EQUILIBRIUM CENTRIFUGATION OF DENSITY LABELLED *Escherichia coli* DEOXYRIBONUCLEIC ACID IN LINEAR AND NONLINEAR CsCl DENSITY GRADIENT

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The banding of density labelled *Escherichia coli* DNA molecules upon centrifugation in linear and nonlinear CsCl equilibrium gradients is described, DNA was density labeled either by growing cells in the presence of glucose-[^{13}C] and $^{15}NH_4Cl$ or by growing in a bromouracil-containing medium. A linear CsCl gradient was formed in a SW 50·1 swinging-bucket rotor which was suitable for the distribution of DNA molecules density labelled with bromouracil with relative density differences of 0·05 g/cm^3. A nonlinear CsCl density gradient formed upon centrifugation of a CsCl solution in an angle-head rotor was suitable for the separation of DNA molecules density labelled with ^{13}C and ^{15}N showing differences of 0·015 g/cm^3.

The method of equilibrium centrifugation in density CsCl gradients has received widespread application in the elucidation of problems of synthesis, structure, and function of bacterial and viral nucleic acids. Equilibrium centrifugation in density CsCl gradients permits changes in buoyant density and thus in the chemical composition of DNA molecules to be recorded. For these reasons this technique has been frequently used both in analytical and in preparative experiments.

The technique of equilibrium centrifugation in CsCl gradients was used first by Meselson and Stahl[1] to obtain a proof of the semiconservative mode of DNA replication; since that time this technique has become more and more useful in studies on the kinetics of DNA replication since it enables mother and daughter DNA strands to be distinguished and their interaction to be examined[2–4].

The most frequent techniques of density labelling of DNA molecules of microbial cells are either the incorporation of 5-bromouracil, a base analog replacing thymine, or the growth of the cells in a so-called heavy medium in which glucose-[^{12}C] and $^{14}NH_4Cl$ have been substituted by their heavy derivates containing stable isotopes, ^{13}C and ^{15}N. The density labeling with bromouracil is advantageous since it offers larger differences in the density of heavy and light DNA yet its application is limited by the harmful effect of the analog on the viability of the cells[5]. Density labeling with [^{13}C]-glucose and ^{15}N-H_4Cl has not been observed to interfere unfavorably with the metabolic activity of cells yet the differences in the density of heavy and light DNA strands are relatively small (Table I).

Preparative equilibrium centrifugation was originally effected in hight-speed swinging-bucket rotors. Fischer and coworkers[7] were the first to demonstrate separation of DNA molecules upon CsCl density equilibrium centrifugation in angle-heads rotors. The advantage of angle-head rotors over swinging-bucket rotors is their higher tube capacity and according to Flamm and co-

workers[8] also a higher loading capacity and a better resolution of DNA strands of similar density. The differences in loading and separation capacity can be accounted for by a different geometry of the rotors and thus by formation of steep linear gradients in swinging-bucket rotors and of shallow, nonlinear gradients in angle-head rotors[8,9].

This study describes a comparison of separation of DNA molecules labelled with bromouracil and with ^{13}C and ^{15}N by CsCl equilibrium centrifugation either in swinging-bucket rotor or in an angle-head rotor.

EXPERIMENTAL

Escherichia coli strains B/r thy$^-$ trp$^-$ Hcr$^+$ and *Escherichia coli* 15T$^-$ 555—7 were used in our experiments. The composition of the synthetic culture medium containing glucose has been described elsewhere[10]. The concentration of the essential components added to the medium was 2 μg of thymine and 14 μg of tryptophan per ml with *E. coli* B/r Hcr$^+$ strain and 2 μg of thymine, 14 μg of tryptophan, 38 μg of arginine, and 30 μg of methionine per ml with the *E. coli* 15T$^-$ 555—7 strain.

The $^{13}C^{15}N$ *density labelling* was effected by growing cells in a culture medium to which 0·1% of $^{15}NH_4Cl$ (atomic purity 99%) instead of 0·2% of $^{14}NH_4Cl$ and 0·1% of glucose-[^{13}C] (atomic purity 50%) instead of 1% of glucose-[^{12}C] had been added. This culture medium was designated as the heavy medium; the culture medium of normal composition was the light medium. For radioisotope labelling of DNA the cells were grown for several generations in the heavy medium containing 1 μCi of thymine-[2-^{14}C] per ml. The cells in the exponential phase of growth ($1 . 10^8$ of cells per ml) were chased for 10 min with 2% of glucose-[^{12}C] and 100 μg/ml of thymine. The cells were subsequently filtered off, transferred to the light medium and irradiated with ultraviolet light in certain cases. Thymine-[6-^3H] (5—10 μCi per ml) was then added to the light medium. Samples (2—5 ml) for the isolation of DNA were taken at individual time intervals of cultivation in the light medium.

TABLE I

Buoyant Densities of *Escherichia coli* DNA in CsCl

The data are given in g/cm^3. The density values of DNA are those reported by Billen and coworkers[3,6].

Type of DNA		Labelling	
		bromouracil	$^{13}C^{15}N$
Double-stranded	LL	1·710	1·710
	HL	1·754	1·726
	HH	1·800	1·741
Single-stranded	L	1·725	1·725
	H	1·815	1·755

The density labelling with 5-bromouracil was effected by growing cells in a medium in which thymine (2 μg/ml) had been replaced by 5-bromouracil (10 μg/ml). The bromouracil-containing medium was designated as the heavy medium. The cells were pre-labelled by being grown for several generations in the light medium containing thymine-[2-^{14}C] (0·1 μCi/ml). When the concentration of the cells was 1 . 10^8 of cells per ml, the cells were filtered off and transferred to a medium in which thymine had been replaced by bromouracil. Following a 15-min incubation in this medium the cells were irradiated by ultraviolet light in certain cases and 5-bromouracil-[6-^{3}H] (1 μCi/ml) was added. Samples for the isolation of DNA were taken at individual time intervals of cultivation in the heavy medium.

Isolation of DNA *and equilibrium* CsCl *gradient centrifugation.* DNA was isolated by the lysozyme--pronase method described by Hanawalt and Cooper[11]. The cells were washed with a buffer (0·1M--NaCl, 0·01M EDTA, 0·1M Tris, pH 8) and suspended in this buffer diluted 1 : 10. The cell suspension was rapidly frozen and thawed and lysozyme (200 μg/ml) was added. Subsequently the suspension was incubated with the enzyme 15 min at 37°C. Pronase (100 μg/ml) and 2 drops of 10% Sarkosyl (detergent) solution were added and the cell lysate was incubated 30 min at 60°C. After cooling to room temperature (25°C), 0·3 ml of the cell lysate was mixed with 2·7 ml of CsCl solution; the final density value was adjusted to 1·725 g/cm^3 in the case of native $^{13}C^{15}N$ density labelled DNA and to 1·735 g/cm^3 in the case of native DNA density labelled with bromouracil. Denatured DNA was prepared by heating the cell lysate 15 min at 95°C and rapid cooling in an ice bath. The cell lysate (0·3 ml) was mixed with 2·7 ml of the CsCl solution; the final density value was adjusted to 1·740 g/cm^3 for $^{13}C^{15}N$ density labelled denatured DNA and to 1·750 g/cm^3 for denatured DNA density labelled with bromouracil. The samples were homogenized with the CsCl solution in glass homogenizers and transferred to polyallomer tubes. After the tubes had been filled up with a mineral oil, centrifugation in the 50 Ti angle-head rotor was carried out 40 h at 35 000 rpm. Alternatively the samples were transferred to cellulose tubes, 1 ml of a mineral oil was added and centrifugation in the SW 50·1 swinging-bucket rotor was performed 65 h at 30000 rpm. In both cases Spinco Model L-2-65B ultracentrifuge was used. After centrifugation at 20°C individual fractions were collected on Whatman No 3 paper strips. The strips were washed with 5% trichloroacetic acid and 96% ethanol and the radioactivity of the high molecular weight fraction was determined in a Tri Carb 3375.

RESULTS AND DISCUSSION

The density CsCl gradient is formed during centrifugation. Its final profile is a function of several parameters such as type of rotor, volume of the sample analyzed, initial density of the CsCl solution, centrifugal force, and length of centrifugation period. Since the buoyant density of DNA molecules in CsCl solutions has a constant value we chose, according to experimental data of other authors[3,4] and from our own experience, such conditions of centrifugation which lead to the desired density range of CsCl. The conditions for the angle head 50Ti rotor and for the swinging bucket SW 50·1 rotor are described under Experimental. The profiles of the gradients obtained are shown in Fig. 1. As obvious from the Figure, centrifugation in the SW 50·1 rotor yields a steep linear gradient whereas a shallow, nonlinear gradient is obtained by centrifugation in the 50Ti angle-head rotor. The density was calculated from refractive index values (n_D) of CsCl solutions, measured in an Abbe refracto-

meter, using the formula: density 25°C = 10·8611 . $n_D^{25°}$ − 13·4974 according to Vinograd and Hearst[12].

When double-stranded DNA molecules labelled with bromouracil were analyzed a good separation was obtained by centrifugation in the SW 50·1 rotor (Fig. 2*a*). Under these conditions a density gradient of 0·05 g/cm³ per 1 ml of the solution collected had formed. Since the differences in the density of double-stranded DNA

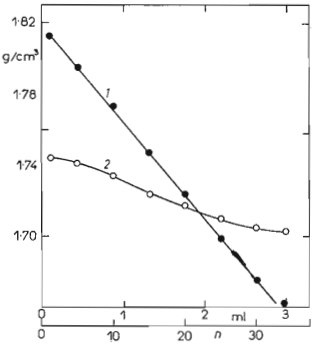

FIG. 1

Density Profiles of CsCl Gradients

1 Centrifugation in the SW 50·1 rotor at 30000 rpm, 65 h; *2* centrifugation in the 50Ti rotor at 35000 rpm, 40 h. *n* number of fractions.

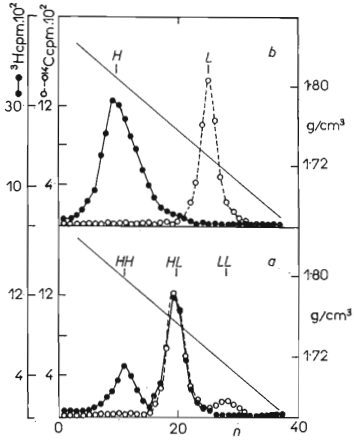

FIG. 2

Density Distribution of *Escherichia coli* 15T⁻ 555—7 DNA Labelled with Bromouracil by Isopycnic Centrifugation in SW 50·1 Rotor

The cells were radioactivity labelled by growing in the light medium containing thymine [-2-^{14}C]. The cells were transferred in the exponential phase of growth to a medium in which thymine had been replaced by bromouracil-[6^{3}H]. After 90 min of cultivation in the heavy medium samples for the isolation of DNA were withdrawn. *a* density banding of double-stranded DNA; *b* density banding of single-stranded DNA. *n* number of fractions.

labelled with bromouracil are 0·059 g/cm³ between heavy-heavy (HH) and heavy-light (HL), and 0·047 g/cm³ between HL and light-light (LL), the gradient is suitable for their separation. Because of sufficiently large differences in density, single-stranded heavy (H) and light (L) DNA molecules can also be well resolved under these conditions

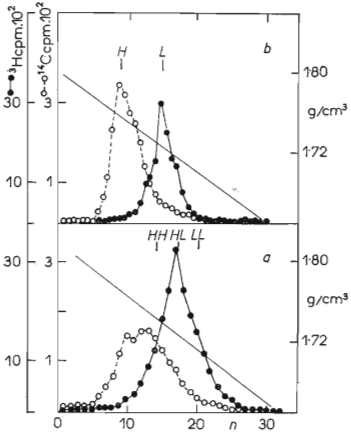

FIG. 3

Density Banding of $^{13}C^{15}N$ Labelled DNA Isolated from *Escherichia coli* B/r Hcr$^+$ Cells by Isopycnic CsCl Centrifugation in SW 50·1 Rotor

The cells were density labelled (with $^{13}C^{15}N$) and radioactivity labelled (with thymine-[-2-^{14}C]) in the exponential phase of growth. They were transferred to the light medium, irradiated with ultraviolet light (300 erg/mm²) and subsequently thymine-[-6-^{3}H] was added. After 120 min of cultivation in the light medium samples for the isolation of DNA were taken. The density banding of double-stranded DNA (*a*) and single-stranded DNA (*b*), *n* number of fractions.

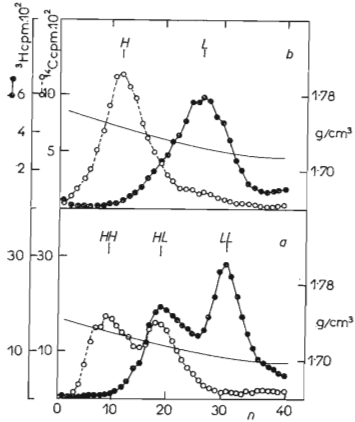

FIG. 4

Density of $^{13}C^{15}N$ Labelled DNA, Isolated from *Escherichia coli* Bcr H'r$^+$, by Isopycnic CsCl Centrifugation in 50Ti Rotor. The treatment of the cells and the designation are the same as those given in Fig. 3. The first *y* scale should be designated in ^{3}H cpm · 10³.

of centrifugation (Fig. 2*b*). The quantity analyzed in one gradient corresponded to DNA isolated from about $1-2 \cdot 10^8$ of cells. HH, HL, and LL double-stranded, $^{13}C^{15}N$ density labelled DNA molecules could not be distinguished by centrifugation in the SW rotor (Fig. 3*a*). The density differences between these molecules, however, are 0·015 g/cm^3 only (Table I) and cannot be recorded in the density gradient formed. The separation of single-stranded H and L DNA (density difference 0·03 g/cm^3) was attained, however, even in this type of gradient (Fig. 3*b*).

A good separation of $^{13}C^{15}N$ density labelled DNA molecules can be achieved by centrifugation in an angle-head 50Ti rotor in which a shallow nonlinear gradient is formed. The maximum density change per 1 ml of solution collected is 0·019 g/cm^3 in the middle part and 0·009 – 0·012 g/cm^3 at the margins. Figs 4*a* and 4*b* show the banding of double- and single-straded $^{13}C^{15}N$ labelled DNA molecules. As can be seen in these figures the entire gradient is exploited and a good separation of both double- and single-stranded DNA molecules is obtained under these conditions of centrifugation. The gradient formed under these experimental conditions was 5-times more shallow (as regards the change of density as a function of volume) and thus the separation was 5 times better and the loading capacity could be increased. The DNA analyzed in the angle-head rotor in our experiments was isolated from $5 \cdot 10^8$ to $1 \cdot 10^9$ of cells.

The results given above show that the advantage of angle-head rotors lies in the formation of a very shallow density gradient which permits DNA molecules of similar densities to be separated from each other. We were able to obtain in 50Ti rotor a good banding of DNA molecules which showed a difference in density of 0·015 g/cm^3 only (Fig. 4*a*). Centrifugation in the SW 50·1 rotor afforded a steep density gradient which is necessary for the separation of DNA molecules showing larger differences in density, *i.e.* 0·050 g/cm^3 (Fig. 2*a*).

The appropriate choice of the conditions of centrifugation, *i.e.* of the centrifugal force and time of centrifugation, as well as of the volume of the gradient analyzed and type of rotor, permits optimum conditions of banding of DNA molecules differing in density to be obtained. The experimental data presented in this paper confirm the advantages of centrifugation in an angle-head for the separation of DNA molecules which differ only little in density upon centrifugation in equilibrium CsCl gradients.

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