LOW-ANGLE LIGHT SCATTERING

OF DEOXYRIBONUCLEIC ACID

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ABSTRACT   An instrument of new design has been built in order to perform low-angle light-scattering measurements to angles as low as 16°. Native deoxyribonucleic acid preparations of different molecular weights have been studied using this apparatus with a new clarification technique. The molecular weights obtained from the low-angle data have been compared with those calculated for the same samples when using the results in the 30–150° range. The two sets of data yield the same molecular weights up to values of about 6×10^6. Higher molecular weights are underestimated to a variable extent when measurements in the usual angular region (30–150°) are used.

INTRODUCTION

Although light-scattering measurements have been widely used in the past to establish the molecular weight and shape of deoxyribonucleic acid (DNA) in solution (1–5), several limitations of this technique as applied to DNA have become apparent in the last few years (5,6). The principal uncertainty concerns the reliability of the determination of the light-scattering molecular weight (M_L) of DNA above $M_L = 5 \times 10^6$ (see reference 7, for a review). This uncertainty is mainly due to the difficulty of extrapolating data to zero angle. Considerations presented by Butler *et al.* (6) suggest that for $M_L > 5 \times 10^6$ the light-scattering method underestimates the molecular weight of DNA. However, an experimental evaluation of the upper limit of validity of M_L would be highly desirable. This can be done by studying the light-scattering envelope of DNA solutions at angles lower than 25–30°, which represent the lower limit of the angular range usually explored. It is the purpose of the present article to report light-scattering data obtained at lower angles (down to 16°) using an instrument of new design and a new clarification technique.

A short communication dealing with the same problem has appeared (8) while this work was already in progress (9).

MATERIALS

Four different DNA preparations were used: (1) DNA B13c was obtained from chicken erythrocytes by the detergent method (10); DNA B13b and B13a were derived from

B13c, respectively, by shearing it in a Waring blendor at full speed in the presence of chloroform–isoamyl alcohol or by digesting it with calf thymus acid deoxyribonuclease; (2) DNA B3 was prepared from calf thymus by a method described elsewhere (11); (3) DNA B11c was obtained from chicken erythrocytes by the same method (11), with the difference that the nucleoprotein was dissolved in M NaCl instead of water and that the deproteinization procedure was stopped earlier than usual; (4) DNA B1a was obtained from calf thymus; it differed from preparation B3 in that the clarified nucleoprotein solution in saturated NaCl was precipitated with alcohol; the precipitate was dissolved in saline solution and submitted to a mild deproteinization procedure by shaking with chloroform–isoamyl alcohol.

As the second virial coefficient of DNA is equal to zero in the solvents which were used in this work (0.13 M NaCl + 0.01 M potassium phosphate buffer pH = 6.8; acetate buffer containing 0.01 M versene, pH = 5.0, μ = 0.15), all the experiments were done, mostly in duplicate or triplicate, on one concentration only.

For the calibration of the low-angle apparatus synthetic polymers were used.

Polystyrene samples of high molecular weights were obtained by fractionation of a styrene sample which was allowed to polymerize at room temperature in the presence of an inhibitor. Polystyrene samples of low molecular weight and polyvinylpyrrolidone were commercial samples.

METHODS

A. *The Low-Angle Light-Scattering Apparatus.* The instrument used for the low-angle light-scattering measurements had the cell immersed in a bath containing a liquid with a refractive index equal or close to that of the cell material. In this respect, it was similar to the apparatus of Wippler and Scheibling (12), which was also used in this work for the angular range 30–150°. Furthermore the photomultiplier used as the detector of the scattered light, could not "see" the cell walls illuminated by the incident beam, a feature which proved to be of the utmost importance. A schematic diagram of the instrument is given in Fig. 1 and its description follows.

The *light source* is a high-pressure water-cooled mercury lamp Philips SP 500. A con-
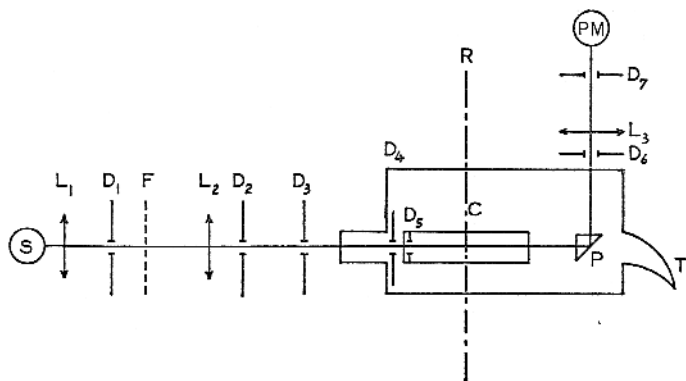

FIGURE 1 Schematic diagram of the low-angle light-scattering apparatus. The prism is in position for measuring the intensity of the transmitted beam. It can be rotated about the axis R. Measurements are made only on the scattered light that passes through one of the side walls.

densing lens L_1 forms an image of the arc on the diaphragm D_1 (0.5 × 5 mm) which acts as an object slit. A Kodak Wratten 77 A filter F, having a transmission of 68 per cent, isolates the green mercury line ($\lambda = 5461$ A). The objective lens L_2, of focal length $f = 170$ mm, forms an image (1.5 × 15mm) of D_1 slightly beyond the exit wall of the cell C. The incident beam then goes through the diaphragm D_2 (1.5 × 15 mm), enters the bath and the cell, and is finally absorbed by a light trap T. Two diaphragms, D_3 and D_4, eliminate respectively the diffraction caused by D_2 and the refraction by the bath wall. They do not play any role in the definition of the light beam.

The *cell*, 150 × 6 × 38 mm (internal dimensions), is constructed of quartz or pyrex of 2.0 mm thickness. The light beam goes through the cell along its major dimension. A platinum diaphragm inside the cell D_5 eliminates the light which is scattered by the entrance wall of the cell.

A total reflection *prism* is immersed with its support in the bath surrounding the cell, and reflects the scattered beam upwards to the photomultiplier *PM* through a lens L_3 of focal length $f = 5$ cm and diaphragms D_6 (2 × 15 mm) and D_7 (1.5 × 15 mm). Lens L_3 is positioned in order to give an image of D_7 having, in the center of the cell, the same dimensions as the image of D_1. This allows an accurate definition of the scattering volume which is the volume of liquid common to both the incident and scattered beams; it varies as a function of $\sin \theta$ (θ being the angle between the scattered and the incident beams). A volume correction will therefore be necessary, as in the conventional instruments. The prism and the photomultiplier are mounted on a common rotatable stand, whose rotation axis goes through the center of the cell. The light which is scattered through a side wall can be observed from 110° down to a very small angle (8–10°), relative to the incident beam.

The photomultiplier is an RCA P 128 operated from a stabilized 1000 volt power supply. The intensity of the current is measured by a galvanometer.

B. *Calibration of the Instrument.* The apparatus was first tested under the optimal condition of having the refractive indices of the external bath, the cell material, and the solution under examination the same, or very nearly the same. This condition was realized by using polystyrene in benzene ($n = 1.506$) as the solution, pyrex glass ($n = 1.51$) as the cell material, and benzene in the external bath. With these values there was practically no refraction at the cell walls.

Three different polystyrene samples of molecular weight 10,500, 2.3×10^6 and 8×10^6 were studied. The sample of lowest molecular weight did not show any dependence of the reciprocal scattered intensity (1/I) upon the angle θ, whereas the other two samples showed a linear dependence of 1/I upon $\sin^2 \theta/2$ (Fig. 2). Furthermore the same molecular weights and for the higher molecular weights samples, the same radii of gyration were obtained in the low angle instrument and in the conventional apparatus. A second test was performed under the more practical condition in which the refractive index was different, yet in a decreasing order going from the external bath, to the cell material to the solution. This was realized by using an aqueous salt solution ($n = 1.33$) or methylethylketone ($n = 1.38$) as the solvent, quartz ($n = 1.458$) as the cell material, and decalin ($n = 1.478$) in the external bath. In this instance a deviation of the scattered beam takes place and the angle α corresponding to the angular displacement of the prism, is different from angle θ (Fig. 3) to which it is related by the relationship $n_1 \cos \theta = n_3 \cos \alpha$ (n_1 and n_3 being the refractive indices of the solution and the external solvent, respectively).

The scattering volume was corrected here as in the former case, by applying the usual
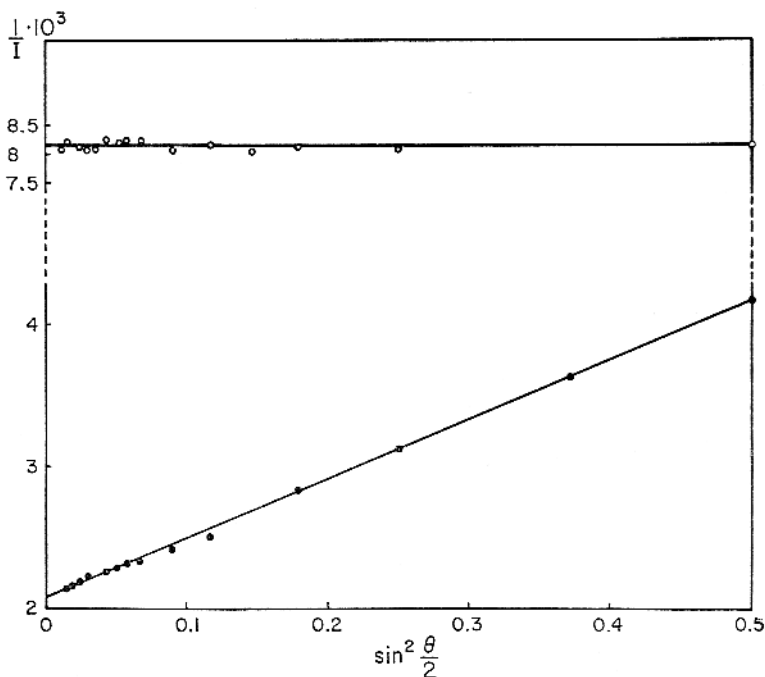
FIGURE 2 Plot of $1/I$ vs.$\sin^2 \theta/2$ for polystyrene in benzene; external liquid is benzene; glass cell. I is the scattered intensity in arbitrary units. ○, polystyrene, $M_L = 10{,}500$; •, polystyrene, $M_L = 2.3.10^6$.
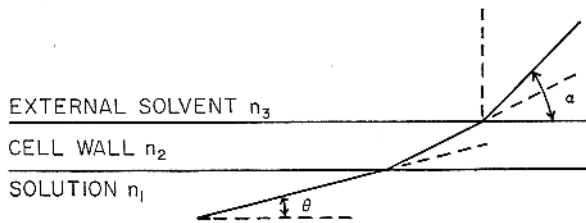

FIGURE 3 Refraction of the scattered beam at the crossing of the cell wall, when refractive indices n_1 and n_3 are different and $n_1 > n_2 > n_3$. θ is the angle between the incident and scattered beams; α is the angle of the prism displacement.

correction factor $\sin \theta \ (1 + \cos^2 \theta)$ (the term $1 + \cos^2 \theta$ results from the fact that unpolarized light was used), although geometrical considerations seemed to suggest that in this instance a correction factor involving $\sin \alpha$ should be used instead. The results given in Fig. 4 show that the usual correction is fully satisfactory, this being probably due either to the multiple reflections which are taking place at the cell walls, or to the convergence of the incident and scattered beams. Indeed, the samples of relatively low molecular weight (polystyrene in methylethylketone, $M_L = 4 \times 10^4$, and polyvinylpyrrolidone in M NaCl, $M_L = 4 \times 10^4$) show that the scattered intensity is not dependent upon the angle θ. A sample of higher molecular weight (polystyrene in methylethylketone, $M_L = 8 \times 10^6$) shows a linear dependence of the reciprocal scattered intensity upon $\sin^2 \theta/2$. Here, too, the same results were obtained by using the conventional instrument.
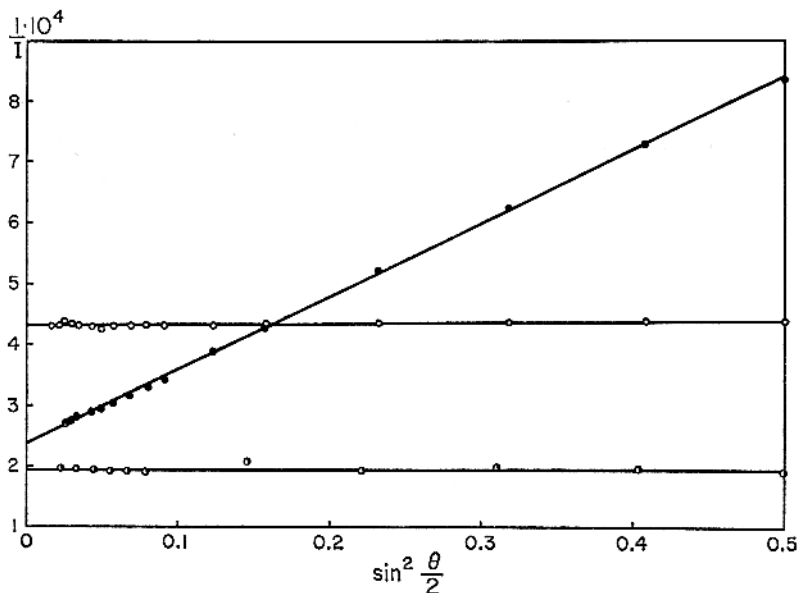
FIGURE 4   Plot of $1/I$ vs.$\sin^2 \theta/2$; external liquid is decalin; quartz cell. ○, polystyrene in methyl ethyl ketone, $M_L = 40{,}000$; •, polystyrene in methyl ethyl ketone, $M_L = 8.10^6$; ◑, polyvinylpyrrolidone in M NaCl, $M_L = 40{,}000$.

C. *Clarification Procedure.*   Solvents and solutions were clarified for measurements with both the low-angle and the conventional instrument according to one of the following procedures.

1. Polystyrene samples in benzene were filtered through Millipore filters of 0.3 and 0.45 μ pore size (Millipore Filter Corporation, Bedford, Massachusetts) directly into the light-scattering cell.

2. Polystyrene samples in methylethylketone were centrifuged 1 hour at 25,000 g in a Phywe "Pirouette" centrifuge. Only the top 25 ml in each nylon centrifuge tube (total volume 50 ml) was transferred to the cell.

3. Polyvinylpyrrolidone in M NaCl was filtered through a very fine sintered glass filter directly into the cell.

The solutions used for the DNA measurements were clarified in the following way (13). They were first shaken with half a volume of a chloroform–isoamyl alcohol mixture 5:1 for 2 hours, using a Jouan wrist–action shaker operated at a rate just great enough to cause complete emulsification. The emulsions were poured into cylinders. The upper aqueous layer while still strongly opalescent due to the presence of suspended droplets of the organic mixture was pipetted into the centrifuge tubes and centrifuged for 2 hours at 25,000 g. The top 25 ml of each tube was transferred into the light-scattering cell with a clean pipette provided with a propipette rubber bulb and a filter paper plug. During the suction process, the pipette tip was always kept 0.5 to 1 cm beneath the surface of the liquid. This avoids contaminating the solution with dust from the surface and from the bottom. Before using the pipette to suck up the solution actually used for the measurements, it was rinsed twice with the top 25 ml from two centrifuge tubes containing the same solution.

The cell that was used for the low-angle studies has a working capacity of about 25 ml and a teflon cover with two small holes for introducing the solution. In every case it was rinsed five times with double distilled water which was filtered directly into the cell through a very fine sintered glass filter. Each separate rinsing took 2 hours. The cell was further rinsed twice with the solvents clarified according to one of the procedures described above and once or twice more with the DNA or synthetic polymer solution.

Table I shows a comparison of the data obtained with the DNA solvents as clarified either according to the above method or to the usual clarification procedure which does not involve the emulsifying treatment. It appears that the dissymmetry of the solvent is about 1.05 when using the chloroform–isoamyl alcohol treatment as compared with about 1.35 when this was not used. Furthermore the scattered intensity at 16° of the solvents which had been emulsified is quite reproducible and at least four times lower than the best values obtained with the untreated solvents.

RESULTS

The results obtained from the measurements of DNA solutions are presented in Figs. 5, 6, and 7 and in Table II. A comparison of the data obtained using the Wippler and

TABLE I

LIGHT-SCATTERING OF SPECIALLY CLARIFIED SOLVENT

θ	16	21	24	30	37.5	45	60	75	90	105	120	135	142.5
I_1	1350	580	425	175	102	70	43	30	29	28	38	66	98
I_2	{7200 / 6000	2200 / 2000	1060 / 1300	273	135	89	52	35	30	31	41	66	101

I, intensity of the light scattered at angles θ between the incident and the scattered beams, by 0.13 M NaCl + 0.01 M potassium phosphate pH = 6.8.
I_1, solvent treated with chloroform–isoamyl alcohol.
I_2, solvent treated by the usual clarification technique (the intensity of light scattered by benzene at 90° was 300).

TABLE II

LIGHT-SCATTERING RESULTS ON DNA SOLUTIONS

DNA samples	Concentration		$\dfrac{Kc}{R} \cdot 10^7$		$M_L \times 10^{-6}$		$\dfrac{\rho}{A}$	
	a	b	a	b	a	b	a	b
	γ/ml	γ/ml						
B13a	211.5	211.5	21.6	21.4	0.465	0.47	700	750
B13b	106.0	132.0	7.5	7.1	1.33	1.40	1100	1100
B3		116.7		1.8		5.5	2260	2260
B13c	36.5	40.5	1.63	1.65	6.15	6.05	2590	2670
B11c	151.3	151.3	1.35	1.10	7.4	9.4	1310	1630
B1a	33.0	33.0	1.18	0.6	8.5	17.0	2260	3910

* Columns a and b give the results obtained by using the Wippler and Scheibling (12) and the low-angle instrument, respectively.
‡ For a description of the methods used in the preparation of DNA samples see text and reference 2.
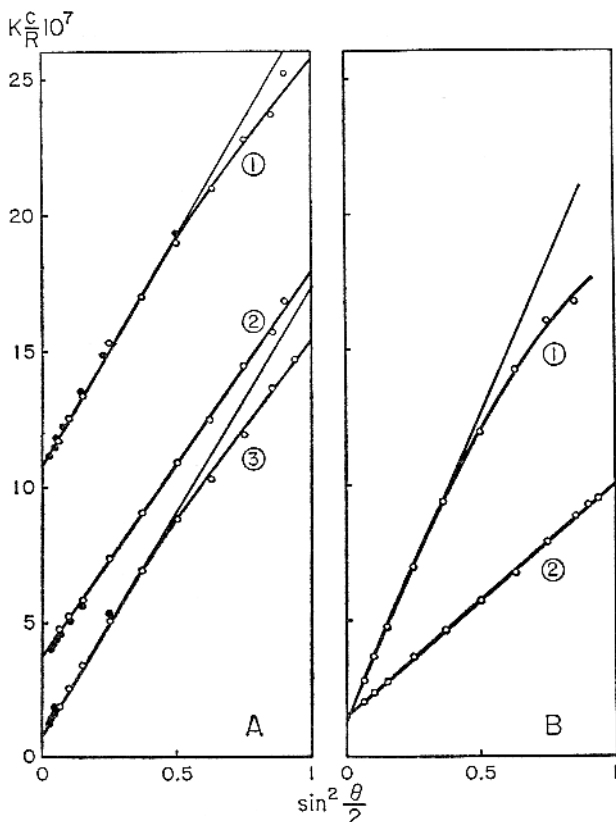
FIGURE 5   Plot of $Kc/R\theta$ vs.$\sin^2 \theta/2$ for DNA samples. o, data obtained in the Wippler and Scheibling instrument; •, some of the data obtained in the low-angle light-scattering instrument. A① B13a; A② B13b; A③ B13c; B① B1a; B② B11c.

Scheibling (12) apparatus with those obtained for the same samples with the low-angle instrument shows that no significant differences are found when the molecular weights are lower than or equal to about 6.10^6. For higher molecular weights an error of variable magnitude is made when extrapolating linearly to zero angle data obtained in the usual angular range 30–150° (see Fig. 7).

DISCUSSION

For DNA solutions the sources of error arising from the use of Zimm plots (14) to perform the linear extrapolation to 0° from angles as high as 30° have already been widely discussed elsewhere (5, 6). Doubts can be raised about the validity of the linear extrapolation mainly on the following grounds: (a) the model to choose for DNA is not exactly known; it is therefore impossible to draw the corresponding theoretical curve; (b) even if a correct model were available, an estimate of the
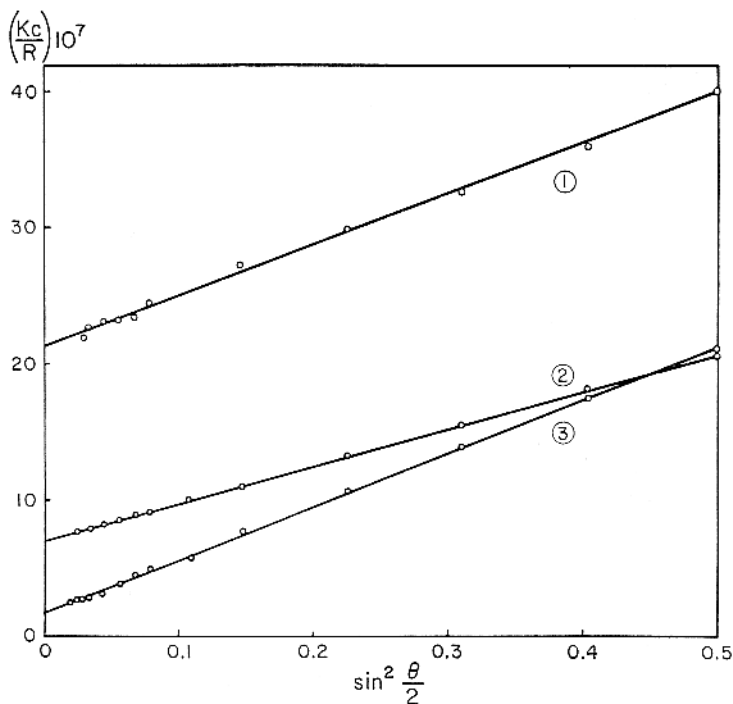
FIGURE 6   Plot of $Kc/R\theta$ vs.$\sin^2 \theta/2$ for DNA samples. Data obtained in the low-angle light-scattering instrument. ① B13a; ② B13b; ③ B13c.

polydispersity of DNA samples is impossible at this time. The results presented above show that DNA preparations, displaying molecular weights up to about 6.10^6 and radii of gyration up to about 2600 A, give essentially the same results when low-angle data are used instead of data obtained in the higher angular region. Fig. 6 shows the range which was explored by the two sets of experiments. The theoretical curves corresponding to Gaussian coils and rigid rods are given (Fig. 8) since it is assumed that DNA has a shape intermediate between these two models. Of course, a curvature in the angular range 0–16° cannot be ruled out, but, if present, it would probably be due to a very small amount of large aggregates of no real interest.

On the other hand, samples like B11c and B1a show a wide discrepancy between the results obtained at the low and the usual angles. Apparently this discrepancy is much greater in the case in which the radius of gyration is larger. Anyway, it is evident that data obtained in the usual angular range for these two preparations do not give even a suggestion as to how large will be the error caused.
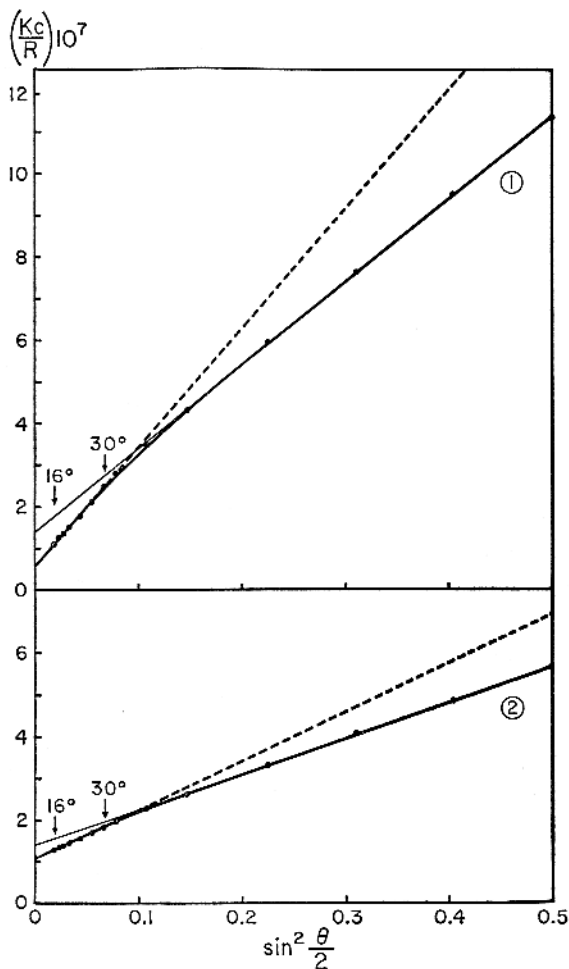
FIGURE 7   Plot of $Kc/R\theta$ vs. $\sin^2 \theta/2$ for DNA samples. Data obtained in the low-angle light-scattering instrument. ① B1a; ② B11c.———, initial slope.

Our results are therefore at variance with those presented by Katz (8) who found essentially no difference in the extrapolated values at the lower angles for a DNA sample having $M_L = 12.10^6$ and $\rho = 3700$ A. This discrepancy, however, might be ascribed to the DNA sample which was used. Finally, it should be pointed out that the shear gradients produced during the clarification procedure were much smaller than those produced during the deproteinization of the DNA samples. Therefore an effect of shearing forces, caused by the mild shaking involved in the clarification tech-
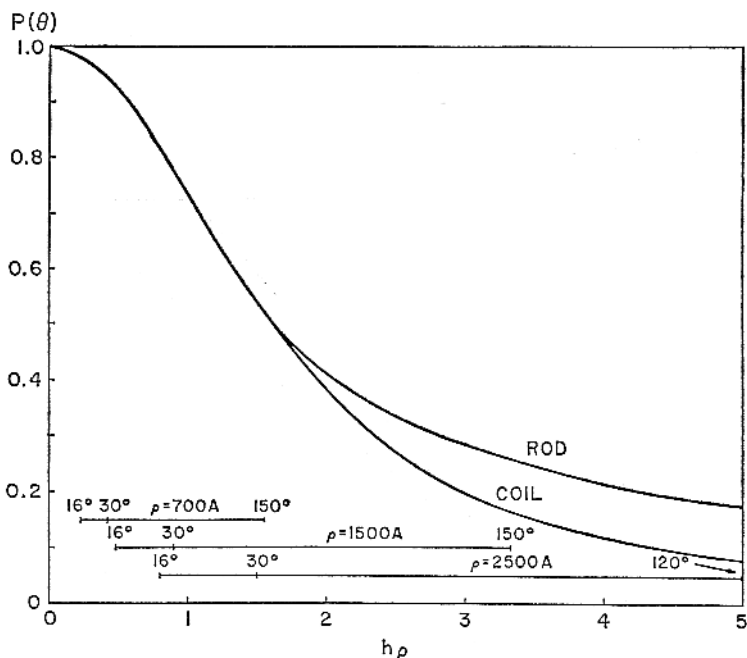
FIGURE 8 Theoretical curve of $P(\theta)$ *vs.* $h \, \rho$ for a coil and a rod; h is equal to $4\pi/\lambda'$ sin $\theta/_2$; λ' is the wavelength of the light in the solvent; ρ is the radius of gyration.

nique, on the molecular weight distribution of the DNA samples is not to be expected here.

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