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Purification of Competent Cells in the *Bacillus subtilis* Transformation System

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Transformed cells have been separated from nontransformed cells by centrifugation on a density gradient of Renografin-76. Separation was achieved both on a linear gradient and on a discontinuous gradient. Under optimal conditions, all of the cells in one band (median density, 1.110 g/ml) were transformants, whereas virtually all cells in the other (median density, 1.131) were nontransformants. In some instances, recentrifugation of the transformant band further enriched the transformant population. The transformed population can also be enriched by zonal centrifugation in a linear gradient of Ficoll. However, this technique is far less efficient than centrifugation in Renografin-76. Since the density of competent cells is identical to that of transformants, we conclude that the low density is a property of competent cells. The significance of this low density to the physiology of competent cells is discussed.

Studies of competence in *Bacillus subtilis* have always been hampered by contamination of competent-cell preparations by noncompetent cells. The fraction of cells in a maximally competent population ranges from a few per cent to probably no more than 20% (24). Thus, any study of the competent culture must reflect, in large part, the properties of noncompetent cells.

Several workers have "isolated" the competent fraction of the population by studying those cells which have specifically taken up transforming deoxyribonucleic acid (DNA). Young (24) and Wolstenholme et al. (23) observed the properties of cells that were shown by autoradiography to have taken up tritiated DNA. Stocker (20), Nester and Stocker (14), and McCarthy and Nester (9) observed the properties of cells that had specifically taken up and expressed transforming DNA. However, each of these approaches has limited applicability.

Separation of competent from noncompetent cells appears feasible, since several workers have shown that these cells differ from noncompetent cells in a number of fundamental respects (9,13). Indeed, Singh and Pitale (19) reported the separation of transformed from nontransformed cells by zone sedimentation on a linear gradient of sucrose.

Recently, isopycnic gradient centrifugation in cesium chloride has been used to fractionate cells of *Escherichia coli* grown under various conditions (15). However, the recovery of viable cells is only about 10%. Using a modification of the method used by Schatz et al. for isolation of mitochondria (18), Tamir and Gilvarg (21) successfully separated cells of *B. megaterium* in various stages of sporulation from vegetative cells by isopycnic gradient centrifugation in Renografin-76, a radiopaque medium employed as an X-ray contrast material. This technique appeared especially promising because the population could be separated into several fractions without any loss of viability. The present paper presents data which illustrate the use of Renografin-76 for the separation of competent from noncompetent cells of *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains. Strains of *B. subtilis* used were 168 (*trpC168*), SBI (*hisA1 trpC168*, markers unlinked, previously designated his_1^-, try_2^-), and WB746 (prototrophic). SB1 and WB746 were derived from strain 168.

Chemicals. Ficoll (obtained from Pharmacia, Uppsala, Sweden) was dialyzed overnight against two changes of distilled water, lyophilized, dissolved in Davis minimal medium (5), and sterilized by autoclaving. Renografin-76 (Squibb Methylglucamine Diatrizoate Injection USP, E. R. Squibb and Sons, New York, N.Y.) was stored at room temperature after dilution with sterile Davis minimal medium. The commercial preparation, a 76% solution of methylglucamine diatrizoate, was designated 100% in concentration to simplify calculations (21). DNA was prepared by the method of Marmur (10), in some cases with the omission of ribonuclease treatment and isopropanol precipitation. Pancreatic deoxyribonuclease (five times crystallized) was obtained from Calbio-

chem, Los Angeles, Calif. Lysozyme was obtained from Worthington Biochemical Corp., Freehold, N.J.

Transformation. Competent cells were prepared by the method described by McCarthy and Nester (9) or by the casein hydrolysate regimen of Nester, Schafer, and Lederberg (13) modified by use of greater aeration during the first growth period. Competent cultures were frozen by the method of McCarthy and Nester (9). Freshly prepared or thawed competent cultures were routinely exposed to saturating concentrations of transforming DNA for 30 min.

Enumeration of cells. Cells were enumerated as colony-forming units. The techniques used have been described (13). The diluted cell suspensions were plated on Davis minimal agar plates with the necessary supplements. Transformants of strain 168 were scored on Davis minimal agar plates containing 0.05% acid-hydrolyzed casein; total viable-cell counts for strain 168 were made on this medium supplemented with L-tryptophan (20 μg/ml).

Formation of gradients. Linear density gradients, usually of 4.6 or 25.0 ml, were formed with a Buchler model 2-5051 or 2-5044 gradient mixer, model 2-5070 stirrer, and model 2-6100 polystaltic pump. For sterilization, it was necessary to disassemble the gradient mixer. The mixing chamber was soaked for several hours in 0.1 N NaOH. The stopcock was soaked in 95% ethyl alcohol. These components were then rinsed with sterile distilled water and reassembled. The O ring on the stopcock was forced into its channel by manipulation with the aid of cotton swab sticks. The apparatus was then rinsed several times with sterile water. The stirring bar and Tygon delivery tubing were autoclaved. Centrifuge tubes were rinsed with 95% ethyl alcohol, and then with sterile distilled water.

Because of the inconvenience and time involved in preparing sterile linear gradients, step gradients, in which the concentration of the supporting medium varied discontinuously, were often used. In most cases, step gradients were prepared in a final volume (including the sample) of 5.0 or 25.0 ml. A layer of 50% Renografin (20 to 30% of total volume) was pipetted into a sterile centrifuge tube. A layer of 30% Renografin (30 to 60% of total volume) was next gently layered onto the lower layer. Then a layer of 12.5% Renografin was layered onto the 30% layer. This 12.5% layer contained about half the final amount of 12.5% Renografin; the remainder of the final volume was the sample, a cell pellet resuspended in 12.5% Renografin. The density of Renografin as commercially supplied seems to vary slightly from vial to vial; in one experiment (Fig. 3), it was necessary to use 31% Renografin for the middle layer of the step gradient.

Application of samples. Transformed cultures were centrifuged, and the cell pellets were resuspended in 12.5% Renografin for application to gradients. The resuspended culture was 10 to 20% of its original volume when applied to a linear gradient, and 50 to 70% of its original volume when applied to a step gradient. It is essential to apply the sample in at least 2% of the total volume of the gradient. If the sample volume is too low, competent and noncompetent cells agglomerate into a clump more dense than the major fraction; the clump may contain 25% of the transformants and 15% of the noncompetent cells.

Centrifugation. Gradients of sucrose and Ficoll were formed and centrifuged at room temperature; they were centrifuged for 30 min at 1,000 rev/min in an International clinical centrifuge equipped with a Lourdes TR-50 four-place trunnion rotor fitted with model 4-10 four-place adaptors.

Gradients of Renografin were formed at room temperature and centrifuged at 4 C for 30 min at 15,000 rev/min in either the SW-39 or SW-25.1 swinging bucket rotor in a Beckman model L preparative ultracentrifuge. In some experiments, the SB-269 swinging bucket rotor was used in an International model B-60 preparative ultracentrifuge.

Collection of fractions. Fractions were collected from the bottom of the gradient into sterile tubes after puncturing the tube with a Buchler model 2-5010 or 2-5020 tube piercing unit. The piercing needle and cleaning wire were autoclaved before use. In some experiments, fractions were collected directly into the diluting buffer. Fractions containing 10 or 20 drops (approximately 0.011 ml/drop) were collected from 5-ml gradients; 80-drop fractions were collected from 25-ml gradients.

Calculation of percentage of competent cells by co-transfer of unlinked markers. N_1 and N_2 = number of single transformants, N_D = number of double transformants, C = number of competent cells, B = total number of viable cells. Markers 1 and 2 are unlinked, i.e., on different DNA molecules. If all competent cells are equally likely to be transformed, then the probability P_1 that a cell will be transformed for marker 1 is: $P_1 = (N_1/C)$. Similarly, $P_2 = (N_2/C)$. If the probability of uptake of DNA is independent of previous uptake, then the probability of a double transformation $P_D = P_1 \cdot P_2$. Thus $P_D = (N_D/C) = (N_1/C) \times (N_2/C) = (N_1N_2/C^2)$, and $C = (N_1N_2/N_D)$. The percentage of competent cells, $(C/B) \times 100$ is $(N_1N_2/N_DB) \times 100$.

RESULTS

Zonal centrifugation on linear gradients of sucrose and Ficoll. The results of Singh and Pitale (19) indicated that a newly transformed population of *B. subtilis* can be separated into two fractions, one rich in transformants and one rich in noncompetent cells. Our initial experiments, aimed at corroborating the results of Singh and Pitale, indicated that only a few per cent of the cells applied to the gradient could be recovered as viable cells. Merely allowing the transformed population to sit in Davis minimal medium containing 15% sucrose for 30 min at room temperature without aeration resulted in the loss of all but 0.2% of the transformants and all but 0.7% of the total population.

We next attempted to use Ficoll as the supporting medium for zonal centrifugation on a linear density gradient, a technique previously employed by Holter and Møller (8) to determine

the specific gravities of amoebae and amoeba fragments. Incubation of a transformed culture in a 30% solution of Ficoll allows recovery of all of the transformants and over 72% of the total population. By zonal centrifugation of a transformed culture on a linear gradient of Ficoll, we were able to achieve a maximal enrichment in transformants of threefold. However, the enrichment is less than threefold for fractions containing more than 45% of the transformants, so this method is not useful for preparative separation. We were encouraged by this partial success to try another suspending medium.

Separation of transformed from nontransformed cells on gradients of Renografin. Figure 1 shows the separation of a transformed culture into two fractions on a linear gradient of Renografin. The majority of the cells occupy a single, relatively sharp, symmetrical band, whereas a peak of lighter cells accounts for the majority of the transformants. Since the fractionation is based on a difference in density between the two populations, a greater degree of separation can be achieved by using a shallower gradient; such a gradient is illustrated in Fig. 2. The most convenient method of separation is by a step gradient, in which the concentration of Renografin varies discontinuously (Fig. 3). If 15 ml or more of the culture is applied to the gradient, the band of competent cells is readily visible (Fig. 4). Under these conditions, the competent cells can conveniently be collected with a pipette.

Recovery of viable cells from the gradient. In 12 experiments, the average recovery of viable transformants was 92% of the viable transformants applied to the gradients; the average recovery of the total viable population in nine experiments was 72%.

Densities of transformed and nontransformed cells. Since Renografin has a high extinction coefficient at 260 mμ (21), the density of any fraction can readily be assayed. The optical density measurements (Fig. 2) show that about 80% of the cells comprising the major peak lie within a density range between 1.127 and 1.136 g/ml and about 90% of the cells in the transformant peak lie within a density range between 1.105 and 1.115 g/ml. It is apparent from Fig. 2 that, after centrifugation, the range of concentrations of Renografin in the gradient is different from the starting range, presumably because the preformed gradient is not at density equilibrium.

Estimate of the enrichment of transformed cells by gradient centrifugation. Clearly, the majority of the cells band in the heavy region of both linear and step gradients of Renografin. In the experiment illustrated in Fig. 1, the second,

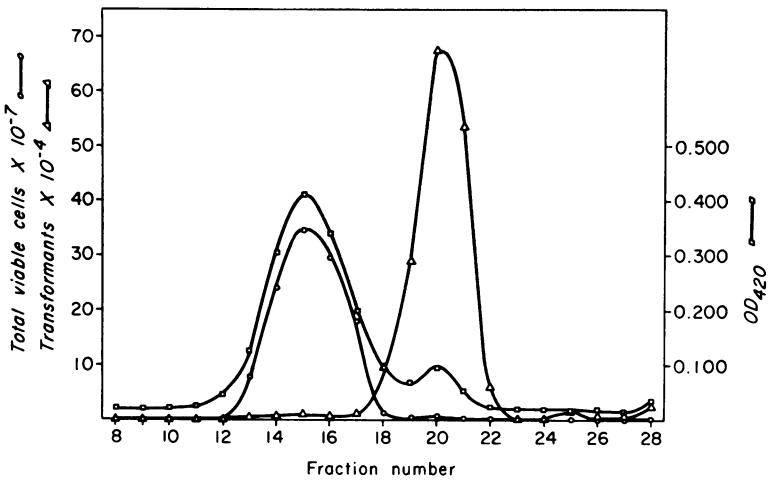

FIG. 1. *Centrifugation of a transformed culture on a linear gradient of Renografin. A 5-ml amount of a culture prepared from 0.5 ml of a freshly thawed suspension of competent cells of strain 168 was transformed by the method of Nester et al. (13) with DNA isolated from WB746. The culture was centrifuged, and the cell pellet was resuspended in 1.0 ml 12.5% Renografin. An 0.8-ml amount was applied to a 25-ml gradient varying linearly in Renografin concentration from 25 to 40%. The gradient was centrifuged for 30 min at 15,000 rev/min in a Beckman model L preparative ultracentrifuge in a SW-25.1 rotor. Fractions of 80 drops each were collected from the bottom of the gradient; each fraction was assayed for colony-forming units and diluted twofold with Davis minimal medium; the optical density at 420 mμ was determined as a measure of total cell number. The transformation frequency of the unfractionated culture was 1.32 × 10⁻³.*

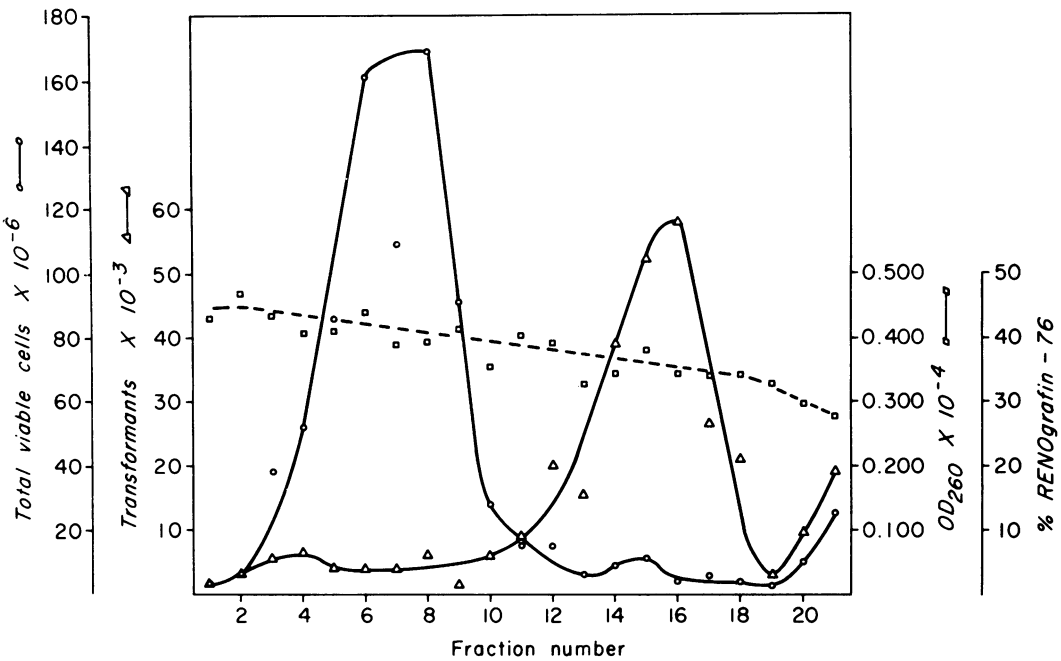

FIG. 2. *Centrifugation of a transformed culture on a shallow gradient of Renografin. A 1-ml amount of a culture prepared from 0.1 ml of a freshly thawed suspension of competent cells of strain 168 prepared by the method of McCarthy and Nester (9) was transformed with DNA isolated from WB746. The culture was centrifuged and the cell pellet was resuspended in 0.2 ml of 12.5% Renografin; 0.1 ml was applied to a gradient consisting of 4.0 ml of Renografin, varying linearly in concentration from 25 to 35%, layered onto 0.6 ml of 40% Renografin. The gradient was centrifuged for 30 min at 15,000 rev/min in a Beckman model L preparative ultracentrifuge in the SW-39 rotor. Fractions containing 20 drops were collected into 9.9 ml of Davis minimal medium; they were assayed for colony-forming units and diluted 100-fold, and the optical density at 260 mμ was determined. The transformation frequency of the unfractionated culture was 2.44 × 10⁻⁴.*

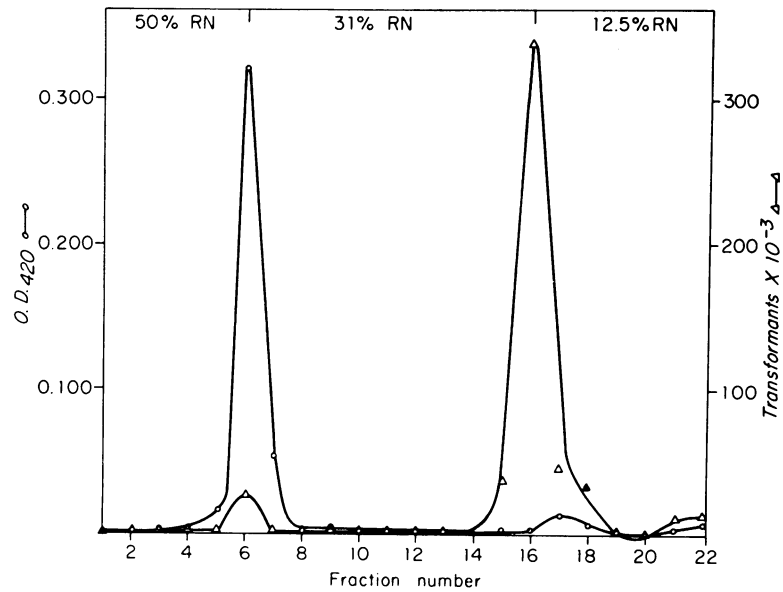

FIG. 3. *Centrifugation of a transformed culture on a step gradient of Renografin. A 4-ml amount of a culture prepared from 0.4 ml of a freshly thawed competent culture (9) of strain 168 was transformed and centrifuged, and the cell pellet was resuspended in 0.4 ml of 12.5% Renografin; 0.2 ml was applied to the gradient. The step gradient consisted of 1.0 ml of 50% Renografin, 3.0 ml of 31% Renografin and 0.8 ml of 12.5% Renografin (see Materials and Methods). The gradient was centrifuged as described in Fig. 2 and 20-drop fractions were collected. Fractions were diluted 10-fold with Davis minimal medium, each was assayed for transformants, and the optical densities at 420 mμ were determined as a measure of cell number. The transformation frequency of the unfractionated culture was 3.2 × 10⁻³. In this experiment it was necessary to use 31% Renografin because of the low density of the commercial preparation (see Materials and Methods).*

minor peak, representing approximately 2% of the total viable population, accounts for 93% of the recovered transformants. Table 1 shows that the maximal transformation frequency is 12 times that of the unfractionated culture, and 4,200 times the transformation frequency in the tube containing the greatest number of noncompetent cells.

It is not possible to determine directly the fraction of competent cells in a population. We are able to detect transformants for a single, or at most, a few markers, whereas, presumably, many competent cells are transformed at regions of the genome identical in both the donor and the recipient strain. Attempts to determine the number of competent cells directly by transfection have had limited success (3,16).

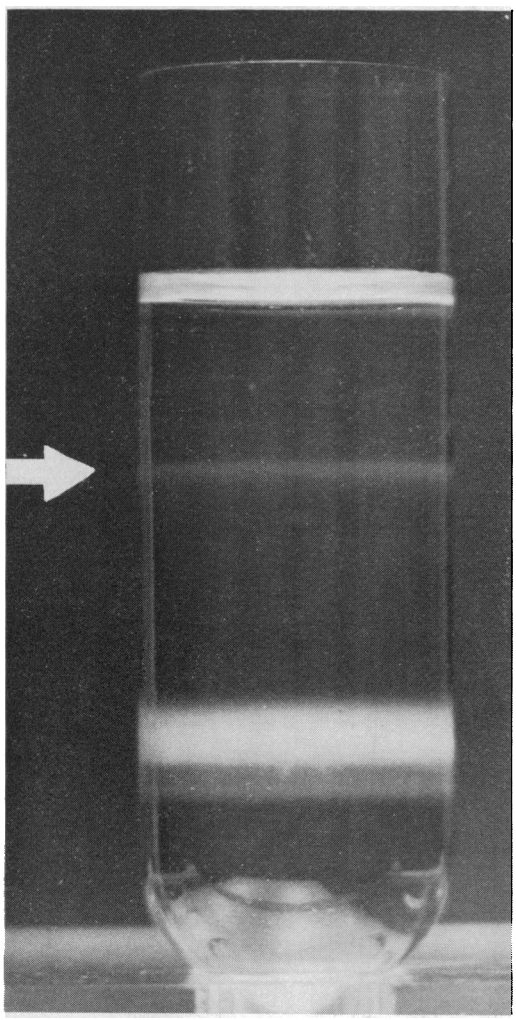

FIG. 4. *Centrifugation of a competent culture on a step gradient of Renografin. A 1.5-ml amount of a freshly thawed suspension of competent cells (9) of strain 168 was diluted twofold with 25% Renografin. This suspension was applied to a step gradient containing 8.0 ml of 50% Renografin, 12.0 ml of 30% Renografin, and 2.0 ml of 12.5% Renografin. The gradient was centrifuged as described in Fig. 1. The arrow indicates the band of competent cells.*

Goodgal and Herriott (7) suggested that the percentage of competent cells in a culture can be calculated from the number of single (N_1 and N_2) and double (N_D) transformants for two unlinked markers and the total number (B) of viable cells: percentage of competent cells = $(N_1N_2/N_DB) \times 100$. According to this formula, competent cultures of *Haemophilus influenzae* can contain 75% competent cells (7) when the transformation frequency of a single marker is 0.5 to 0.75%; the calculated percentage of competent cells in a transformed culture of pneumococcus may be greater than 100% (6).

Figure 5 illustrates the separation of transformants from nontransformants. Strain SBl, which carries the unlinked markers *trpC168*, *hisA1*, was used as the recipient. Using the formula given above, we found that only 21% of the cells in the tube containing the most transformants were competent, whereas the preceding and following tubes contained 94% and 87% competent cells. By subtracting this number of competent cells in each tube from the total number of cells, we estimated that 0.6% of the total number of noncompetent cells occur in these three tubes. Similarly, some transformants were found in the major peak. The occurrence of noncompetent cells in the low-density band can be explained in two different ways: (i) cells may actually have a low density and still not be competent, or (ii) cells of high density might be physically trapped in the transformant band. If the noncompetent cells are actually heavy, they might, in theory, be removed from the transformant band when this band is recentrifuged. Figure 6 shows the distribution of transformed and nontransformed cells when the light region of a step gradient is centrifuged on a second step gradient. There are three clear-cut peaks representing (i) the heavy noncompetent cells, (ii) the light competent cells, and (iii) competent cells with an intermediate density. In the light peak, the calculated percentage of competent cells averaged 123% in all but the leading tube. In the heavy region transformants were separated from noncompetent cells on the steep gradient formed at the interface between 50 and 30% Renografin. The fact that recentrifugation further enriched the low-density peak for competent cells indicates that the noncompetent cells must have been trapped in the light region

TABLE 1. *Levels of transformation in the unfractionated culture and in selected gradient fractions (Fig. 1)*

Source of sample	Transformants/ml	Viable cells/ml	Transformation frequency
Unfractionated culture	2.35×10^6	1.78×10^9	1.32×10^{-3}
Peak tube of major peak (no. 15)	9.0×10^3	3.46×10^9	2.60×10^{-6}
Tube between peaks (no. 18)	8.0×10^4	1.04×10^8	7.69×10^{-4}
Peak tube of transformant peak (no. 20)	6.75×10^5	6.2×10^7	1.09×10^{-2}

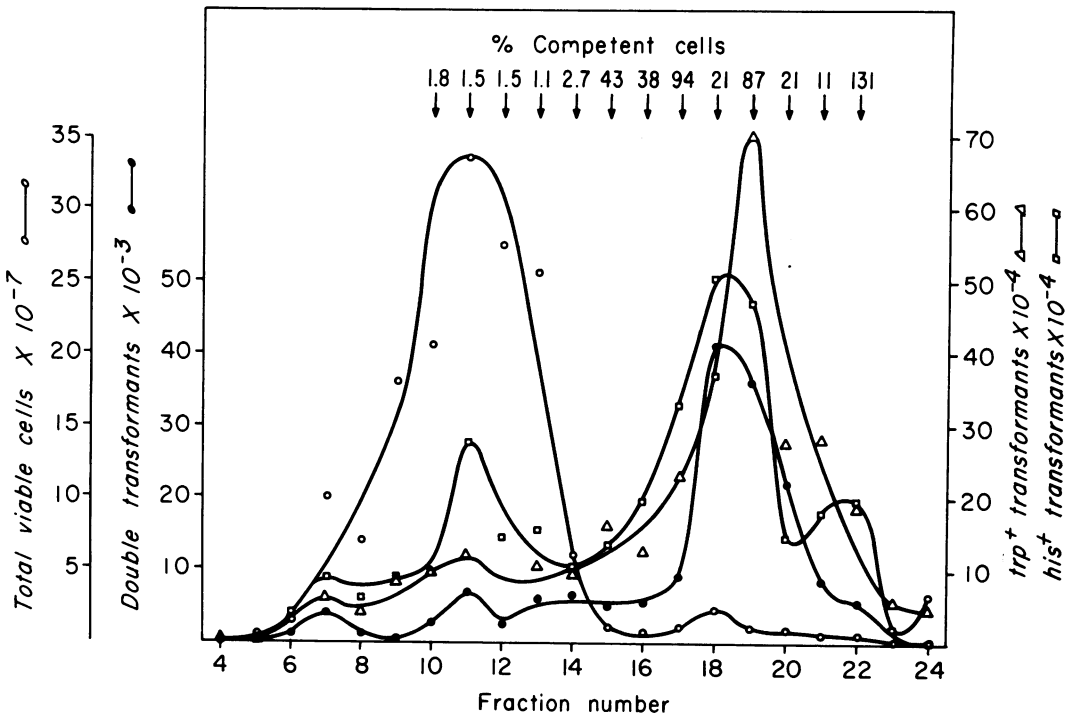

FIG. 5. *Estimation of level of competence in gradient fractions. A 2-ml amount of a competent culture (9) of SB1 (his_1^- trp_2^-) was transformed as described in Fig. 2. The culture was centrifuged and the cell pellet was resuspended in 0.4 ml of 12.5% Renografin. A 0.3-ml amount of the cell suspension was applied to a 12-ml linear gradient varying in concentration of Renografin from 25 to 40%. The gradient was centrifuged for 30 min at 15,000 rev/min in an International model B-60 preparative ultracentrifuge in the SB-269 rotor. Fractions containing 20 drops were collected. Alternate fractions were collected in 9.9 ml of Davis minimal medium and assayed for colony-forming units; the remaining fractions were diluted with 1.0 ml of Davis minimal medium for determination of optical density at 420 mμ. The percentage of competent cells in fractions was calculated by the formula given in the text; the values are given in the figure. Values were not calculated for fractions giving less than four colonies per plate at the dilutions tested. The unfractionated culture was calculated to contain 6.5% competent cells.*

during the first centrifugation. The two most dense fractions in the heavy peak accounted for 27% of the cells recovered from the second gradient. The transformants that banded in a region intermediate between heavy and light were probably cells that were losing or had lost competence and were returning to the density of noncompetent cells. At the beginning of the second centrifugation, the culture was about 3.5 hr past the time of maximal competence. This is about the time biosynthetic latency in competent cells ends and growth resumes if the culture is left in the medium used for the second growth stage of the transformation procedure (14, 20). The low percentage of competent cells in the most dense fraction of the major transformant peak may indicate that some heavy noncompetent cells were still trapped there.

Density of transformed versus nontransformed competent cells. The low density of transformants

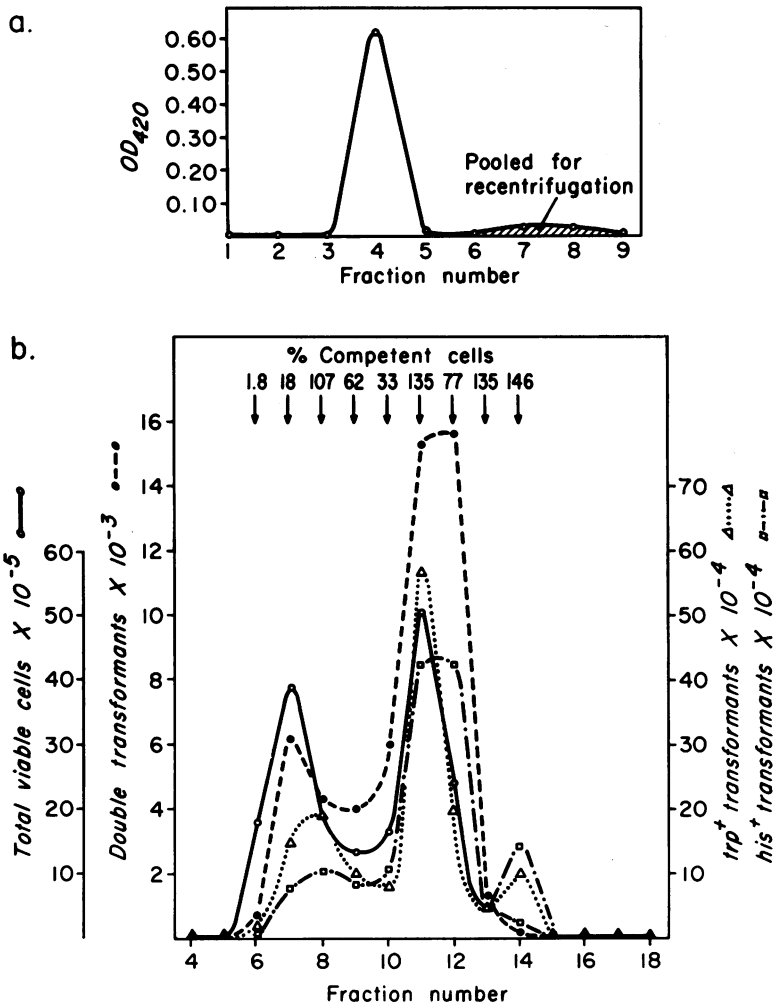

FIG. 6. *Recentrifugation of the transformed fraction from a step gradient.* (A) *A 5-ml amount of a competent culture* (9) *of SB1* (*hisA1 trpC168*) *was prepared and transformed as described in Fig. 1. The culture was centrifuged; the cell pellet was resuspended in 3.5 ml 12.5% Renografin. The suspension was layered on top of a step gradient of 8.0 ml of 50% Renografin, 10.0 ml of 30% Renografin, and 3.5 ml of 12.5% Renografin; the gradient was centrifuged as described in Fig. 1. Nine fractions of 240 drops each were collected; a portion of each fraction was diluted sixfold with Davis minimal medium, and the optical density at 420 mμ was determined. Fractions 6 through 9 inclusive were pooled, diluted with two volumes of Davis minimal medium, and filtered through a sterile membrane filter. Cells were washed from the filter with 1.5 ml of Davis minimal medium; 1.0 ml of this suspension was diluted with 1.0 ml of 25% Renografin.* (B) *This suspension was layered on top of a step gradient containing 1.2 ml of 50% Renografin and 1.6 ml of 30% Renografin. The gradient was centrifuged as described in Fig. 2. Fractions containing 20 drops were collected and assayed for colony-forming units. The percentage of competent cells in fractions was calculated as in Fig. 5. The culture used in the first centrifugation* (A) *was calculated to contain 9.5% competent cells; the culture applied to the second gradient* (B) *was calculated to contain 73.4% competent cells.*

might conceivably be a property of all competent cells, or a result of DNA uptake. To distinguish between these two possibilities, competent cells of strain 168 were incubated with or without prototrophic DNA under otherwise identical conditions. Each population was centrifuged through a linear gradient of Renografin.

Fractions of the untransformed culture were diluted with modified CHT-2 (13) lacking tryptophan and exposed to prototrophic DNA. Figure 7 shows that the density of competent cells transformed before and after centrifugation was identical. Thus, we conclude that the low density is a property of the vast majority of competent cells.

If Renografin was present in the medium during

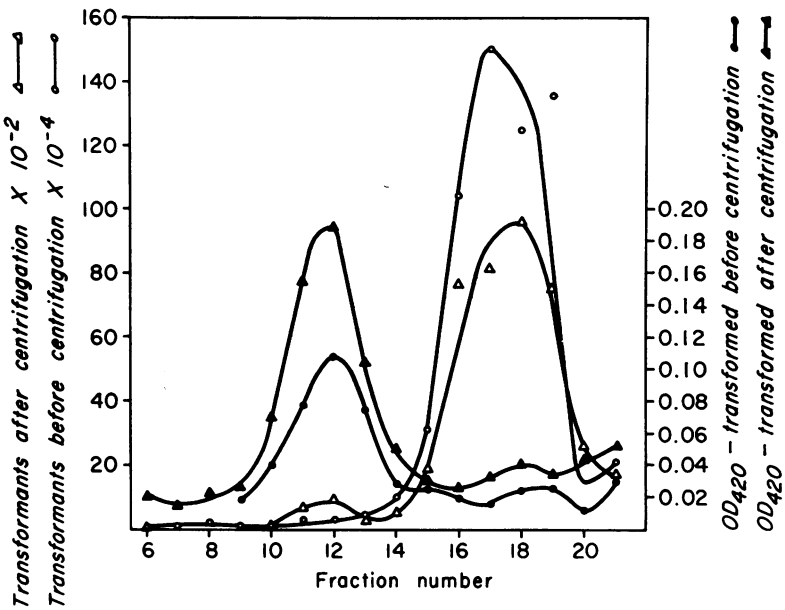

FIG. 7. *Centrifugation of transformed and nontransformed competent cultures. A 0.3-ml amount of a freshly thawed competent culture (13) of strain 168 was diluted to 3.0 ml with modified casein hydrolysate medium (13). A 1-ml amount of this suspension was transformed as in Fig. 1; 2.0 ml were incubated under identical conditions except that DNA was omitted. Both cultures were centrifuged; each cell pellet was resuspended in 0.2 ml of 12.5% Renografin and layered onto a 4.6-ml gradient of Renografin varying in concentration from 25 to 40%. The gradients were centrifuged as described in Fig. 2; 20-drop fractions were collected. Fractions from the gradient containing the transformed culture were diluted with 1.3 ml of Davis minimal medium and assayed for transformants and optical density at 420 mμ. Fractions from the gradient containing the non-transformed culture were diluted with 2.0 ml of modified casein hydrolysate medium (13) lacking tryptophan and containing DNA isolated from strain WB746. These cultures were transformed as in Fig. 1 and assayed for transformants and optical density at 420 mμ.*

exposure of the competent culture to DNA, it inhibited transformation at concentrations above 2.5 to 3.5%, and slightly stimulated transformation at lower concentrations (Fig. 8). Since the difference in stimulation by Renografin in the concentration range covered by the transformant peak in Fig. 7 was less than 2%, the results presented in Fig. 7 have not been corrected to the values expected if Renografin had no effect on transformation. The reasons for these effects are not known. At a 100% concentration, Renografin contains 0.01 M ethylenediaminetetraacetate; (EDTA); the concentration of EDTA in medium containing 30% Renografin might be enough to reduce the concentration of Mg^{++} below the minimal level required for transformation (25). However, when a competent culture was transformed in medium containing 30% Renografin and a total of 0.15 M $MgSO_4$, the level of transformation was still less than 1% of the control lacking Renografin.

DISCUSSION

The results presented convincingly demonstrate that centrifugation in a density gradient of Renografin does enrich for the competent cell fraction. However, because there is no way of directly determining the number of competent cells in a population, the maximal attainable extent of purification is not yet clear.

Our calculation of the percentage of competent cells in a population is based on three assumptions: first, that all competent cells are equally likely to incorporate, integrate, and express a molecule of DNA; second, that the probability that a cell will incorporate a molecule of DNA is independent of whether it has previously taken up a DNA molecule; and, third, that all gene loci are equally likely to be transformed. There are no data which contradict any of these assumptions. Indeed, using the above assumptions to calculate the number of competent cells in a population of *Haemophilus*, Goodgal and Herriott (7) were able to predict very accurately from the frequencies of single transformation the number of double and triple transformations for unlinked markers. On the other hand, Ephrussi-Taylor (6) reported that in pneumococcus double transformation of unlinked markers was rarer than expected; if $(N_1/B) \times (N_2/B) > (N_D/B)$,

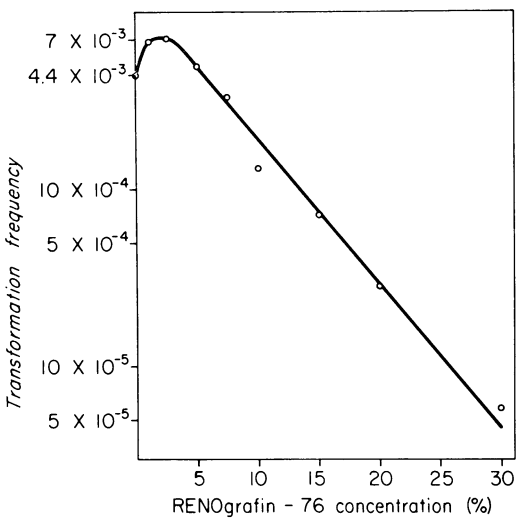

FIG. 8. *Effect of Renografin on transformation frequency. Various amounts of Renografin were added to 0.2 ml of five times concentrated CHT-2 (13) and diluted to 0.8 ml with sterile distilled water. A 0.1-ml amount of a solution of DNA isolated from WB746 and 0.1 ml of a freshly thawed concentrated competent culture of strain 168 (9) were added to each tube. The cultures were transformed (13) and assayed for colony-forming units. There was no loss of the total viable cells as the concentration of Renografin increased.*

then the calculated percentage of competent cells, $(N_1N_2/N_DB) \times 100$, is greater than 100%. Similarly, we found that, in a recentrifuged competent fraction (Fig. 6), the percentage of competent cells as calculated from the coincident transfer of unlinked markers reached a maximum of around 145%. Thus, the model implied by the assumptions listed above is, under some conditions, probably an oversimplification. Possible reasons for these overly high values for the competent fraction include a low observed number of double transformants because of competition for DNA adsorption sites (17) or incorporation of a very small number of DNA molecules by cells that are only slightly competent.

It is obvious that in some experiments we have not achieved a complete separation of the transformed from the nontransformed cells (Fig. 3, 5, 6). These results point up some of the limitations of the technique. For example, there remains some contamination of one type of cell by the other. These cells might be in density equilibrium with the gradient or physically trapped by the surrounding cells. In a population calculated to be 9.5% competent, the trapped noncompetent cells are not actually light, since they band in the heavy region on a second centrifugation (Fig. 6). No long chains are visible by phase-contrast microscopy of the gradient fractions; furthermore, it seems likely that chains would band at some intermediate density. It seems plausible that cells could accumulate at the interface between the 12.5% Renografin sample layer and the top of the next layer, perhaps because of a difference in surface tension between the two layers of liquid. The more dense noncompetent cells would probably overcome this surface resistance more readily than competent cells (which would not go through the interface at all in a step gradient). The competent cells, temporarily held up at the interface and thereby forced into a tight band, would become increasingly packed, and could trap some heavy, noncompetent cells. As the band of competent cells moves through the interface onto the linear gradient, it would tend to diffuse slightly as it moved to its equilibrium position, but perhaps not enough to release the trapped heavy, noncompetent cells. Similarly, some light competent cells might be carried along by the concentrated band of noncompetent cells, and thus band in the major, heavy peak. This would account for the relatively large number of transformants in the major peak of the gradient shown in Fig. 5. The fact that agglomeration of cells occurs when the volume of the sample applied to the gradient is too small (*see* Materials and Methods) can be understood by this same reasoning.

Zonal centrifugation of two populations of sample material with sucrose or Ficoll as the density gradient medium separates the species on the basis of sedimentation coefficient, which is determined by the density as well as by the size and shape of the material (1). It is not possible to deduce the reason for fractionation of competent cultures by zonal centrifugation (19). Isopycnic gradient centrifugation shows that a difference in bouyant density does occur, although size differences have not been ruled out. Competent cells are not dividing during the period of competence (14, 20), nor do they synthesize appreciable quantities of ribonucleic acid (RNA) or DNA (9). In *E. coli*, the rate of bulk RNA synthesis is correlated with cell size (11), suggesting that the nondividing competent cells might be smaller than the more rapidly growing noncompetent cells. On the other hand, the ratio of RNA to protein in rapidly growing cells is higher than in slowly growing cells (4, 22), so on this basis competent cells might be expected to be less dense than noncompetent cells (10); indeed, we found that the density of rapidly growing cells of *B. subtilis* was slightly higher than that of slowly growing cells (*unpublished data*). Perhaps the two populations differ in their permeability to water;

this would contribute to the difference in bouyant density. Wolstenholme et al. (23) suggested that large membranous bodies occur in competent cells of *B. subtilis* and are involved in the incorporation of transforming DNA.

The relationship between the difference in density of competent and noncompetent cells and the differences in their physiological properties is currently being pursued. The loss of competence after several hours (14, 20) is accompanied by a return to the higher-density peak (Fig. 6). We know that frozen cells lose competence when they are stored for some time. We are interested in knowing what causes the loss of competence, and whether a change in density also occurs.

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