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# DENSITY-GRADIENT PATTERNS OF *STAPHYLOCOCCUS AUREUS* CELLS AND CELL WALLS DURING GROWTH AND MECHANICAL DISRUPTION

ESKIN HUFF, HARRIET OXLEY, AND CAROL S. SILVERMAN

*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Bethesda, Maryland*

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ABSTRACT

HUFF, ESKIN (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), HARRIET OXLEY, AND CAROL S. SILVERMAN. Density-gradient patterns of *Staphylococcus aureus* cells and cell walls during growth and mechanical disruption. J. Bacteriol. 88:1155-1162. 1964.—Procedures capable of rapid disruption of *Staphylococcus aureus* cells with optimal release of intact cell walls were investigated. This search was implemented by observation of the flotation patterns of cells and subcellular particulate matter after centrifugation in a cesium chloride density gradient. A quantitative evaluation of the light-scatter throughout the gradient was achieved by transfer of the entire density gradient into an optical cell with wedge-shaped cross section. When this cell was photographed under indirect illumination, each band of light-scattering material appeared on the negative as a shaded curve, with an area proportional to amount of that material present. A series of photographs of known amounts of cells and cell walls was used to estimate the amounts of these materials in mixtures of the two occurring during mechanical disruption. With the methods employed, time studies established the optimal time for release of cell walls as 5 min in a Braun shaker. The use of sucrose gradients in the further purification of cell walls, and chemical analysis of the isolated walls, are described.

Salton (1960) pointed out that the preferred method for preparation of undegraded cell walls involves disruption of cells by means of shaking with glass beads, followed by differential centrifugation. The devices of Mickle (1948), Cooper (1953), Nossal (1955), Shockman, Kolb, and Toennies (1957), and Merkenschlager, Schlossmann, and Kurz (1957) were used to disrupt the cells of *Staphylococcus aureus* and other bacteria. However, no careful study has been made of the optimal conditions for release of cell walls. This appears to be due to lack of a satisfactory method for characterization of cell walls other than by examination in an electron microscope.

Meselson, Stahl, and Vinograd (1957) observed that the deoxyribonucleic acid (DNA) from an organism has a specific density when distributed in a cesium chloride equilibrium density gradient. It seemed conceivable that cell walls would have a similar specific density in CsCl, and could thus be distinguished from whole cells. An experiment was carried out in which cell walls were prepared from staphylococcal cells harvested at various stages during a 12-hr growth period. Throughout this period, the density of the cell walls was constant at 1.415 ± 0.005. This result encouraged the development of a new procedure in which cell walls and cells can be characterized and quantitated in a cesium chloride gradient. This paper describes methods which can be utilized to follow the time course of release of cell walls, destruction of cells, and formation of intermediate particles. The isolation of cell walls and their characterization by electron microscopy, chemical composition, and sedimentation characteristics in sucrose and flotation in CsCl are described.

## MATERIALS AND METHODS

*Organism. S. aureus* 8507 (Oeding) was used in all experiments. Slant cultures of this organism were maintained on a medium consisting of 1.5% agar in Trypticase Soy Broth (BBL). A 7-hr Trypticase Soy Broth culture grown at 37 C was used to inoculate 100 ml of broth, which in turn, after 16 hr was used to inoculate 1 liter of broth. Growth was continued aerobically without agitation for 5 hr. A batch of 30 liters of broth culture was harvested in a Sorvall continuous-flow centrifuge over a 2-hr period. The effective growth time here was defined as the sum of the time from inoculation to harvest plus one-half the harvest time. Thus, the effective growth time

of the above preparation would be 6 hr. The cells were washed in 1,700 ml of distilled water at 0 C. The packed cells were then resuspended in 90 ml of distilled water and frozen overnight. After thawing, the cells were centrifuged for 10 min at 20,000 rev/min in a Spinco swinging-bucket rotor (SW-25.1). Cells were resuspended in a small volume of water to a calculated optical density of 100 at 660 mμ [50 mg (dry weight) of cells per ml]. The yield of diluted cells was 125 to 130 ml, with a viable count of $9 \times 10^{10}$ per ml. These suspensions were then frozen in 20-ml batches until just before use.

*Glass beads.* Superbrite glass beads (0.003 in. in diameter) were purchased from the Minnesota Mining and Manufacturing Co., St. Paul, Minn. Magnetic particles were removed by several passages over a strong magnet and flotation of the beads on bromoform. Bromoform was removed by washing with acetone, and the beads were then air-dried at room temperature. Beads recovered from a Nossal or Braun shaker run were washed with water, treated with a sodium dichromate-sulfuric acid mixture, washed with water, washed with acetone, and finally air-dried. Because of the presence of colloidal stainless steel, beads used with a Branson 20-kc sonifier were discarded.

*Nossal disintegrator.* A large-scale mechanical shaker constructed by the Instrument Shop, National Institutes of Health, according to Nossal (1955) was fitted with tightly capped, cylindrical nylon inserts (2.17 cm, inside diameter, by 9.8 cm long; total volume = 36.6 ml). For disintegration, 20 ml of cell suspension [22 to 44 mg (dry weight) per ml] were mixed with 24 g of glass beads. Because of the rapid frictional heating of 1 C per sec, each nylon insert was shaken for no longer than 20 sec at a time, and then was removed to an ice bath for 15 to 20 min to cool back to 0 C before the subsequent shake.

*Braun shaker.* Reagent bottles (60 ml, Corning no. 1500; total volume 67 ml) were loaded with 36 ml of cell suspension and 44 g of glass beads. Shaking was carried out in the apparatus described by Merkenschlager et al. (1957) and manufactured by The Braun Co., Melsungen, Germany. In this method, the temperature of the cell suspension is maintained at 0 to 5 C throughout disintegration.

*Branson 20-kc sonifier.* A suspension of 20 ml of cells was treated in a stainless-steel jacketed chamber made to fit the probe of the Branson 20-kc sonifier. Water at 5 C was circulated through the jacket, and the temperature within the chamber was thus maintained at less than 10 C.

*Sucrose gradients.* Linear sucrose gradients were prepared as described by Yoshida et al. (1961), with the use of a gradient mixing device similar to that of Britten and Roberts (1960). All sucrose solutions were filtered through Millipore HA filters. A volume of 18.6 ml of 5% sucrose in 1 M KCl was placed in the left unstirred chamber of the gradient mixing device. The right stirred chamber contained 16.7 ml of 40% sucrose in 1 M KCl. Each gradient was delivered into a centrifuge tube (Sorvall no. 204). After centrifugation at 3,100 rev/min in an International PR-1 centrifuge for 30 or 70 min, the gradient tubes were punctured in a device similar to that described by Martin and Ames (1960), and a total of 37 1-ml fractions was collected. The turbidity of cell walls measured at 660 mμ was reduced almost threefold when measured in 40% sucrose. Therefore, it was necessary to multiply the optical density measurements at 660 mμ of cell walls in sucrose by a correction factor which varied from 1 to 3, depending on the final sucrose concentration in which the optical density was measured.

*Chemical analyses.* Analyses were carried out on samples of cell-wall suspensions dried in a vacuum desiccator over anhydrous $CaSO_4$. Total phosphate was determined by the procedure of Chen, Toribara, and Warner (1956) as modified by Ames and Dubin (1960), as well as by the method of Gomori (1942). Determination of ribonucleic acid (RNA) and DNA was by the orcinol and diphenylamine methods, respectively, as described by Schneider (1957).

## RESULTS

*Establishment of flotation patterns of cells and cell walls.* After varying periods of disruption, a sample of 0.10 ml or less was placed in a test tube in ice. Water was added to bring the volume to 0.10 ml, and then CsCl (0.90 ml; density, 1.390) was added to bring the final density to 1.352; 12 hr to 7 days were allowed for equilibration with CsCl. After longer periods of time, two bands instead of one were present in the whole-cell region of the CsCl gradient. After equilibration, a CsCl step gradient was prepared by

placing 0.33 ml of tetrabromoethane in the bottom of a cellulose nitrate tube, followed by the sequential addition of 1.00 ml of each of the cesium chloride solutions of densities 1.472, 1.409, 1.352 (containing the disrupted cells), and 1.306. To prevent evaporation of water, the gradient was then overlaid with light paraffin oil. The tubes were centrifuged (Beckman model L ultracentrifuge) for 3 hr at 33,000 rev/min in a Spinco SW-39 rotor. Control experiments showed that equilibrium had been reached by this time, since further centrifugation for 22 hr did not change the distribution patterns.

*Transfer of gradient to optical cell.* After centrifugation, the tubes were removed from the rotor, and the bulk of the paraffin oil was removed by suction. Remaining oil was removed by overlaying the gradient with benzene, followed by suction removal of the benzene. The bottom of the centrifuge tube was punctured by a no. 21 hypodermic needle connected via polyethylene tubing to a reservoir of tetrabromoethane (Fig. 1). A specially constructed optical glass cell (Optical Cell Co., Inc., Brentwood, Md.) of wedge-shaped cross section, adapted to fit the centrifuge tube, was placed above the centrifuge tube, forced onto it to provide a water-tight seal, and held in place by means of a clamp. The entire gradient was raised up into the wedge-shaped section of the cell by lifting the reservoir above this cell and allowing the tetrabromoethane to displace the gradient. When the gradient was properly positioned for photography, the flow of tetrabromoethane was stopped by a clamp. The cell was then illuminated by indirect light (Fig. 1b). Photographs were taken with Kodak Panatomic X roll film, and were developed with a fine-grained developer under standardized conditions of time and temperature. The image size on the negative was 1.5 times normal, allowing direct observation of the patterns on the negative. Only the wedge-shaped portion of the optical cell, indicated by the thick lines above the dashed line in Fig. 1a, was reproduced in the photographs presented here. Figure 2 shows examples of the patterns of cells and cell walls obtained in CsCl by this procedure.

*Determination of cells and cell walls in a gradient.* Known amounts of whole cells (1 to 40 $\mu$g, dry weight) and cell walls (14 to 140 $\mu$g, dry weight) were centrifuged in a CsCl step gradient, and photographs of the gradients were prepared as

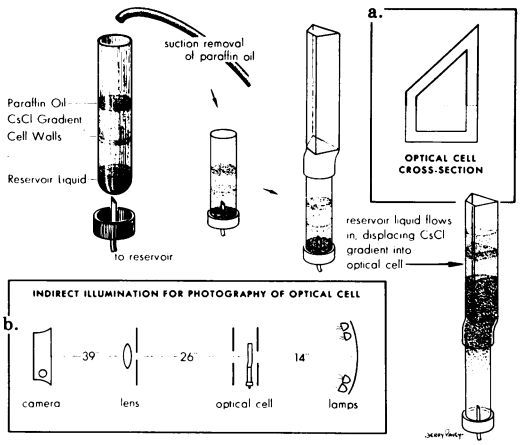


FIG. 1. *Transfer of CsCl gradient from centrifuge tube to optical cell.*

described above. In the range studied, there was a direct relationship between the shaded area on the photographic negative and the dry weight (Fig. 3). Unknown amounts of cell walls and cells in a mixture could be determined with an accuracy of $\pm 7\%$ by comparison of the photograph of the CsCl gradient to these standard photographs.

*Time study of the disruption of cells by glass beads.* Three devices were studied for their ability to release cell walls from *S. aureus* in the presence of beads: the Braun shaker, the Nossal disintegrator, and the Branson 20-kc sonifier. Of these, the Braun shaker breaks the largest number of cells (1.5 g, dry weight) in the shortest length of time (5 min of total shaking time over a 15- to 20-min period) without the temperature of the suspension rising above 5 C. Consequently, most time studies of disruption were carried out with this apparatus. The time course of disruption of *S. aureus* cells in a Nossal disintegrator was similar to that observed in the Braun shaker. The presence of an air space (equivalent to 20% of the total volume) and of glass beads was essential to the disruption of cells in both machines. Disruption of cells in the Branson 20-kc sonifier resulted in low yields of cell walls. Here, the formation of cell walls was dependent on the presence of glass beads, and was directly related to the amounts added in the range of 0.13 to 1.2 g of beads per ml of cell suspension; whole cell walls were released only transiently, followed by further breakage to particles not

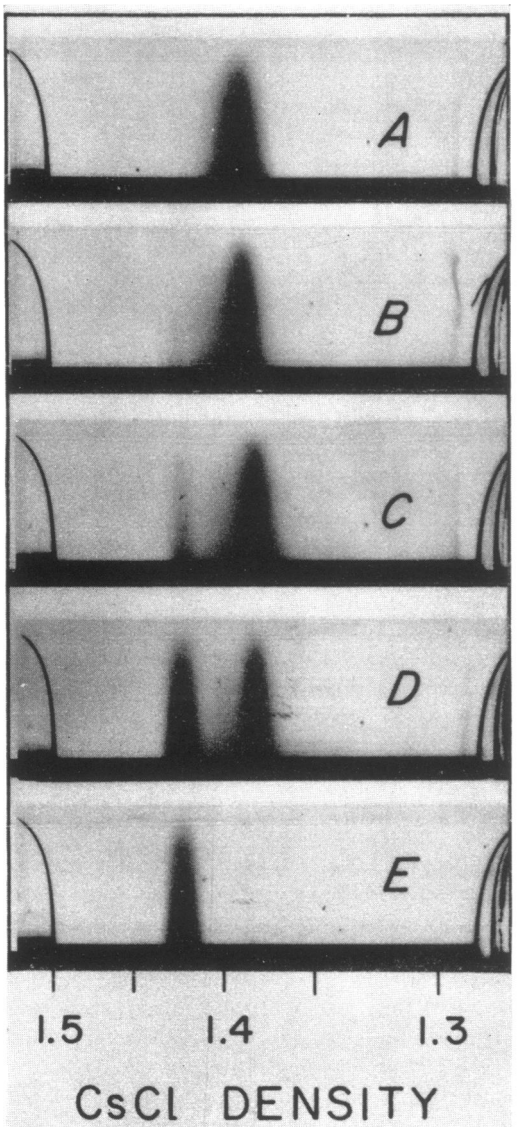


FIG. 2. *Light-scattering patterns observed after disruption of Staphylococcus aureus cells by shaking for various periods of time in a Braun shaker. Samples were removed at intervals during disruption and placed in CsCl for 2 to 3 days prior to flotation in CsCl. The volume of original material placed in the gradient and time of removal were as follows: (A) 0.9 μliter, 0 time; (B) 2.1 μliters, 0.61 min; (C) 4.0 μliters, 1.5 min; (D) 13.3 μliters, 2.67 min; (E) 13.3 μliters, 4 min. The single peak in A and the single peak in E demonstrate the positions of whole cells (of effective growth time of 6 hr) and cell walls, respectively.*

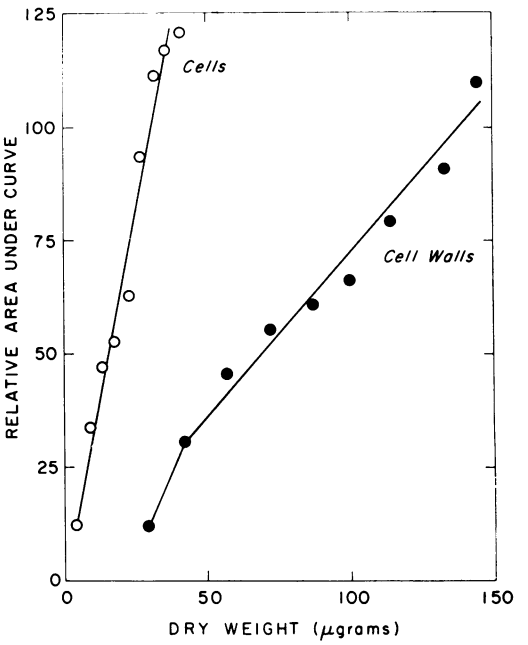


FIG. 3. *Quantitative relationship between dry weight of cells or cell walls and the intensity of light-scattering of these materials distributed in a cesium chloride gradient. Relative area under the curve refers to the shaded area measured on photographs, taken under standard conditions of illumination, when the indicated amount of cell walls (or cells) was distributed in a cesium chloride gradient in a wedge-shaped optical cell. A relative area value of 100 equals 1 in.² on a final enlargement 5.1 times normal size.*

detectable in an indirectly illuminated CsCl gradient.

To study the time course of breakage, a standard suspension of *S. aureus* cells (37 mg, dry weight, per ml) was shaken with glass beads. At intervals, the beads were allowed to settle, and samples of the supernatant fluid were taken for determination of the optical density at 660 mμ, viable count, and CsCl gradient pattern. A representative series of CsCl gradient patterns observed during disruption of staphylococcal cells in a Braun shaker is shown in Fig. 2. The amounts of cell walls and unruptured cells were determined from these patterns by measurement of the area under the light-scatter curve and comparison with the standard curves of Fig. 3. These data are presented in Table 1. In four of five experiments, it was observed that

after 4 to 6 min of shaking in the Braun shaker there was no band other than the main density 1.426 cell-wall band detectable, all traces of the density 1.380 band of unruptured cells having disappeared. The positions of the indicated CsCl densities throughout the cell in Fig. 2 were calculated from data given by Ifft, Voet, and Vinograd (1961). All densities were determined from such patterns by measurement of the distance from the peak to the uppermost portion of the lower meniscus, at which point the density is approximately 1.50.

The disappearance of the density 1.380 band of unruptured cells and the decrease in the viable counts are first order. In one experiment, the cells were not completely disrupted, even after 10 min of shaking. However, these cells did differ from the others used in having an effective growth time of 5.1 hr as compared with 6.0 to 6.8 hr for all other experiments.

During the early stages of disruption in most experiments, a band of density 1.402 appeared (faintly visible in patterns B, C, and D of Fig. 2), reaching a maximum by 1 min and disappearing by 4 min of shaking. The nature of this band, which may be an intermediate stage in the release of wall material, is unknown.

A logarithmic plot of the rate of reduction in viable counts during disruption in a Braun shaker revealed that a resistant portion of the population (1 in $10^6$ organisms) was killed at $\frac{1}{12}$ the rate of the others. This observation is similar to that of King and Alexander (1948), who observed that in a mechanical shaker 1 in $10^6$ *S. aureus* cells was killed at a rate $\frac{1}{16}$ that of the bulk of the organisms. In our experiments with 50 times as many cells per ml, decrease in viability in a Braun shaker was at a rate threefold greater than that reported by King and Alexander (1948).

A comparison of the viable counts and unruptured cells in Table 1 shows that after 1 min of shaking the unruptured cells are predominantly nonviable. This result is in contrast to the results of Cooper (1953) who, with a mechanical shaker, found a close correlation between viable count and rate of disruption of *S. aureus*. In the case of the Branson 20-kc sonifier, it was observed that disappearance of the unruptured cells parallels the loss of viable count.

*Whole-cell and cell-wall densities during growth.* The density of *S. aureus* cell walls has been found to be 1.412, with little or no change during the course of growth. Cell walls reisolated from a CsCl gradient showed no change in the ratio of total phosphate to hexosamine released on hydrolysis. This would indicate little change in the chemical nature of cell walls in a CsCl gradient. In contrast to the cell walls, the density of the whole cell changes considerably during growth. Stationary and lag-phase cells have an average density which varies from 1.375 to 1.384. Early logarithmic phase cells have an average density of 1.450. By the time the cell count is 90% of the final value, this average density has dropped to 1.425, and another species of density 1.400 appears. This species becomes dominant, and is replaced by the 1.375 to 1.384 species after the viable count becomes stationary. It is of interest that no gradual change of one density type to another was observed. Instead, two bands of material were present during the period in which cells were changing from one density type to another. This would suggest that individual cells rapidly change from one density type to another, so that few cells of

TABLE 1. *Release of cell walls from Staphylococcus aureus by disruption of the cells in a Braun shaker with glass beads*[a]

| Shaking time | Viable count | Cells remaining[b] | Walls released[c] | Optical density[d] |
|---|---|---|---|---|
| *min* | % | % | % | % |
| 0 | 100 | 100 | 0 | 100 |
| 0.6 | 1.8 | 32 | 53 | 50 |
| 1.5 | 0.014 | 15 | 83 | 27 |
| 2.6 | $3 \times 10^{-4}$ | 4.1 | 93 | 13 |
| 4.0 | $1 \times 10^{-4}$ | 1.1 | 96 | 7.7 |
| 5.0 | $7 \times 10^{-5}$ | 0.2 | 100 | 6.3 |

[a] Cell and cell-wall gradient patterns were prepared as in Fig. 2, and dry weights were calculated by comparison with the standard patterns used to prepare Fig. 3.

[b] The percentage of cells remaining was calculated from the dry weights; the initial dry weight of the cells was assumed to be 100%.

[c] The dry weight of cell walls determined after all traces of cells had disappeared was considered to be 100%.

[d] The per cent optical density (OD) at 660 mμ $= \frac{(100)\ (\text{final OD at } 660\ \text{m}\mu)}{\text{starting OD at } 660\ \text{m}\mu}$.

TABLE 2. *Chemical analysis of cell-wall subfractions obtained from a sucrose gradient**

| Region of gradient | Fraction no. | Total dry wt | Per cent dry weight as | | $\frac{OD_{660}}{\text{dry wt}}$ |
|---|---|---|---|---|---|
| | | | RNA | Phosphorus | |
| | | *mg* | | | |
| Precipitate | 37† | 8.6 | 0.51 | 2.72 | 0.42 |
| A, lower | 12–16 | 9.3 | 0.16 | 2.70 | 0.47 |
| B, middle | 17–19 | 17.3 | 0.17 | 2.63 | 0.36 |
| C, upper | 20–26 | 20.9 | 0.22 | 2.72 | 0.29 |

* Material from the indicated regions of four identical gradients was pooled, washed twice with water, and resuspended in 1.00 ml of water. Samples were dried and weighed, and RNA and phosphorus were determined.

† The precipitate came off partially with the last milliliter of sucrose, and the remainder of the precipitate was combined with this fraction.

intermediate density are observed at any one time.

*Isolation and characterization of cell walls.* Cell walls were prepared by shaking in a Braun shaker with glass beads, followed by washing with KCl and fractionation in a sucrose gradient. These cell walls were examined for size heterogeneity in sucrose gradients, with an electron microscope, and for phosphorus and RNA content. In a typical experiment, 1 g (dry weight) of cells in 36 ml of water was shaken in a Braun shaker with glass beads for 5 min. The disrupted cell suspension was decanted from the beads and combined with a 1 M KCl wash of the beads to give a final volume of 30 ml. This suspension was centrifuged 15 min at 11,000 × *g*, the supernatant fluid was decanted, and the residue was washed three additional times with 30 ml of 1 M KCl, followed by centrifugation for 10 min at 46,000 × *g*. The final pellet was resuspended in 8 ml of 1 M KCl buffered with 0.05 M tris(hydroxymethyl)aminomethane (tris) at pH 6.4 to 6.8 and frozen at −15 C. At lower pH and at a lower molarity of KCl, clumping of cell walls occurred to a greater extent during a freeze-thaw cycle. The frozen cell-wall suspension was thawed, and was treated for 2 min in a Raytheon 10-kc oscillator at 5 C to break up clumps which formed during the freezing process. Remaining clumps were removed by centrifugation for 10 min in a conical centrifuge tube at 700 × *g*. The supernatant suspension was removed, and a 2-ml sample was placed on a sucrose gradient and centrifuged for 30 min at 3,100 rev/min as described under Materials and Methods.

Sucrose gradients of completely disrupted organisms contained a single band of turbidity. Material from this band was washed free from sucrose by centrifuging at 46,000 × *g* and suspending in distilled water three separate times. Electron micrographs revealed the presence of cell walls and cell-wall fragments, with no evidence of cytoplasmic contents. When placed in a CsCl gradient, this material gave a single symmetrical band of density 1.426.

Incompletely disrupted organisms gave rise to two bands of turbidity in sucrose gradients. Material from the upper band appeared to be identical to that observed in completely disrupted organisms, and consisted of cell walls. Material from the lower band was washed free from sucrose. This material was nonviable and indistinguishable from whole cells when placed in a CsCl gradient. The amounts of remaining cells, as determined by the turbidity in a sucrose gradient, checked well with the values obtained from a CsCl gradient.

Although the cell-wall material was distributed symmetrically in a sucrose gradient, it was of interest to see whether there were physical and chemical differences between cell walls isolated from lower and upper portions of the cell-wall band. A small batch of cell walls from cells with an effective growth time of 6.8 hr was shaken for 7.8 min in a Braun shaker and washed four times with 1 M KCl. This material was placed on four identical sucrose gradients. Centrifugation time was extended to 70 min to bring the cell-wall peak into the center of the gradient. In all gradients of cell walls, 10 to 20% of the starting material forms a precipitate on the bottom of the sucrose gradient tube, owing to the presence of clumps, which tend to form as cell walls stand in concentrated suspensions. Material from the precipitate and the A, B, and C regions (Table 2) of each gradient was pooled, washed twice with water, and suspended in 1.0 ml of water. Samples were dried and weighed, and RNA and total phosphorus were determined (Table 2). It can be seen that the phosphorus content was constant at about 2.72% phosphorus, as has been observed for similar preparations. The RNA content was rather constant at less than 0.2%, except in the case of the precipitate.

Material from the A, B, and C regions of

Table 2, when rerun in separate sucrose gradients, came off respectively in the A, B, and C regions of the second sucrose gradient. This would suggest that material from the A region is of different size than that from the C region. This was confirmed by electron micrographs which show whole cell walls in the A region and cell-wall fragments in the C region. As yet, it is not known whether cell-wall fragments form directly by breakage of cells or are preceded by whole cell walls. When whole cell walls from the A region of the sucrose gradient were treated for 5 min in a Braun shaker with glass beads, no decrease in the optical density at 660 mμ was observed, and there was little or no change in the pattern of sedimentation in sucrose. This would suggest that the smaller fragments may arise only during rupture of the whole cells or shortly thereafter. Further work is being carried out to determine whether cell-wall fragments differ chemically from whole cell walls, and to determine whether these fragments are related to any newly synthesized portion of cell-wall material or are merely formed in a random fashion from all parts of the cell wall during disruption.

## DISCUSSION

Because of their large size (500 mμ in diameter) it has been difficult to characterize cell walls by standard physical and chemical methods used for proteins and nucleic acids. The only method which has been employed extensively with success for characterization of cell walls has been observation of electron micrographs, as discussed by Salton (1960). Virus particles of 300 mμ lengths were separated by Steere (1963) on 1% agar-gel columns, but separation of cell walls and cell-wall fragments has not been reported. Electrophoresis has not been used to characterize cell walls, although Roberson and Schwab (1960) carried our electrophoresis of sonically treated streptococcal cell walls. Diffusion, light-scatter, and size distribution measurement have not been used.

In considering the possible methods for characterizing cell walls by physicochemical means, there appeared to be an absence of any study of the flotation patterns of cell walls in a CsCl gradient. It is clear from the present study that cell walls from a single strain of *S. aureus* have a specific density, regardless of the growth phase of the parent organism. Although the densities of cell walls of different bacterial species have not been determined, it seems probable that these also may be specific and constant, and may allow a characterization of cell-wall type.

Purification of staphylococcal cell walls was accomplished by Yoshida (1961), with sucrose density gradients. Roberson and Schwab (1960) separated streptococcal cell walls from chemically similar particles with the use of sucrose gradients. In the present study, it was possible to separate cell-wall material into subfractions of different sizes with the use of sucrose density gradients. The kinetics of formation of fragments and source of origin will be reported in a future publication.

In the present study, it was possible to determine conditions necessary for the complete breakage of cells to cell walls by observation of CsCl flotation patterns. A further study of these patterns revealed a possible intermediate in the disruption of cell walls. Pattern changes during autolysis were also observed, and will be reported elsewhere.

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