PHYSICAL METHODS FOR OBTAINING SYNCHRONOUS CULTURE OF *ESCHERICHIA COLI*[1]

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Recently the techniques of synchronous culture of microorganisms have attracted special attention. The methods used for this purpose differ according to the organism employed. The early phase of growth resulting from inoculation of aged cells of Protozoa into a fresh medium was shown to represent rhythmic growth (Browning *et al.*, 1952). The same has been demonstrated for bacteria (Houtermans, 1953) and for yeast (Ogur *et al.*, 1953). Temperature cycling of a culture also has resulted in the synchronous growth of bacteria (Hotchkiss, 1954; Lark and Maaløe, 1954; Szybalski and Hunter-Szybalska, 1955) and of Protozoa (Scherbaum and Zeuthen, 1954; Zeuthen and Scherbaum, 1954; James, 1954). Proper regulation of the growth medium using wild type *Escherichia coli* strain B (Yanagita, 1954) and a deficient mutant of *E. coli* strain B (Barner and Cohen, 1955) also induced synchronization of cell division. Intermittent illumination proved to be a useful method for obtaining synchronous growth of an autotrophic alga, *Chlorella* (Tamiya *et al.*, 1953).

Since all these methods are based on the physiological conditioning of the microbial cells, it is possible that the synchronous cells obtained might possess some abnormality in their physiological pattern. Our plan was to improve the method for obtaining a synchronous culture without any influence on the normal physiological nature of the cells. Two physical methods, fractional sedimentation and fractional filtration, were tested for the separation of larger (mature) and smaller (immature) cells present in a logarithmic phase culture of *E. coli*. Satisfactory results were obtained by the method of fractional filtration of the culture.

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MATERIALS AND METHODS

E. coli strain B was cultured in a medium containing NH_4Cl, 1 g; $Na_2HPO_4 \cdot 12H_2O$, 18 g; KH_2PO_4, 1 g; $MgSO_4 \cdot H_2O$, 0.2 g; glucose, 1 g; and 0.01 g of "tween 80" in 1 L of distilled water adjusted to pH 7.4. Cells grown in this medium were inoculated into a fresh medium and when the number reached 10^8 cells per ml at 37 C, the culture was centrifuged at 4,000 rpm for 12 min. The sedimented cells were used for the experiments to be described. Viable counts were carried out by the capillary tube method of Yanagita (1956). The standard error of this method was 11 per cent under the present experimental conditions.

RESULTS

Fractional centrifugation. When a logarithmic culture of *E. coli* was centrifuged at the proper centrifugal force (about 400 × gravity for 4 min under our experimental conditions), rough separation of larger and smaller cells could be obtained (Yanagita, 1954). Much more clear-cut separation of these cells was obtained by controlling the specific gravity of the medium in which the cells were suspended. Cells from 1 L of culture were suspended in 5 ml medium without added glucose. One-half ml of this suspension was gently layered on the top of 2 ml sucrose solutions of specific gravities ranging between 1.10 and 1.30 with intervals of about 0.02; care was taken to prevent mixing of these two layers. Each of these systems was centrifuged at 10,000 rpm for 8 min. At a specific gravity of 1.28, the cells did not sediment but remained at the boundary of the two layers, whereas at 1.18 or below, all of the cells went to the bottom. At a specific gravity of 1.245 the cells were separated quite clearly. The majority of the cells were found to lie either at the top or at the bottom of the sucrose solution, a slight turbidity remaining in the solution. The

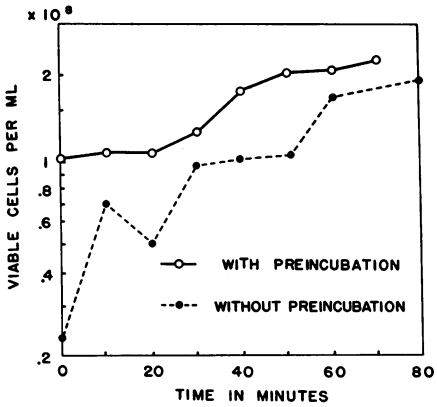

Figure 1. An example of experiments demonstrating stepwise multiplication of small cells obtained by fractional centrifugation in a sucrose solution. The broken line represents the growth curve of cells obtained from the top of the sucrose solution without any pre-incubation. The solid line represents the growth curve of cells preincubated for 20 min in a synthetic medium without added glucose. Irregular fluctuations observed during the course of initial increase of treated cells were also observed in other experiments of the same type.

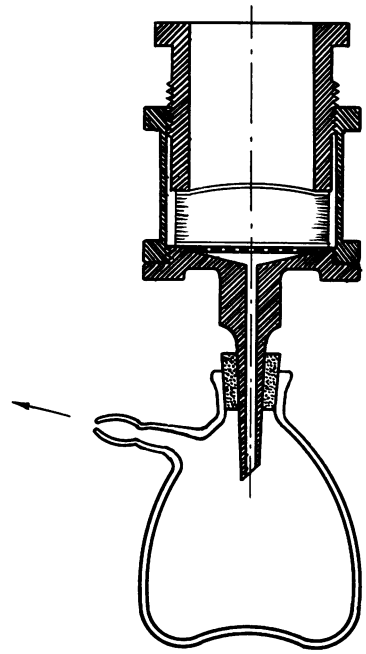

Figure 2. Paperpile apparatus used for the fractional filtration of cells. Inside diameter, 55 mm.

top cells were smaller and the bottom cells larger in size.

When the top cells were inoculated into a fresh medium, the number of viable cells was very small during the first 20 min and was followed by a stepwise increase. This is shown in figure 1 as a broken line. The initial low counts might have been due to an osmotic injury to the cells resulting in the failure of these cells to form colonies in the capillaries used for cell counting. Consecutive treatments consisted of washing, resuspension, and incubation of cells in a medium, without added glucose, at 37 C for 30 min. The cells thus treated seemed to recover from the injury and showed a characteristic stepwise growth upon inoculation into a medium (a solid line in figure 1). A glucose solution of a specific gravity of 1.27 could be substituted for the sucrose solution. Various materials, such as gum arabic, dextran, a dialysis residue of peptone and tris-buffer, also were tested for their ability to minimize the osmotic injury to the cells. None of these solutions proved satisfactory.

Fractional filtration. Mechanical separation of smaller and larger cells by fractional filtration yielded satisfactory results. The filtration procedure was based on the paper chromatopile technique used in chemical analysis. Particles of different sizes placed on the top of a paper pile and developed with a constant flow of a solvent may migrate to a different extent through the paper pile. To apply this hypothesis to *E. coli*, a Seitz filter apparatus (diameter: 36 mm) can be used. A more suitable filtration apparatus, permitting the treatment of more cells, was especially designed and is shown in figure 2.

Various kinds of filter papers were checked for the rate of filtration and for the retention of cells in the paper pile. Twenty sheets of Toyo filter paper[3] No. 126 together with one sheet of No. 1 filter paper on the bottom were placed in the apparatus and were wetted with synthetic medium. The thin paper on the bottom was used to prevent the passage into the filtrate of fibers originating from the heavy papers in the pile. One ml of a suspension containing 10^{10} cells was absorbed on to two sheets of No. 1 paper which were placed on the top of the paper pile, and then the whole paper pile was tightened with an inner screw ring

[3] Toyo filter papers No. 1 and 126 are comparable to Whatman filter paper No. 1 and Eaton Dikeman filter paper No. 623 or 624, respectively.

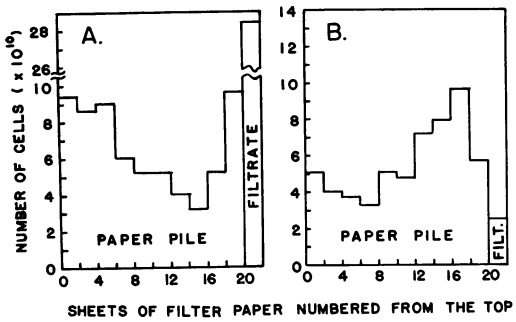

Figure 3. Retention of cells in paper piles and filtrates. *a*. Eighteen No. 126 filter papers were packed together between two No. 1 filter papers. Filtration was stopped after 20 ml of filtrate were obtained. Cells applied: 8.4×10^{10}, and cells recovered: 4.7×10^{10}. *b*. Twenty No. 126 filter papers were packed together. Filtration was stopped after 12 ml of filtrate were obtained. Cells applied: 8.8×10^{10}, and cells recovered: 5.9×10^{10}.

(see figure 2). Vacuum filtration was carried out while a small amount of medium was continually applied at the top of the pile until turbidity appeared in the filtrate. Then the top two sheets of No. 1 filter paper were taken out of the apparatus and were eluted with synthetic medium. The average yield of the larger cells thus obtained was about 10 per cent of the initial cell suspension applied at the top of the paper pile. A mass of smaller cells was obtained upon centrifugation of the filtrate at 10,000 rpm for 8 min.

Two examples of experiments demonstrating the retention of cells in the paper pile and filtrate are shown in figure 3. In the first experiment (figure 3*a*) the pile was composed of two kinds of filter papers as described above and the development was stopped after 20 ml of filtrate were obtained. In the second experiment (figure 3*b*), the pile was composed of 20 sheets of No. 126 filter paper only and the development was stopped after only 12 ml of filtrate were obtained. Histograms shown in figure 3 describe the retention of cells in the paper piles in both experiments. On the basis of these experiments it was decided that the first procedure would be more desirable than the second for the separation of larger and smaller cells since two distinct peaks at both ends of the histogram were observed in *a* but not in *b*.

When the larger cells obtained from the top

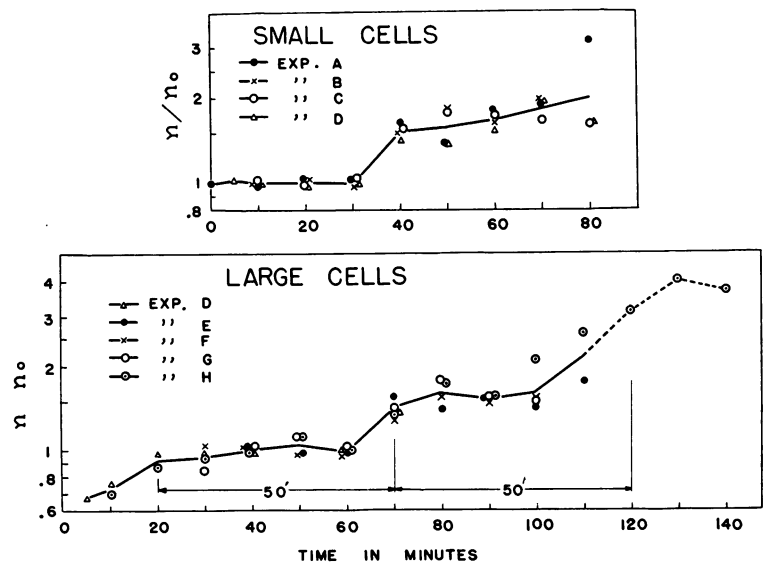

Figure 4. Growth curves of cells obtained by fractional filtration. Upper and lower curves represent growth curves obtained after inoculations of small cells and large cells, respectively. All experiments from A to H were carried out under the same experimental conditions using different batches of cells. Average numbers (n_o) of cells during the first flat phase in each experiment were as follows: A, 0.65; B, 0.57; C, 0.72; D, 2.33 (large cells) and 0.83 (small cells); E, 1.28; F, 1.00; G, 3.02; and H, 4.57 cells per ml ($\times 10^8$). In each experiment the ratio of number (n) of cells at a given time interval to the average number, n/n_o, was plotted logarithmically against time. Curves were drawn through the average points at successive time intervals.

papers were used as inoculum, they began to divide almost immediately. This was followed by a period without increase in cell numbers. On the other hand, incubation of the smaller cells obtained from the filtrate resulted in an initial period of constant viable counts, followed by division of cells. Examples of growth curves are shown in figure 4. In cultures inoculated with larger cells, distinct periods that could be interpreted as three successive rhythmic multiplications—three steps of the growth curve—were observed as shown in the figure. The generation time deduced from this experiment was 50 min. The period of the initial increase in cell number after the inoculation of larger cells was found to be about 20 min. Therefore pre-incubation of the larger cells for 20 min prior to the initiation of the synchronous culture resulted in growth curves starting from a physiologically immature state comparable to that found after inoculation of smaller cells.

DISCUSSION

Physical separation of larger and smaller cells in a bacterial culture previously was tried by Hershey (1939). One-day-old aerated broth cultures of *E. coli* and Shigella were centrifuged and the growth curves of cells obtained from the supernatant and from the sediment compared. No significant difference between lag periods of both cultures could be observed. The cells used were considered to be at a stationary or declining cultural phase. As was shown by Martin (1932) and other workers, cells in such aged cultures tend to be more homogeneous in size than those in the logarithmic phase where wide distributions in cell sizes can be observed. In the present studies, cells of the logarithmic phase were used with considerable success for the separation of larger and smaller cells. Although separation through fractional sedimentation in a sucrose solution of proper specific gravity yielded two types of cells, abnormality in the growth curve resulting from the injury caused by high osmotic pressure could not be avoided. Such cells recovered from the injury after 30 min incubation in a medium without glucose; nevertheless, these effects may be undesirable for further physiological studies. On the other hand, a paper chromatopile technique was applied quite satisfactorily to obtain suspensions containing cells of relatively homogeneous size. No drastic physiological changes in the cells should result from this method. The method should be applicable to organisms other than *E. coli*, especially to the rod-shaped bacteria.

The paper chromatopile technique also could be used for the study of size distribution patterns of cells in a bacterial culture. As shown in figure 3*b*, when a homogeneous paper system was used in the paper pile, the retention of cells in the pile was much higher at the bottom of the pile than near the surface. This indicates that a population from the logarithmic phase of cultural growth contains many more small cells (immature cells) than large cells (mature cells). This conclusion also is supported by the experiments of Martin (1932).

SUMMARY

Two types of cells, a larger type (mature) and a smaller type (immature), were separated by means of physical methods from logarithmic phase cultures of *Escherichia coli* strain B. Fractional sedimentation in a sucrose solution of a specific gravity of 1.245 yielded good separation, but the cells thus obtained showed an abnormal growth curve presumably due to osmotic injury. In contrast, fractional filtration, based on the paper chromatopile technique used in chemical analysis, had no injurious effects and, therefore, constitutes a more desirable method. A stepwise growth curve, indicative of synchronous divisions, has been obtained with the cell fractions after inoculation into fresh media.

REFERENCES

BARNER, H. D. AND COHEN, S. S. 1955 Synchronization of division of a thymineless mutant of *Escherichia coli*. Federation Proc., **14,** 177.

BROWNING, I., BRITTAIN, M. S., AND BERGENDAHL, J. C. 1952 Synchronous and rhythmic reproduction of protozoa following inoculation. Texas Repts. Biol. and Med., **10,** 794–802.

HERSHEY, A. D. 1939 Factors limiting bacterial growth. V. Fractional sedimentation of *Shigella*. J. Bacteriol., **38,** 485–490.

HOTCHKISS, R. D. 1954 Cyclical behavior in pneumococcal growth and transformability occasioned by environmental changes. Proc. Natl. Acad. Sci. U.S., **40,** 49–55.

HOUTERMANS, VON T. 1953 Ueber den Einfluss des Wackstumszustandes eines Bakterium auf seine Strahlenempfindlichkeit. Z. Naturforsch., **8b,** 767–771.

JAMES, T. W. 1954 Quantitative studies on the

ribonucleic acid content in *Amoeba proteus* in relation to the nucleus and the division cycle. Dissertation for Ph.D. in Zoology, University of California, Berkeley, California.

LARK, K. G. AND MAALØE, O. 1954 The induction of cellular and nuclear division in *Salmonella typhimurium* by means of temperature shifts. Biochim. et Biophys. Acta, **15,** 345–356.

MARTIN, D. S. 1932 The oxygen consumption of *Escherichia coli* during the lag and logarithmic phases of growth. J. Gen. Physiol., **15,** 691–708.

OGUR, M., MINCKLER, S., AND MCCLARY, D. O. 1953 Desoxyribonucleic acid and the budding cycle in the yeast. J. Bacteriol., **66,** 642–645.

SCHERBAUM, O. AND ZEUTHEN, E. 1954 Induction of synchronous cell division in mass cultures of *Tetrahymena piriformis*. Exptl. Cell Research, **6,** 221–227.

SZYBALSKI, W. AND HUNTER-SZYBALSKA, M. E. 1955 Synchronization of nuclear and cellular division. Bacteriol. Proc., **1955,** 36–37.

TAMIYA, H., IWAMURA, T., SHIBATA, K., HASE, E., AND NIHEI, T. 1953 Correlation between photosynthesis and light-independent metabolism in the growth of *Chlorella*. Biochim. et Biophys. Acta, **12,** 23–40.

YANAGITA, T. 1954 Single step growth method for *Escherichia coli* B. Bacteriol. Proc., **1954,** 29–30.

YANAGITA, T. 1956 Capillary tube method for counting viable bacteria. J. Bacteriol., **71,** 381–382.

ZEUTHEN, E. AND SCHERBAUM, O. 1954 Synchronous divisions in mass culture of the ciliate protozoon *Tetrahymena piriformis*, as induced by temperature changes. *Recent developments in cell physiology*, pp. 141–157. Edited by J. A. Kitching. Academic Press Inc., New York, N. Y.