ANOMALOUS SEDIMENTATION OF HIGH MOLECULAR WEIGHT DENATURED MAMMALIAN DNA

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SUMMARY. High molecular weight mammalian DNA (of greater than 5×10^8 daltons) appears to sediment anomalously in alkaline sucrose density gradients if centrifugation is at high speed. More accurate sedimentation profiles of this DNA may be obtained following low speed centrifugation.

Velocity sedimentation of both native and denatured DNA has proven to be a powerful tool not only in the study of DNA physical chemistry, but also in the study of radiation-induced DNA breakage and its repair *in vivo*. The technique of DNA sedimentation in alkaline sucrose density gradients, introduced by McGrath and Williams (1,2), has permitted sedimentation analysis of very large molecules of DNA (molecular weight greater than 10^8 daltons) released from bacterial and mammalian cells layered directly onto the top of alkaline sucrose gradients. In the present study, data to be presented indicate that high speed centrifugation of large single stranded DNA (greater than 5×10^8 daltons) gives rise to anomalous sedimentation profiles which give a false impression of molecular homogeneity. This apparent homogeneity may lead to the erroneous conclusion that DNA *in vivo* exists in the form of subunits of relatively uniform size (2-5). However, low speed centrifugation of the same DNA produces a broader sedimentation profile which appears to be a much more accurate representation of the true distribution of DNA molecular sizes. Data concerning radiation induction of single strand breaks suggest

that the equations relating DNA molecular weight with sedimentation coefficient, derived empirically for single stranded bacteriophage DNA (6), can also be used for DNA of much higher molecular weight provided that the centrifugation speed and DNA concentration are held below certain limits.

MATERIALS AND METHODS

The sedimentation properties of high molecular weight mammalian cell DNA have been studied in alkaline sucrose density gradients using a modified technique for "gentle" cell lysis (7), which minimizes DNA breakage during the cell lysis and denaturation period preceding centrifugation. L cells, labelled for 24 hours in medium containing 0.05 μCi/ml ^{14}C-thymidine (specific activity 54 mCi/mM), are layered at 4°C onto a thin layer (about 0.5 cm thick) of 2% sucrose in water which had been placed on top of a 10 to 30% linear alkaline sucrose density gradient made up in 0.3 N NaOH, 0.5 M NaCl, and 0.01 M EDTA. Intact cells fall slowly through the 2% sucrose layer before undergoing disruption near the interface with the alkaline sucrose. The gradients are maintained at 4°C for 16 to 20 hours before being centrifuged and fractionated. The DNA of each fraction is precipitated in trichloroacetic acid and collected on glass fiber filters for scintillation counting.

RESULTS AND DISCUSSION

Sedimentation of unirradiated DNA is discussed in detail elsewhere (7). It is sufficient to say that under the conditions used for "gentle" cell lysis, the cells must be irradiated with 700 to 1000 rads or more in order to obtain sedimentation profiles characteristic of single stranded DNA. If centrifugation is carried out at high speed (23,000 rpm in the SW-27 rotor of the Beckmann L2 ultracentrifuge), DNA from cells given x-ray doses of 700 to 1000 rads sediments in a narrow peak at about 180 S. Since the DNA of irradiated cells would be expected to contain random

single strand breaks, such a narrow profile is unexpected (9). DNA sedimentation profiles from cells given x-ray doses greater than 1780 rads are shown in Fig. 1. Analogous profiles have been reported by other investigators (3,8). It has been suggested (3) that such DNA sedimentation profiles result from x-ray induced, random degradation of a uniform population of molecules with single stranded molecular weight about 5×10^8 daltons.

From the weight average molecular weights of DNA sedimentation profiles similar to those shown in Fig. 1, obtained with high speed

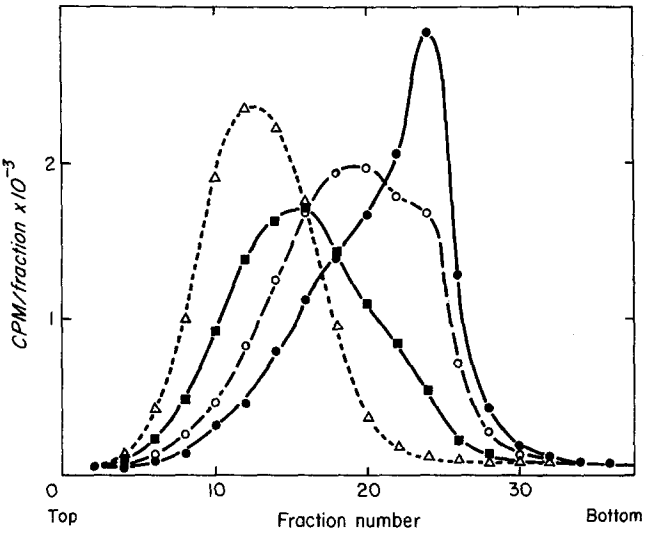

Figure 1.

Alkaline sucrose gradient sedimentation profiles of ^{14}C-DNA released from L-cells exposed to various x-ray doses. Cells were grown for 24 hours in medium containing 0.05 μCi/ml ^{14}C-thymidine before being exposed to various doses of 280 kilovolt x-rays. About 10^5 cells (containing about 1 μgm of DNA) were then placed on a 2 ml layer of 2% sucrose in water which was on top of a 36 ml 10 to 30% linear sucrose density gradient made up in 0.3 N NaOH, 0.5 M NaCl, and 0.01 M EDTA (7). These gradients were maintained at 4°C for 18 hours after cell loading but prior to centrifugation which was in the SW-27 rotor (Beckman Instruments Inc., Palo Alto, California) for 6 hours at 23,000 rpm and 4°C. The contents of each centrifuge tube were fractionated from the top and the acid precipitable material of each fraction was collected on glass fiber filters (Whatmann, GF/C) for scintillation counting. The direction of sedimentation is from left to right. X-ray doses given to cells: 1780 (____.____), 2670 rads (__ __o__ __), 4450 rads (___□___), and 8900 rads (- - -△ - - -).

centrifugation following doses in the range 890-4450 rads, it was calculated that, on the average, one single strand break was produced by 103 ev deposited in the DNA. The fact that this value does not agree with the 60 to 70 ev/break quoted by investigators working in higher dose ranges (3,10,11) suggested that there might be an error in the determination of the molecular weights of large single stranded DNA molecules, and prompted the investigation described below.

If, as some autoradiographic evidence suggests (12-14), the mammalian chromosome contains a single large double stranded DNA molecule, then cells irradiated with 700 to 1000 rads and treated with alkali should release single stranded DNA with a broad size distribution (9). Thus, the narrow 180 S DNA peak observed following 1000 rads might be composed of DNA highly dispersed in size. It is purposed that if, under the high speed centrifugation conditions used, single stranded DNA molecules larger than approximately 5×10^8 daltons sediment at about 180 S regardless of size, and smaller DNA molecules sediment approximately according to empirical relations derived for bacteriophage DNA (6), then the sedimentation profiles of irradiated DNA would be similar to those observed in Fig. 1. This hypothesis could also account for the apparently high value for ev/break as the calculated weight average molecular weights would be too small when an appreciable amount of DNA is larger than 5×10^8 daltons.

It has been shown that anomalies in the sedimentation behaviour of native DNA become more serious as the molecular weight, centrifugation speed, or concentration are increased (15). The fact that these anomalies can be eliminated at low rotor speed together with recent observations of M.M. Elkind (16) suggested to us that low speed centrifugation might produce the expected profiles of randomly-fragmented DNA. The sedimentation profiles of DNA released from irradiated cells and centrifuged at 8,000 rpm are shown in the panels on the right side

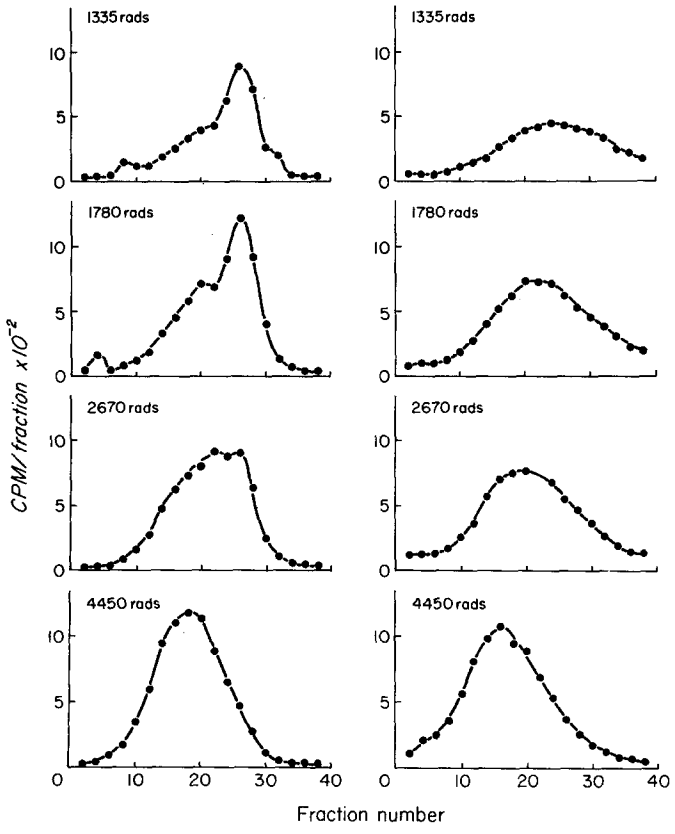

Figure 2.

Alkaline sucrose gradient sedimentation profiles of ^{14}C-DNA released from L-cells irradiated with the doses shown in each panel. Irradiated cells were placed onto a layer of 2% sucrose on alkaline sucrose gradients (see legend of Figure 1) and then stored at 4°C for 18 hours prior to centrifugation. The profiles in the left column were from gradients centrifuged at 23,000 rpm for 6 hours at 4°C while those in the right column were centrifuged at 8,000 rpm for 49.75 hours at 4°C.

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of Fig. 2. These gradients were centrifuged for 49.75 hours so that the product of $(rpm)^2$ x time for the 8000 rpm centrifugation was equal to that for the 23,000 rpm situation. The shapes of all 8000 rpm profiles are those expected for randomly-fragmented single stranded DNA whereas a distinct 180 S peak was present in the DNA profiles following low doses in the gradients centrifuged at 23,000 rpm and shown on the left side of Fig. 2. While the less rapidly sedimenting portions of the

high and low speed profiles are essentially identical it appears that DNA sedimenting at greater than 180 S during 8000 rpm centrifugation was held up at about 180 S during centrifugation at 23,000 rpm. The difference between high and low speed centrifugation profiles of DNA released from cells given low doses of x-rays cannot be accounted for merely by the increased diffusion during the 50 hours of centrifugation at 8000 rpm because there is close agreement between the high and low speed DNA profiles from cells irradiated with 4450 rads.

The weight average molecular weights of the DNA from the 8000 rpm centrifugation have been used to calculate the efficiency of single strand break production by x-rays and the value obtained was 72 ev/break. Since 72 ev/break compares well with values obtained in much higher dose regions (3,10,11), it would appear that the relationship between sedimentation coefficient and molecular weight derived for single stranded bacteriophage DNA in alkali (6) is applicable to much larger DNA molecules provided the centrifugation speed is sufficiently low to avoid the sedimentation anomaly.

It seemed possible that the anomalous sedimentation behaviour might be due to aggregation of DNA molecules. To partially exclude this possibility H^3 labelled DNA from cells irradiated with 4450 rads was sedimented in the same gradient as ^{14}C labelled DNA from cells irradiated with 1335 rads. The results indicated no detectable aggregation. Therefore it would appear that the anomalous sedimentation behaviour may be due to a velocity dependent coefficient of viscosity for single stranded DNA in alkaline gradients.

The findings reported above, along with similar findings made recently in other density gradient sedimentation systems with high molecular weight native (17) and denatured DNA (16) suggest that accurate determination of molecular weights of very large DNA may be obtained from sedimentation data only if centrifugation speed is sufficiently low

to avoid the anomalous sedimentation. If the centrifugation conditions are correctly chosen then it would appear that the equations which relate sedimentation coefficients of bacteriophage DNA to their molecular weights may be used for much larger DNA. A measurement of the efficiency of single strand break production by radiation may be a useful test for the suitability of such centrifugation conditions.

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