ANALYTICAL DETERMINATION OF THE BUOYANT DENSITY OF DNA IN ACRYLAMIDE GELS AFTER PREPARATIVE CsCl GRADIENT CENTRIFUGATION

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1. Introduction

The use of buoyant density centrifugation in CsCl gradients generated during centrifugation is a well-established technique for determining the base composition of DNA [1–4]. The analytical determination of base composition is usually carried out with an analytical ultracentrifuge which when equipped with ultraviolet optics and film detection permits the analysis of 0.1 μg of DNA. The use of CsCl gradients generated in a preparative ultracentrifuge required 1–10 μg of a given DNA species to be detected by ultraviolet absorption upon fractionation of the gradient through conventional flow cell systems.

Several laboratories [5–7] have demonstrated that CsCl gradients may be generated in the presence of acrylamide, bisacrylamide, secondary catalyst and riboflavin and that DNA banded in the gradient may be fixed upon exposure of the gradient–acrylamide mixture to light. However, no information has been presented which has characterized the accuracy of this technique for the determination of the buoyant densities of various DNA species. We have used this technique to determine the buoyant densities of various DNA's and have shown that gradients may be generated in the preparative ultracentrifuge which may be gelled, stained, and analyzed permitting the accurate determination of base composition on as little as 0.2 μg of DNA.

2. Experimental

2.1. *DNA standards*

DNA was purified from exponential cultures of *Bacillus cereus* (a strain isolated, identified, and carried at the Department of Microbiology, University of Florida, Gainesville, Fl.) and *Escherichia coli* MRE 600 (kindly provided by J.P. Richardson, Department of Chemistry, University of Indiana, Bloomingston, Ind.) as previously described [8]. *Micrococcus luteus* DNA was obtained from Miles Laboratories Inc., Kankakee, Illinois. Chloroplast DNA was purified from chloroplasts of *Euglena gracilis* isolated from renografin gradients as previously described [9]. DNA from the bacteriophages ϕ25 and SP15 and 4 'unknowns' (No. 2159, 3819, 4213, 3903) were kindly provided by M. Mandel, M.D. Anderson Tumor Institute, Houston, Texas. DNA concentrations were determined by the indole method [10].

2.2. *Chemicals and reagents*

Reagent chemicals were: optical grade CsCl, Harshaw Chemical Co.; enzyme grade Tris, Schwarz/Mann; acrylamide, *N*,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), and 1-ethyl-2-[3-(1-ethylnaphtho[1,2d]-thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2d]-thiazolium bromide, commonly called 'Stains-all' [11], Eastman Organic Chemicals Inc.; formamide (99%), Matheson-Coleman-Bell; riboflavin, Nutritional Biochemicals Co. Acrylamide was recrystallized twice from chloroform, the bisacrylamide twice from acetone.

Reagent solutions for preparing gradients have been described by Cole [6]. Solution B was prepared by

adding 11.4 g Tris and 1.2 ml TEMED to 33 ml distilled water. Upon dissolution, the pH was adjusted to 6.9 with 85% H_3PO_4. Solution C was prepared by dissolving 0.735 g bisacrylamide and 24 g acrylamide in water to a final volume of 50 ml. Solution BC was prepared by mixing solutions B and C in the ratio 1.49 ml B to 1.00 ml C. Reagents were filtered through glass fiber discs and stored in the dark at room temperature.

Saturated riboflavin solutions were prepared fresh within two weeks of use. Three mg riboflavin were mixed for 1 hr in 10 ml diluted water at room temperature in the dark. The saturated solution was filtered through a glass fiber filter and stored in a dark bottle at 4°C.

'Stains-all' solutions were prepared according to Dahlberg et al. [11]. A stock solution of 0.1% 'Stains-all' in formamide was distilled with water and formamide to give a final concentration of 0.005% in 50% formamide. This final staining solution was used once and then discarded.

2.3. *Preparation of gradients*

To each 2 ml cellulose nitrate centrifuge tube (Beckman #303369) were added 1.76 g solid CsCl followed by 0.73 ml of distilled water and 0.50 ml solution BC. DNA samples in 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) or 0.1 × SSC were added along with 1 × SSC so that the combined volume of sample and SSC was 0.220 ml. The final concentrations for acrylamide and bisacrylamide were 4.8% and 0.15%, respectively. In a dimly illuminated room (a red safe light may be used), 0.025 ml of saturated riboflavin was added. The contents of each tube (capped with parafilm) was mixed vigorously with inversion. A few drops of mineral oil were layered over the top, the tubes were placed in adapters (Beckman #303376) and the caps (Beckman #303624) were secured with a wrench. The adapters were placed in a type 50 Ti rotor and centrifuged at 35000 rpm for 44 hr (36 hr is adequate) in a Beckman L2 preparative ultracentrifuge, 23°C, brake off.

2.4. *Analysis of gradients*

After centrifugation the caps were removed from the tubes and the adapters containing the tubes were placed in a vertical position in a rack in ice. The tubes were illuminated with a 250 W incandescent bulb placed 4 inches directly above the tubes for 30 min. The polymerized gels were removed from the the tubes by sliding a 20 guage needle between the gel and the tube and forcing the gel out with water from a syringe. The gels were washed in distilled water for 1.5 hr, rinsing continuously for three-15 min intervals in test tubes. The water was decanted and the gels were covered with 'Stains-all' and placed in the dark for 12 hr. The gels were destained with several changes of water for 6 or more hours.

After destaining, blue bands of the stained DNA were visible in the gel. These were quantitated by analysis at 600 nm using a Gilford Model 2400 Spectrophotometer equipped with a Model 2410 linear transport. Relative positions were recorded as fractions of gel length to allow for slight differences due to swelling of the gels during washing.

3. Results and discussion

Fig. 1 demonstrates the resolution of this technique. While resolution of *Euglena* chloroplast DNA (ρ 1.683 g/cc) and *B. cereus* DNA (ρ 1.696 g/cc) was not quantitatively complete (fig. 1A), the peak concentrations were resolved to permit accurate identification. The background which is most prominent at the bottom of the gel is due to stained RNA present in the *B. cereus* and chloroplast DNA preparations. Fig. 1B is a profile analogous to fig. 1A except that the sample containing *B. cereus* DNA was omitted. The staining capacities of the various DNA samples are similar if not equivalent. Thus the staining of DNA by 'Stains-all' may permit the quantitation of the DNA when an appropriate standard is used.

Fig. 2 demonstrates a curvilinear relationship between relative position in the gradient and the density of DNA. The curve was drawn to give the best fit to points plotted from the peak positions in fig. 1A. Points representing peak positions for fig. 1B (•) fall on a curve only slightly different from that presented. Using the presented curve, the error for interpolating densities from the positions in fig. 1B is not greater than 0.001 g/cc. The pronounced curve in the denser portion of the gradient may be explained by the decreasing ratio of volume to distance found in the curved portion of the centrifuge tube, as observed by Flamm et al. [12]. The fact that a slight curvilinear relationship exists in the less dense portion of the gradient is difficult to explain. Assuming a completely

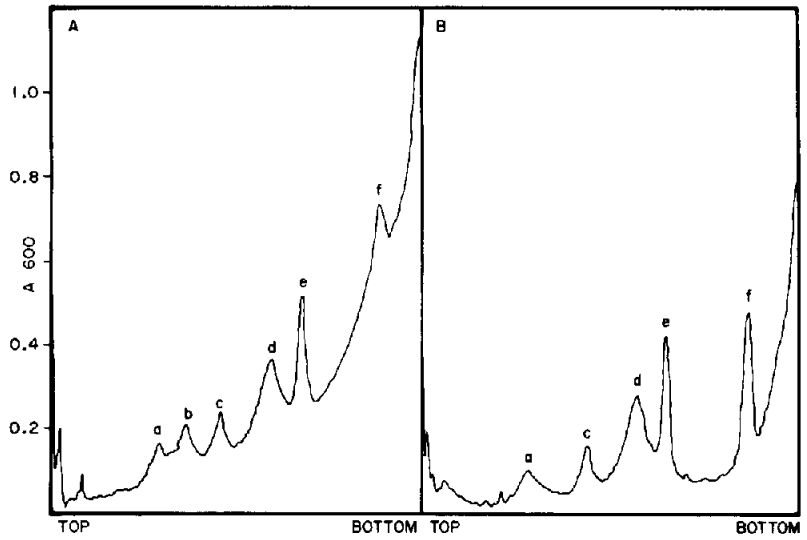

Fig. 1. Scans of stained CsCl gradient gels A: a) *Euglena gracilis* chloroplast DNA, 0.7 μg, 1.683 g/cc; b) *Bacillus cereus* DNA, 0.5 μg, 1.696 g/cc; c) *Escherichia coli* DNA, 0.5 μg, 1.710 g/cc, d) *Micrococcus luteus* (*M. lysodeikticus*) DNA, 3.4 μg, 1.731 g/cc; e) ϕ25 DNA, 1.4 μg, 1.742 g/cc; f) SP15 DNA, 1.7 μg, 1.761 g/cc. B: as in A except that *B. cereus* DNA was omitted. Listed density values are based upon CsCl buoyant density centrifugation in a Beckman Model E analytical ultracentrifuge [3, 9, or M. Mandel, personal communication].

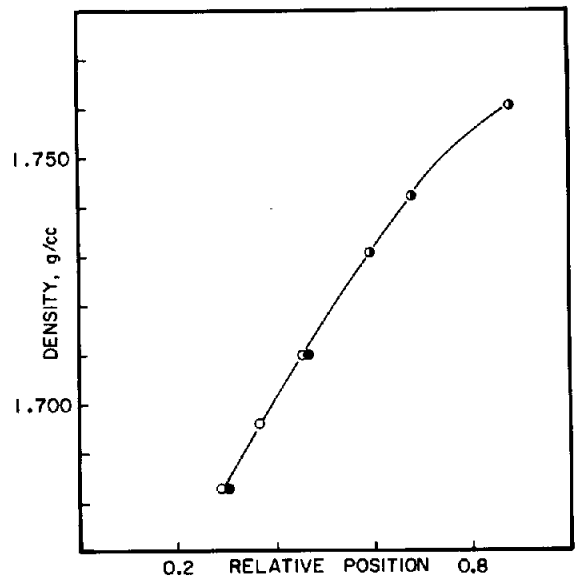

Fig. 2. Relationship of relative position of DNA in CsCl gradient gel to DNA density. Peak positions of fig. 1A are given by open circles; those of fig. 1B are closed circles. Where peak positions are equivalent, points are given as half closed circles.

linear relationship between relative position and density between 1.683 g/cc and 1.731 g/cc will result in an error no greater than ±0.001 g/cc. For determinations of density accurate to the third decimal point, a French Curve has been calibrated for a given gradient

Table 1
Comparison of density values obtained after CsCl gradient centrifugation in a Model E with those obtained after CsCl-acrylamide gradient centrifugation in a Model L2.

No.	Source	Density, g/cc	
		Mandel Model E	Model L2
2159	Para-1 induced tumor in Syrian hamster	1.6995 (2)*	1.699
3819	*Bdellovibrio stolpii* UK 1.2	1.7007 (3)*	1.700
4213	*Thiocapsa* sp. CA2210	1.725	1.724
3903	*Pseudomonas aeruginosa* 385	1.727	1.726

* Number in parentheses refers to number of replicate analyses.

profile which may be used with two standards to identify the position of an unknown.

The accuracy of this method relative to that obtained by buoyant density centrifugation in a Beckman Model E analytical ultracentrifuge was determined by analyzing four DNA samples characterized by Dr. M. Mandel and provided as unknowns to us. The results are presented in the table 1 and demonstrate that the values obtained in CsCl-acrylamide gradients all agree within 0.001 g/cc with those obtained in CsCl gradients analyzed in the Model E.

The technique described here has the following advantages over currently used methods for the analytical determinations of the buoyant densities of DNA; a) as many as 12 samples may be processed at once (as many as 20 may be processed at once in a Beckman 30.2 rotor). Where time of centrifugation is rate limiting to a given experimental design, a large number of samples may be processed in a given amount of time; b) gels may be analyzed by radioautography or slicing and radioactive counting to determine the position of labelled components, permitting specific radioactivity determinations on analytical quantities of specific DNA species; c) a large number of samples are convenient to analyze relative to liquid gradient fractionation, since the DNA bands are stable either stained or unstained for many days after centrifugation and polymerization and d) the cost of the system used here is only a small fraction of that for a system utilizing the analytical ultracentrifuge. Experiments are in progress to detect the DNA fluorometrically as a complex with ethidium bromide. This should increase the sensitivity to permit the detection of as little as 0.01 μg of DNA.

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