:\bar{M}_z$ etc. is as 1:2:3 etc. The weight-average molecular weight detected under these conditions after 3 h is 35 000, significantly lower than that produced by any other technique or by any

other sonication procedure. The inflexibility of the DNA tertiary structure during the sonication process appears to be an important factor affecting the extent of the degradation.

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