DENSITY GRADIENT RELAXATION:

A METHOD FOR PREPARATIVE BUOYANT DENSITY SEPARATIONS OF DNA

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SUMMARY

A multi-speed centrifugation procedure, called the "density gradient relaxation method", has been applied to buoyant density separations of milligram quantities of a DNA mixture. With relaxation, larger quantities of DNA can be separated in shorter times than with conventional one-speed centrifugations.

The goal of preparative buoyant density centrifugation of a mixture of two species of DNA is to band the species with least overlap, thus facilitating the usual drop-collecting methods. In practice, this is often difficult to achieve when moderate (milligram as opposed to microgram) quantities of a DNA mixture need to be separated. We have devised a multi-speed "density gradient relaxation method" which alleviates the problem of overloading by reducing the overlap between two bands. The method is illustrated by the separation of about 3 milligrams of an approximately 70:30 mixture of two species of DNA ($\Delta\rho_B$ = 0.02 g/ml) in a single run, using a conventional swinging-bucket rotor. Although experimental conditions, such as time, have not been optimized, the ease with which such separations can be carried out, and the possible usefulness of this method in other buoyant density separations prompt us to report our results.

The method involves a short period of high-speed centrifugation, followed by a long period of centrifugation at reduced speed. The first period serves to

concentrate and segregate the two species of DNA according to their buoyant densities. The second period serves two purposes: the density gradient "relaxes" and the DNA species seek new buoyant density positions. It is known that density gradients due to cesium chloride are established rapidly (Van Holde and Baldwin, 1958); thus the time required in the second period is due to the movement of the macromolecules to their appropriate density positions. Since the new gradient profile can be calculated according to the equation $\frac{d\rho}{dr} = K\omega^2 r$ (Ifft, Voet and Vinograd, 1961), it is clear that at reduced speed the two DNA species move farther apart. As centrifugation can be stopped at any stage, it is not necessary, nor even desirable, that the two species reach their new equilibrium positions in the gradient. Banded DNA is collected at a position which is not its equilibrium position, thus effecting a saving in time.

MATERIALS AND METHODS

DNA was extracted (Marmur, 1961) from a culture of Escherichia coli 600-1 after a growth sequence of > 15 generations in a labeled medium (99% D_2O, 99.5% $^{15}NH_4Cl$ as the sole nitrogen source) and about 1.9 generations in a non-labeled medium. The growth experiment was carried out in connection with a project on DNA replication (Anet, Strayer and Boyer, work in progress). This DNA was expected to contain equal amounts of non-labeled and hybrid DNA, that is one D-^{15}N-containing strand. T_2 DNA was prepared by Dr. O. C. Richards (Richards and Boyer, 1965) and Micrococcus lysodeikticus DNA was a gift from Mr. P. Poon.

Analytical bandings were performed on a Beckman Model E, and preparative runs on Beckman-Spinco Model L and Model L-2 centrifuges. Beckman-Spinco swinging-bucket rotors SW 50L and SW 25.1 were used for preparative centrifugations. U.V. films were analyzed on a Beckman Analytrol densitometer, and preparative gradients analyzed at 260 mμ on a Zeiss spectrophotometer with 0.5 ml cells (1 cm path length), using the first fraction in the reference cell.

Preparative centrifugations were performed with CsCl solutions (ρ = 1.716 - 1.7222) in 0.01 M EDTA-Tris (pH 8) containing 75-120 μg/ml of

D-^{15}N-containing DNA. Cesium chloride solutions, 2.2 ml (SW 50L) or 8.3 ml (SW 25.1) were topped with mineral oil (washed to remove water-soluble impurities) and collected (100 drops/ml) in 3 drop (SW 50L) or 10 drop (SW 25.1) fractions in 0.6 ml 0.015 M NaCl-0.0015 M Na Citrate (Baldwin and Shooter, 1963). Single strand separations were performed with the SW 50L rotor but at pH 12.4 and ρ_{CsCl} =1.778 (Baldwin and Shooter, 1963).

RESULTS AND DISCUSSION

Analytical banding (44,770 r.p.m., 25° C) of D-^{15}N-containing DNA is shown in Fig. 1a. An approximate 70:30 mixture of non-labeled:hybrid (one D-^{15}N strand) is revealed. The buoyant density difference ($\Delta\rho_B$) is calculated as 0.02 g/ml, using ρ_B for non-labeled DNA as 1.710. Confirmation of $\Delta\rho_B$ is shown in Fig. 1b with T_2 DNA (ρ_B = 1.700) and Micrococcus lysodeikticus DNA (ρ_B = 1.731) as markers (Schildkraut, Marmur and Doty, 1962). Hybrid DNA is not resolved from Micrococcus lysodeikticus DNA.

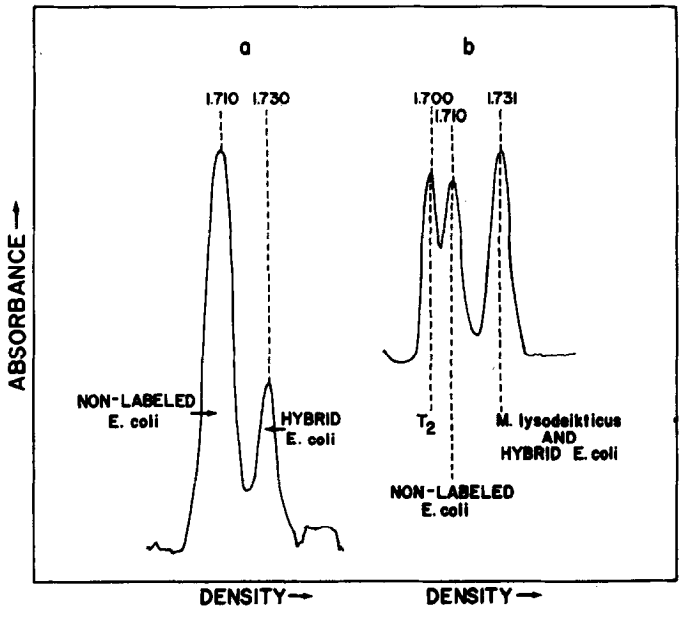

Fig. 1. Demonstration of a) the composition of D-^{15}N-containing DNA mixture from Escherichia coli; b) the buoyant density difference between non-labeled and hybrid E. coli DNA. Hybrid DNA is not resolved from M. lysodeikticus DNA.

Density gradient relaxation centrifugation is compared with high-speed and low-speed analytical centrifugations in Fig. 2. Densitometer traces (set 1) show that the D-^{15}N-containing DNA mixture (18 μg) is barely separable after 17 hours at 44,000 r.p.m., even though the two species have banded at their equilibrium positions. Traces B, C and D (set 1) show that upon reducing speed to 26,000 r.p.m., non-labeled DNA and hybrid DNA move farther apart. It is clear that after 36 hours at 26,000 r.p.m. (total time, 53 hours), the mixture is easily separable (trace C), even though the species have not reached

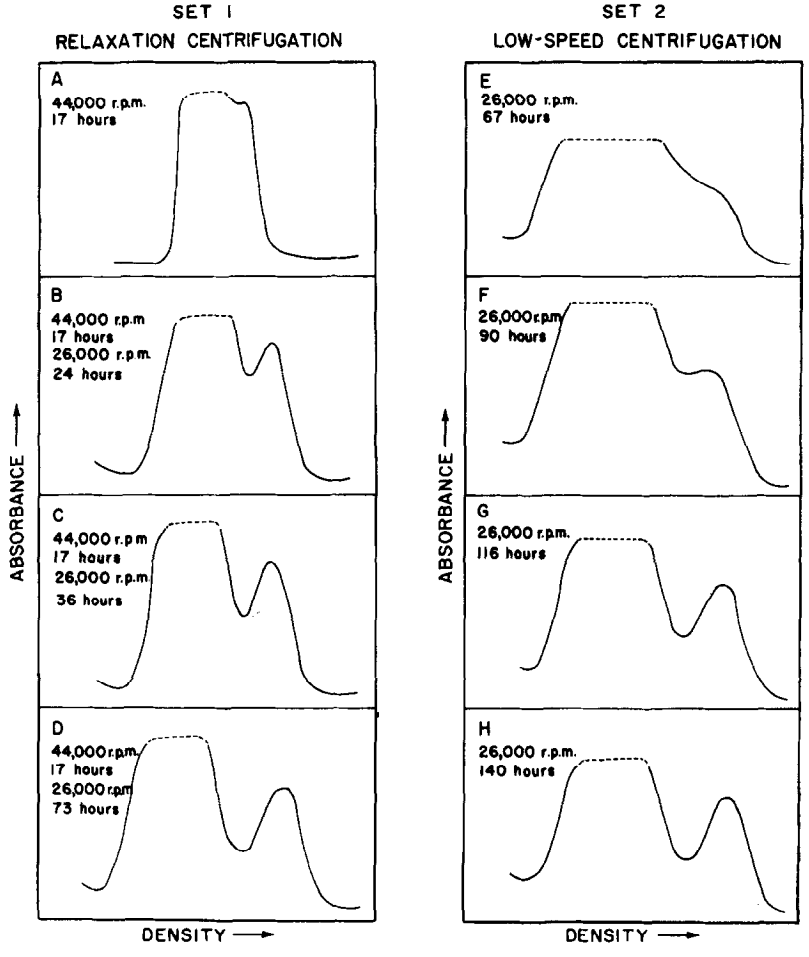

Fig. 2. Comparison of relaxation centrifugation (set 1) with low-speed analytical centrifugation (set 2) on D-^{15}N-containing E. coli DNA (18 μg). The maximum of non-labeled strands is not obtained from the densitometer traces. The traces show a flat region, depicted in dotted lines.

their new equilibrium positions [trace C compared with trace H (set 2)]. Since the maximum absorbance of the non-labeled DNA could not be obtained from the densitometer traces, two preparative centrifugations, each with 200 μg of the DNA mixture were carried out, and the profiles of the collected gradients (92.5 hours, SW 50L) are shown in Fig. 3a (low-speed) and Fig. 3b (relaxation). Thus for the same time and the same concentration, relaxation centrifugation gives better separation than low-speed centrifugation alone.

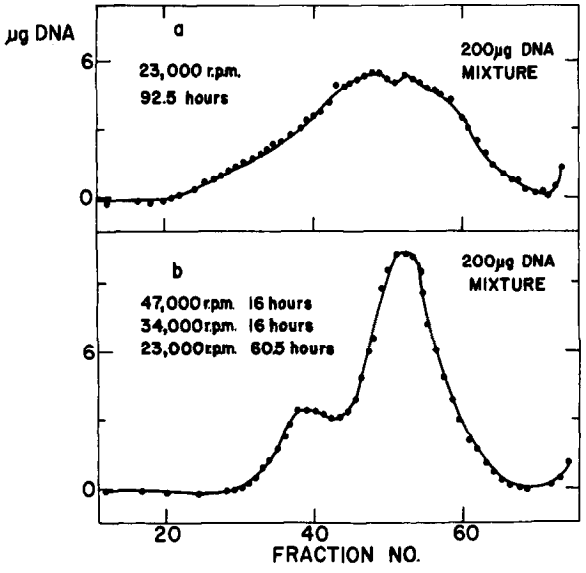

Fig. 3. Comparison of preparative low-speed (a) and relaxtion (b) centrifugations with a D-^{15}N-containing DNA mixture. The micrograms of DNA were calculated from measured absorption at 260 mμ according to Burgi and Hershey (1961).

The separation of 1 mg/ tube (SW 25.1) of the D-^{15}N-containing DNA mixture is shown in Fig. 4a, using relaxation centrifugation (24,000 r.p.m., 76 hours followed by 15,000 r.p.m., 168 hours). Similar separations have been obtained with 600 μg/tube (SW 25.1) in 6.7 days (24,000 r.p.m., 63 hours, 17,000 r.p.m. 97.5 hours); and 150 μg/tube (SW 50L) in 4.7 days (39,000 r.p.m., 36 hours, 23,000 r.p.m., 78 hours). The purity of fractions 20-48 (Zone I) (Fig. 4a) is shown by pooling the corresponding fractions from different runs and preparative single strand separation (SW 50L) at pH 12.4 (39,000 r.p.m., 22 hours,

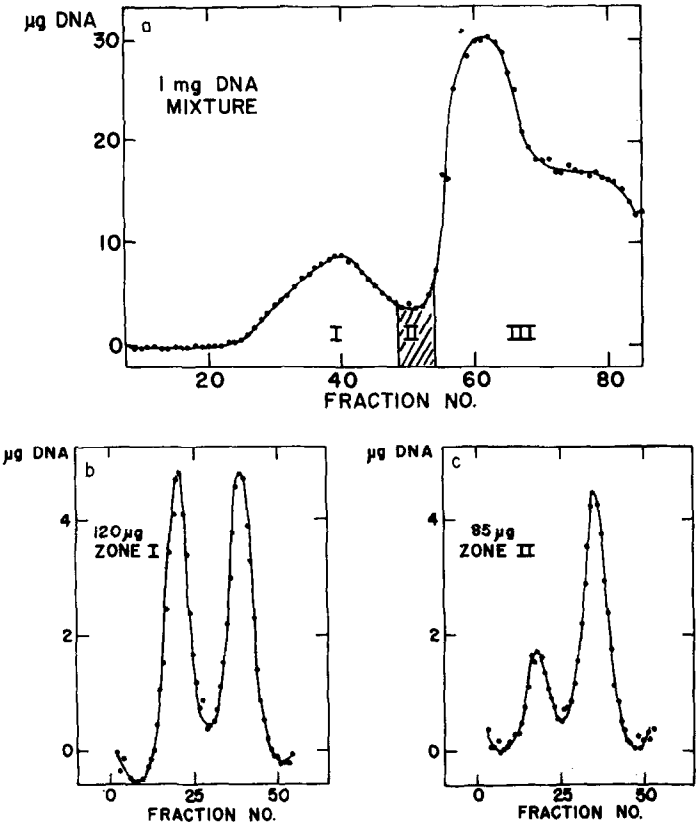

Fig. 4. Relaxation centrifugations: a) Separation of 1 mg/tube of D-^{15}N-containing DNA mixture; b) Demonstration of purity of zone I (4a); c) Distribution of non-labeled and hybrid DNA in zone II (4a); the micrograms of DNA were calculated according to Burgi and Hershey (1961) for double-stranded DNA and a correction applied for the known hyperchromicity of DNA on denaturation for single-stranded DNA (Marmur and Doty, 1959).

29,000 r.p.m., 78 hours). The 1:1 distribution of D-^{15}N-labeled and non-labeled strands (Fig. 4b) clearly shows that nearly pure hybrid DNA was obtained after relaxation centrifugation. The proportions of hybrid DNA present in fractions corresponding to fractions 49-53 (Zone II) can be judged from the single strand separation (SW 50L) shown in Fig. 4c. In all preparative separations, we have consistently recovered at least 70% of the original DNA.

The results presented in this paper show that relaxation centrifugation can be useful in reducing overlap. Relaxation centrifugation is advantageous in time over low-speed centrifugation, and thus may be applicable to mixtures

with small buoyant density differences. The time required for the first period may possibly be reduced with the rapid equilibrium isopycnic cesium chloride gradients developed by Bruck and Leick (1968). The time required for the second period will depend on the flotation (sedimentation) velocity and hence the molecular weight distribution in the DNA species.

An extension of the density-gradient relaxation method is the separation of two DNA bands at interfaces with immiscible liquids. We have performed such experiments on an analytical centrifuge with a hexane-CsCl-fluorocarbon system. These results, as well as the details of the relaxation method (including optimum relaxation speeds, which appear to be about 0.7 times initial speed) will be published in a fuller account.

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