CsCl DENSITY GRADIENT CENTRIFUGATION STUDIES OF INTACT BACTERIAL CELLS

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ABSTRACT Cells of *Escherichia coli* have been successfully banded in CsCl density gradients and a portion of the population reclaimed in a viable state. Differentiation between two strains of this organism in a CsCl density gradient has been demonstrated also. Several studies were undertaken to see whether differences could be detected between two samples of cells of the same strain which had been subjected to different conditions. The results were as follows: (*a*) Introduction of a heavy label (5-bromouracil) into the DNA during a 90 minute period did not produce an observable change in cell density. (*b*) Removal of a required amino acid from the growth medium of an *E. coli* auxotroph resulted in an increase in both the density and heterogeneity of the cells. (*c*) Exposure of cells to 27 kr of gamma radiation, followed by a period during which portions of both DNA and RNA were lost, yielded two distinct bands, one at the normal position in the gradient and the other shifted to a lighter region.

INTRODUCTION

In studies of the action of various kinds of agents on bacterial cells an important question asked concerns the population of cells which have been altered *versus* those which are unchanged. For example, an interpretation of an effect of radiation on cells would be different if it were known whether all cells are equally altered or whether some are altered and others are not. The ability to select molecules and viruses on the basis of density has been of great use and it seemed possible that it could be extended to cells. The present investigation, therefore, was undertaken to determine whether the CsCl density gradient procedure of Meselson, Stahl, and Vinograd (1) could be used to study intact bacterial cells. While this work was in progress, two other laboratories reported successfully separating microbial cells using density gradient-forming materials other than CsCl. Juhos (2) employed the colloidal silica sol Ludox (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) to separate *E. coli* from T4 bacteriophage, and the separation of vegetative cells and spores of *Bacillus megaterium* was achieved by Tamir and Gilvarg (3), using

preformed Urografin gradients (Schering-Kahlbaum, Berlin, Germany). These authors were also able to separate *E. coli* W from vegetative cells of *B. megaterium*. In the CsCl studies reported here, the concern was not only with trying to band cells and determine the number remaining viable, but also to ascertain whether cells subjected to various experimental conditions could be distinguished from untreated cells of the same strain.

MATERIALS AND METHODS

Bacteria

The bacterial strains employed were *E. coli* B, ATCC 11303 and a thymine- and leucine-requiring auxotroph of *E. coli* 15 obtained from Dr. Stanley Person of this laboratory.

Media

The synthetic C minimal medium described by Roberts *et al.* (4) was used as the liquid culture medium in all experiments. To permit growth of *E. coli* 15 T^-L^- it was necessary to supplement this medium with 2 mg/liter thymine and 40 mg/liter leucine (both Calbiochem, Los Angeles, Calif., A grade). When ^{32}P was used to label the cells, the phosphate in the medium was reduced by a factor of 10.

Viability assays after centrifugation were conducted by plating on a medium which contained per liter of distilled water: 6 g Difco Bacto-Tryptone, 16 g Difco yeast extract, 13 g Difco Bacto-Agar, and the same thymine supplement as above.

Radiochemicals

Thymine-2-^{14}C, specific activity 24 mc/mM, and 5-bromouracil-2-^{14}C, specific activity 31 mc/mM, were obtained from Calbiochem. Radioactive phosphorus in the form of $Na_2{}^{32}PO_4$, specific activity 50 mc/mM, was acquired from New England Nuclear, Corp., Boston, Mass.

CsCl Centrifugation

The CsCl used was Trona UV-grade (American Potash and Chemical Corp., Los Angeles, Calif.). Samples for centrifugation were prepared by mixing, in a 5.0 ml cellulose nitrate centrifuge tube, a concentrated solution of CsCl in distilled water brought to pH 8.4 with sodium hydroxide, with a sufficient volume of cell suspension to yield a final volume of 3.0 ml and an initial density of approximately 1.40 g/cc. Although there was some variation between experiments, these initial conditions could usually be fulfilled by mixing 2.7 ml of a 0.689 g/ml CsCl solution with 0.3 ml of cell suspension (or with 0.3 ml of a mixture of cell suspensions when more than one strain, or two samples of the same strain treated differently, were centrifuged in the same tube). Each tube was finally topped with a 1.1 ml layer of mineral oil. Centrifugation was for 72 hours at 30,000 rpm and 6°C with the SW39L rotor in the Spinco model L2 ultracentrifuge. Very probably shorter times would have been adequate.

At the end of the centrifugation period, each tube was in turn mounted firmly in a special fixture and connected, by means of rubber tubing and a one hole rubber stopper, to an apparatus which permitted variation of the air pressure above the liquid column and thereby provided control over the flow rate of the solution from the tube.

The bottom of the tube was then pierced and a series of approximately 50 fractions were

collected. The density of the fractions was determined on the basis of refractive index measurements.

Total Cell and Viability Analysis

To determine the viability of the cells after centrifugation, the osmotic strength of the fractions was slowly lowered by first adding 0.1 ml of 3 × concentrated normal growth medium to each fraction and then by slowly adding normal growth medium drop by drop until a final volume of approximately 5.0 ml was reached. The total number of cells per fraction was estimated with a Coulter counter Model A (Coulter Electronics, Chicago). The number of viable cells was assayed by plating a series of dilutions for each fraction. The plates were incubated for 24 hr at 37°C after which they were scored for colonies.

Radioactivity Analysis

To each fraction was added 1.0 ml of ice-cold 10% trichloroacetic acid (TCA). After being held in an ice bath for about 1 hr, each sample was filtered through a Schleicher and Schuell type B-6 filter, washed with 10 ml of cold 5% TCA, glued to a planchet, and allowed to dry. The radioactivity per sample was measured with a G-M counter (Nuclear-Chicago Corporation, Des Plaines, Ill.). In those experiments in which cells labeled with ^{14}C and ^{32}P were centrifuged together in the same tube, it was necessary to count each sample twice in order to ascertain how much of the radioactivity was due to each isotope. First, the total counts per sample (^{14}C + ^{32}P) were obtained. Next, sufficient aluminum foil was placed between the sample and the G-M tube to eliminate the ^{14}C, and the ^{32}P counts alone were determined. The difference, corrected for absorption of some ^{32}P in the foil, gave the ^{14}C counts.

Experimental Procedures

The cells were grown at 37°C with aeration and growth monitored by observing changes in turbidity at 420 mμ with a Bausch and Lomb Spectronic 20 colorimeter.

In the initial experiments, *E. coli* 15 T^-L^- was grown to midlog phase in the presence of thymine-^{14}C. The cells were harvested by centrifugation and resuspended in fresh, isotope-free medium. Samples were prepared for centrifugation using 0.3 ml aliquots of this resuspension in the manner previously described.

To compare the banding characteristics of *E. coli* B with those of *E. coli* 15 T^-L^-, the former was grown in medium containing ^{32}P and the latter in medium supplemented with thymine-^{14}C. Upon reaching midlog phase, the cells in each culture were collected by centrifugation and resuspended in fresh medium lacking the isotopes. Portions of the two resuspensions were mixed and 0.3 ml samples of this mixture were used for CsCl centrifugation.

The effect of a density label in the DNA of the cells was investigated by growing *E. coli* 15 T^-L^- to a titer of approximately 10^8 cells/ml, rapid filtering (0.45 μ Millipore), and resuspending in fresh prewarmed medium containing 5-bromouracil-^{14}C in place of the thymine supplement. The cells were allowed to grow in the presence of the heavy label for 90 min (about 1.3 generations), then were collected by centrifugation and resuspended in fresh isotope-minus medium at a concentration of 10^9 cells/ml. A portion of this resuspension was mixed with part of a control culture of *E. coli* 15 T^-L^- which had been grown in a ^{32}P-containing medium to a titer of 10^8 cells/ml, then harvested and also resuspended at 10^9 cells/ml in fresh medium. As previously, 0.3 ml aliquots of this mixture were used to prepare samples for centrifugation.

In order to examine the effect on banding of "completing the chromosome" [Maaløe and Hanawalt (5)], *E. coli* 15 T^-L^- was grown in thymine-^{14}C supplemented medium to a titer of 10^8 cells/ml. The culture was rapid filtered, washed with fresh prewarmed medium, resuspended in fresh prewarmed medium lacking the leucine supplements (+T −AA), and incubated for 70 min at 37°C with aeration. At the end of this period, the usual harvesting procedure was employed and the cells finally resuspended at a concentration of 10^9 cells/ml. A ^{32}P-labeled control culture was prepared as before and portions of the control and experimental cultures were mixed. Samples of this mixture were taken for centrifugation in the usual manner.

To observe the effect of ionizing radiation on the banding pattern, the T^-L^- auxotroph was grown in the presence of thymine-^{14}C to a titer of 10^8 cells/ml. The culture was then rapid filtered, washed twice with fresh medium, and resuspended in fresh isotope-free medium

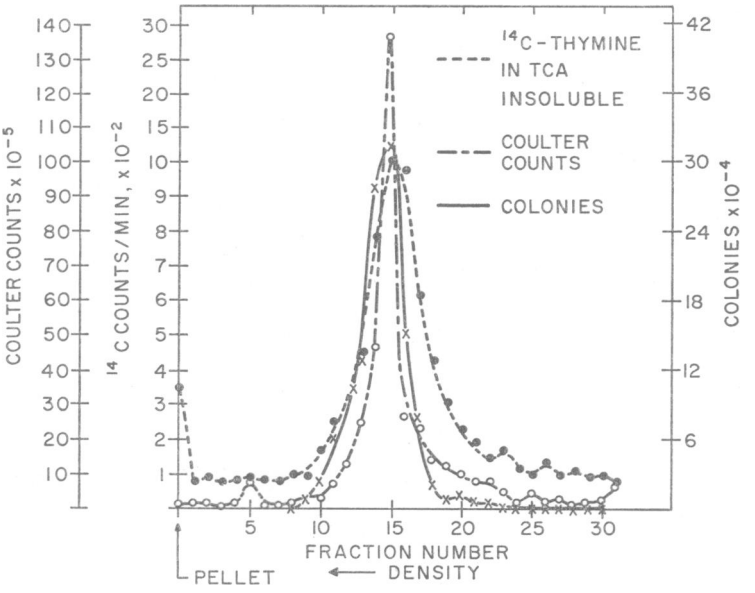

FIGURE 1 Distribution of *E. coli* 15 T^-L^- centrifuged in CsCl. Fractions analyzed for radioactivity, colony-forming, and Coulter counts.

at the original titer. The resuspension was oxygenated vigorously for 1 min and then exposed to 27 kr of gamma radiation, from a ^{60}CO source. After a 2 hr incubation at 37°C with aeration, during the course of which both DNA and RNA underwent various degrees of degradation (6–8) a portion of the irradiated suspension was mixed with part of a ^{32}P-labeled control culture and samples of this mixture taken for centrifugation.

RESULTS

The banding profile from an experiment in which the fractions were assayed for radioactivity, Coulter counts, and viability is shown in Fig. 1. The similarity of the three patterns is quite clear and establishes the fact that cells of *E. coli* cannot only be banded in a CsCl density gradient, but also that a proportion of these cells can

be reclaimed in a viable state. In Fig. 1 the colony counts are given in terms of colonies per milliliter of cells of the 5 ml dilution made from each fraction, whereas the Coulter counts are given on a per fraction basis. To compare, therefore, it is necessary to multiply colony counts by a factor of 5 and when this is done it can be seen that approximately 10% of the cells remained viable after the centrifugation treatment. Since it was desirable to reclaim as many cells as possible in the viable state, some additional studies of the effect of CsCl on cell viability were undertaken. In these, cells were suspended in CsCl at various densities for varying times and temperatures. It was found that cells held at near to zero were recoverable to about 30% while as the temperature was raised to 37°C the proportion fell to 2%. If the

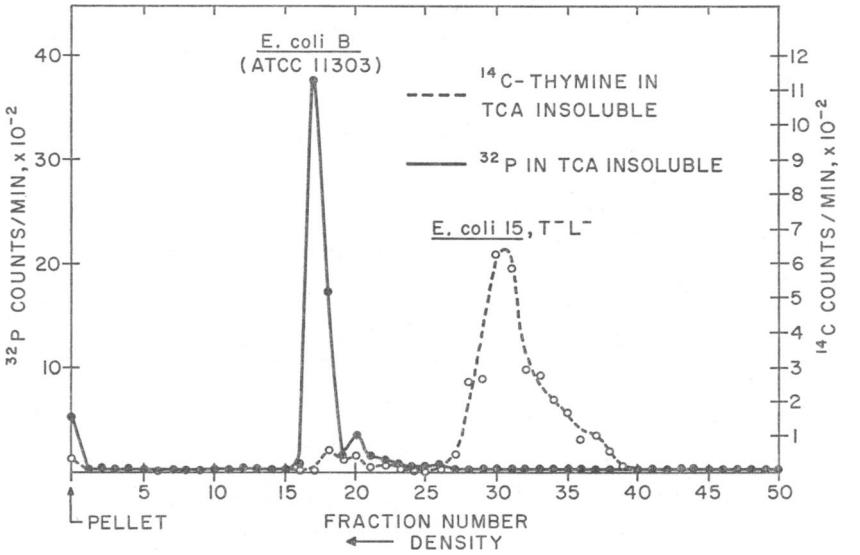

FIGURE 2 Density difference between *E. coli* B and *E. coli* 15 T⁻L⁻ in CsCl density gradient.

cells were in lag phase (over 7×10^8 per ml) the recovery approached 100% even at 37°C. Subjecting the cells to intermediate osmotic stress, as described above, increased the yield of viable cells appreciably.

The data in Fig. 2 show that *E. coli* B bands more sharply and in a more dense region of the gradient than *E. coli* 15 T⁻L⁻. The approximate density at the center of the band is 1.48 g/cc for the former and 1.42 g/cc for the latter. Perhaps the most interesting feature is the extremely sharp band formed by *E. coli* B. This phenomenon has been investigated in several experiments (data not shown) and it has been concluded that, for some still unknown reason, cells of *E. coli* B have a tendency to aggregate which results in an illusion of greater density homogeneity than actually exists. This does not seem to be true in the case of *E. coli* T⁻L⁻.

The insertion of a density label into the DNA of the T⁻L⁻ auxotroph failed to

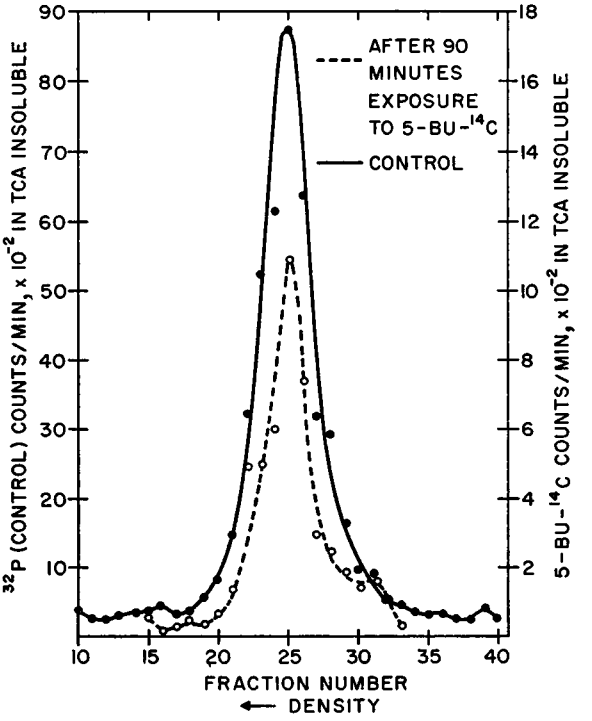

FIGURE 3 Cells exposed to 5-bromouracil for 90 min compared to normal cells. Insertion of density label into cellular DNA produced no detectable change in either the banding position or the sharpness of the band.

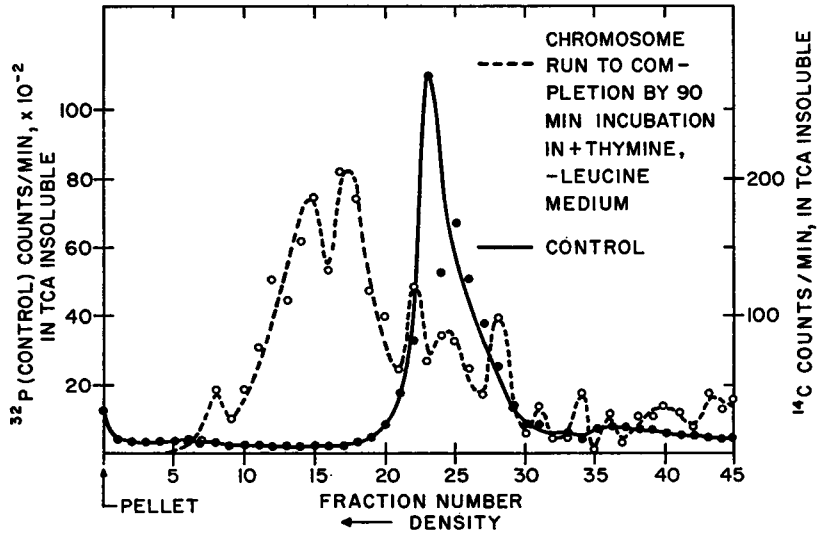

FIGURE 4 *E. coli* 15 T^-L^- allowed to "complete the chromosome" by incubating for 70 min in $^-$AA medium exhibit an increase in density and also in heterogeneity.

increase the density of the cells sufficiently to cause a shift in the banding position (Fig. 3). It is possible that, although the cells incorporated 5-bromouracil-^{14}C under the conditions described, the amount of incorporation may have been relatively small and that more extensive incorporation might have resulted in a detectable density change. In any event, we believe that whole cell density gradient sedimentation may not be sensitive enough to be used to observe the effect of density labels in individual macromolecules.

The removal of a required amino acid from the growth medium of an auxotroph has generally been accepted as permitting the round of DNA replication in process

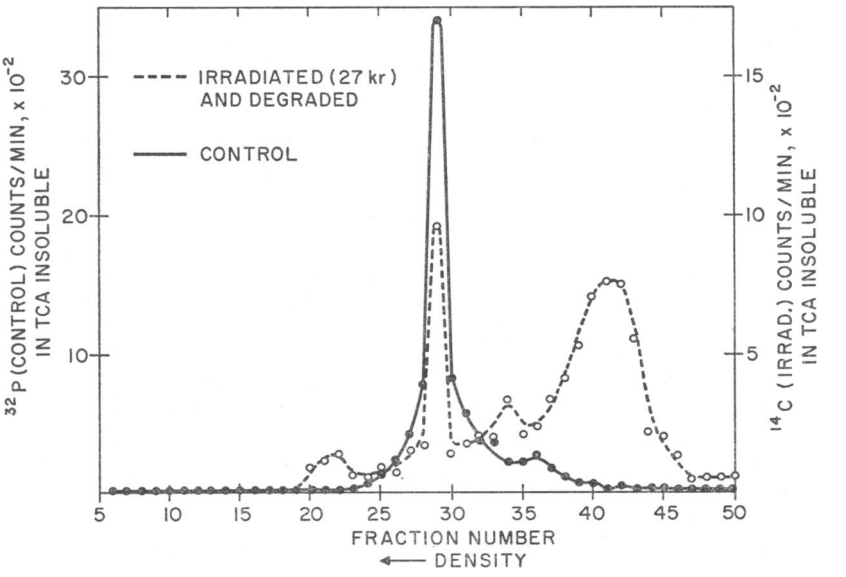

FIGURE 5 Irradiation of *E. coli* 15 T^-L^-, followed by an incubation period during which portions of the DNA and RNA are degraded, yields two bands upon subsequent centrifugation in CsCl.

in each cell at the time of amino acid removal to continue to completion, but to prevent the initiation of a new round of replication (5). The CsCl banding pattern of cells so treated is shown in Fig. 4. Although the nature of the density gradient technique is such that no conclusions can be drawn as to the state of the DNA, it is evident that a large amount of heterogeneity exists in regard to the density of cells which have been subjected to this treatment. A possible explanation, of course, is that various amounts of residual RNA synthesis occurs in cells incubated under $^-$AA conditions.

The degradation of DNA in microorganisms following exposure to ionizing radiation was initially reported by Stuy (6). The degradation of a portion of the RNA as well was noted by Drakulic and Kos (7). These phenomena have also

been studied in our own laboratory (8–10) and both types of nucleic acid loss have been observed in *E. coli* 15 T^-L^-. When a culture of these cells was irradiated, the DNA allowed to degrade to an extent measured separately as 67%, and then banded in CsCl, the results illustrated in Fig. 5 were obtained. The peak of radioactivity from degraded cells which is associated with the control peak was at first suspected to be the result of an aggregating tendency similar to that in *E. coli* B. However, repetition of the experiment omitting the control cells from the gradient also yielded two distinct peaks similar to those in Fig. 5.

DISCUSSION

The experiments reported demonstrate that bacterial cells can be banded in CsCl density gradients, that a portion of the cells remain viable throughout the treatment, and that differences between cells of the same strain subjected to different conditions can be detected. The feasibility of the technique, therefore, would seem to have been amply demonstrated. However, a priori determination of the applicability of the procedure to a given experimental situation is at the present time hindered by the fact that its resolving power remains uncertain. One example in which density differences could not be detected has been given.

The usual values quoted for the density of a bacterial cell are in the neighborhood of 1.1 g/cc (11), whereas the observed densities for the two strains of *E. coli* are considerably higher. Although there is as yet no direct supporting data, it is believed that the increased density may be the result of an osmotic effect due to the high concentration (4.1 M) of CsCl required to band the cells. It should perhaps also be noted that no gross changes in cell morphology could be detected when cells in CsCl solution were examined with a phase-contrast microscope (× 2000).

The two experiments on altered cells seem to us to be significant. In the case of the chromosome completion test the presence of a population of increased density is gratifying, but at the same time a heterogeneity in the heavy population can be seen which should repay more study. Also there is about 15% of the population which retains the normal density, a fact which clearly calls for interpretation, and which should also be considered when experiments based on the chromosome completion procedure are, in their turn, interpreted.

The radiation studies indicate that while a large fraction of cells have undergone degradation and hence loss of DNA and RNA, there does exist a fraction of the population which retains the original density. In these experiments the average DNA degradation was 67%. Since roughly 9% have no density change, or very little, those at the peak of light cells must presumably have degraded to the extent of 73%. This figure does not exclude the possibility that some cells actually degrade all their DNA so that this effect on cells could have more of the "all or nothing" character than is indicated by the average amount of degradation.

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