SEDIMENTATION ANALYSIS OF NEUTRALIZED ALKALI-DENATURED DNA

David Freifelder

Graduate Department of Biochemistry
Brandeis University, Waltham, Massachusetts

Received November 21, 1967

Single-strand DNA can be studied to advantage at pH ≫ 12 because (i) in such solutions the strands show no tendency to renature or aggregate, thus avoiding the necessity to add stabilizing substances, which prevent hydrogen-bonding (e.g., formaldehyde), and (ii) the ionic strength can be varied from 0.01 molar upwards. This technique has been used to measure the degree of single-strand breakage in normal bacteriophage DNA (Davison et al., 1964) and in DNA which has been damaged by several types of radiation (Freifelder, 1966)(Freifelder and Uretz, 1966). However, for measurements requiring more than about one-half hour, alkaline hydrolysis can become serious (Freifelder, in preparation). Also, for larger DNA molecules the single strands are sensitive to hydrodynamic shear degradation (Davison and Freifelder, 1966). If alkali-denatured DNA is neutralized at high ionic strength, a rapidly sedimenting form of DNA results which presumably consists of internally hydrogen-bonded and hence highly compact single-strand DNA molecules (Studier, 1965). The use of this form of DNA whenever possible would be advantageous since alkaline hydrolysis is avoided and this DNA is highly resistant to hydrodynamic shear (Davison and Freifelder, 1966). In order to be a useful technique it is necessary that each molecule collapse independently, i.e., without any intermolecular aggregation. This is likely

since an appreciable fraction of this neutralized, denatured DNA sediments homogeneously (Studier, 1965). Whether this is rigorously true can be determined quantitatively by measuring the size distribution of a heterogeneous mixture before and after neutralization. To do this, one can make use of the fact that denatured bacteriophage DNA is almost always heterogeneous with respect to molecular weight because of the natural single strand breaks (Davison et al., 1964) and thereby compare the percentage of broken strands in samples of several bacteriophage DNA's which have been denatured and denatured-and-neutralized. In the present paper such an analysis has been performed for normal denatured DNA as well as DNA with an increased number of strand breaks resulting from X-irradiation (Freifelder, 1966).

MATERIAL AND METHODS

The growth and purification of each of the phages has been described (Davison et al., 1964). For X-irradiation the phages were suspended in 0.01 M PO_4, pH 7.8, containing 10^{-3} M L- histidine and irradiated with 150 kv, unfiltered X-rays (Freifelder, 1966).

DNA solutions were prepared as follows: 20 µl purified phage suspended in 0.01 M Tris, pH 7.8, 10^{-3} M $MgSO_4$, 0.5 M NaCl was added to 1.5 ml 0.01 M PO_4, pH 7.8, 0.002 M Versene. Then 0.3 ml 1 M NaOH was added and the solution was incubated for 2 minutes at room temperature. To make neutralized, denatured DNA a 0.9 ml aliquot was taken and 0.2 ml 1 M KH_2PO_4 and 0.2 ml 4 M NaCl were added sequentially; to make HCHO-denatured DNA, 0.2 ml neutralized 37% formaldehyde and 20 µl 1 M KH_2PO_4 was added to the remaining 0.9 ml. In some cases the DNA was centrifuged in the original alkaline solution. Each of these samples was centrifuged at a concentration of 15-20 µg/ml in a 30 ml Kel-F cell using a Spinco Model E Ultracentrifuge equipped with UV optics. The exposure time for UV photography was always 6 seconds and the speed was adjusted so that for all conditions the boundary moved approximately the same distance during the exposure.

RESULTS AND DISCUSSION

For each of the DNA's examined the sedimentation diagrams for centrifugation of denatured DNA in alkali and neutral solutions consisted of a main boundary representing intact, single strands and slower material consisting of smaller fragments. (For numerous figures depicting these boundaries, see Davison et al., 1964.) For equivalent distances sedimented, the boundaries have nearly the same shape, although in neutral solution the main boundary is slightly broader. The important observation is that the percentage of material in the main boundary (intact molecules) is the same for the two conditions. In comparisons of this sort, several complications can arise: (i) If the solution is neutralized after only a short exposure to alkali (the time depending on the molecular weight of the phage), a sharp boundary with the sedimentation coefficient of native DNA is found. This represents molecules whose hydrogen bonds were broken by the alkali but whose strands had not fully separated, and has been used to measure the length of time required for strand separation (Davison, 1966). In the present experiments the incubation period was always sufficiently long that native DNA did not appear in the neutralized sample. (ii) In some ionic conditions (for example, DNA denatured in 1 M NaCl and 0.25 M NaOH, followed by neutralization with 0.15 M KH_2PO_4 final concentration), some native DNA is found after neutralization. The amount of native DNA found depends somewhat upon the time of incubation in alkali; this amount is not in general reproducible and can be markedly reduced by pre-incubation of the native DNA in 0.1 M Versene, pH 7.1. This phenomenon is not understood. It is recommended that, if ionic conditions other than those in use in this work or those of Studier (Studier, 1965) are to be used, the system be checked for the presence of native DNA following neutralization.

It was thought that renaturation might be a complication because of the high ionic strength of the neutralized solution. To check this, denatured and neutralized T4 DNA was incubated for 2 hours at 25° in ionic strength

ca. 1 and centrifuged; no renatured material could be detected, either by a decrease in optical density (260 mμ) of the solution or the appearance material having a sedimentation coefficient less than or equal to native DNA.

Table I summarizes the data for the denatured DNA of phages B3, T7, T4, and α, examined in alkaline solution or, following alkaline denaturation, in formaldehyde or neutral solutions. In each case, the percentage of material sedimenting as a homogeneous, sharp boundary is the same for the various sol vent conditions. Since it might be expected that the larger molecules would have the greater tendency to aggregate with one another when hydrogen bond reformation is permitted (i.e., in neutral solution), the identity of the percentage of homogeneous material suggests that intermolecular aggregation does not occur. Hence neutral sedimentation will yield the same results as alkaline sedimentation without the complications of alkaline hydrolysis. Also, since all three conditions of centrifugation yield the same result also for X-irradiated DNA, the use of neutralized alkali-denatured DNA to examine induced strand breakage is probably justified.

In the present experiments the DNA was in the concentration range of 20-40 μg/ml at the time of neutralization. It might be expected that at higher concentrations intermolecular aggregation would compete with the intramolecular folding described in the present work. To test this, 20 μl phage T7, 2 mg/ml, in phosphate-Versene was added to 10 μl 1 M NaOH and incubated for 5 minutes to allow strand separation. Then 10 μl 1 M KH_2PO_4 was added for neutralization. Hence, denaturation was at 1 mg/ml and neutralization at 750 μg/ml. This solution was then diluted for centrifugation. The boundary obtained was similar to that obtained at low concentration; there was no sign of intermolecular aggregation. This surprising result should be taken with caution since under certain ionic conditions this may not always be the case. For instance if DNA is heat-denatured in low ionic strength, intermolecular aggregation does occur if salt is added.

TABLE I

Percentage of Denatured DNA Sedimenting More Slowly Than the Principal, Homogeneous Boundary*

Type of Phage DNA	Percentage* Condition 1	Condition 2	Condition 3
E. coli phage T4	31, 34, 36, 30, 35	31, 33, 34, 32, 30	31, 36, 35, 34, 32
E. coli phage T7	50, 47, 55, 50, 51	50, 51, 48, 50, 50	46, 51, 48, 50, 50, 51, 50, 50, 45
Pseudomonas aeroginosa 1C, phage B3	26, 24, 23, 25, 26	27, 28, 22, 24	22, 20, 26 24
B3, X-irradiated	———	71, 71, 73	71, 71, 70, 74
B. megaterium, Parigi, Phage α	33, 36, 30	33, 36, 36	33, 37, 33

Condition 1 - DNA denatured in alkali and sedimented in alkali

Condition 2 - DNA denatured in alkali and sedimented in neutral formaldehyde

Condition 3 - DNA denatured in alkali and sedimented in neutral solution without formaldehyde

*Each value represents a separate sample

This work was supported by grant No. GM-14358 from the National Institute of General Medical Sciences, U.S. Public Health Service. This is contribution No. 533 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts.

REFERENCES

Davison, P. F., J. Mol. Biol., 22, 97 (1966).

Davison, P. F., and Freifelder, D., J. Mol. Biol., 16, 490 (1966).

Davison, P. F., Freifelder, D., and Holloway, B. W., J. Mol. Biol., 8, 1 (1964).

Freifelder, D., Radiat. Res., 29, 329 (1966).

Freifelder, D., and Uretz, R. B., Virology 30, 97 (1966).

Studier, F. W., J. Mol. Biol., 11, 373 (1965).