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Biophysical Studies of Microbial Cell Walls

Part 1 - The Preparation of Isolated Cell Walls

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ABSTRACT

A biophysical characterization of microbial cell walls can be greatly facilitated by the availability of walls free from other cell constituents. Isolated cell walls for this purpose are best prepared by rupturing the microbial cell by mechanical means, removing soluble cytoplasmic constituents by washing, and subsequently separating the walls from unbroken cells and particulate debris by centrifugation. In this investigation methods were explored for the efficient rupture, without undue fragmentation, of typical microorganisms using violent agitation with small glass beads effected by high-speed stirring or by shaking in a commercial cell homogenizer. The extent of rupture of the microorganisms was measured by viability loss, microscopic appearance, turbidity, and the ultraviolet adsorption of released cytoplasmic constituents. Clean fractionations of cell walls with minimum losses were accomplished by centrifugation on preformed density gradients of solutions of selected salts and nonelectrolytes. The purity of the final cell-wall product was assessed by light and electron microscopy and by the ultraviolet light adsorption of acid extracts. Autolytic degradation of the walls was minimized by maintaining low temperatures during all stages of preparation. The cell walls of Gram-positive organisms met all of the criteria of purity; those from Gram-negative organisms were less homogeneous.

PROBLEM STATUS

This is an interim report; work on the problem is continuing.

AUTHORIZATION

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BIOPHYSICAL STUDIES OF MICROBIAL CELL WALLS

PART 1 -- THE PREPARATION OF ISOLATED CELL WALLS

INTRODUCTION

The growth of knowledge of the composition, structure, and function of many of the components of the living cell has depended to a considerable extent on the development of convenient methods for their isolation from other cellular constituents. Among the structural features of the microbial cell of which this is particularly true is the cell wall. The relative ease with which this morphological entity can be prepared has undoubtedly facilitated and encouraged the rapid expansion of biochemical work on it which has occurred during the last two decades (1). For some phases of our work on the physicochemical properties of the surfaces of bacteria it has also appeared desirable to use isolated cell walls. Electrophoresis, for example, could not meaningful be carried out on intact cells under conditions where the cell is damaged sufficiently to release substances from the cytoplasm or cytoplasmic membrane which would subsequently absorb on or react with the wall surface and, thus, change the mobility. Such artifacts might be avoided by working with purified cell walls. Similarly, studies of the interaction of water and other agents with the cell surface can be made in a more meaningful way, if cell walls free of other cellular constituents are used (2).

The major objectives which we have endeavored to achieve in a method of cell-wall preparation are that alterations in the chemical and physical properties of the walls be minimal, that the process be capable of yielding gram amounts of cell walls quickly and easily, and that the apparatus involved be simple to operate and maintain.

The preparation of isolated cell walls can be divided naturally into three steps: the production of a break in the surface layers of the cell through which cytoplasmic constituents can escape, isolation of the cell walls from these constituents and from unbroken cells, and assessment of the purity of the final product. These steps are discussed in detail, followed by a presentation of typical results obtainable by the procedures evolved.

DISRUPTION OF MICROBIAL CELLS

Mechanical Methods of Rupture

Chemical and enzymatic (autolytic) methods were among the earliest ways of producing a break or lesion in the surface layers of microbial cells. Such methods are now recognized to be objectionable because of the probability of degrading the cell wall (1). They have been replaced almost exclusively by mechanical methods of disintegration. Various approaches have been tried (1,3,4): rapid decompression (5,6), ultrasonication (7,8), application of shearing stresses to liquid or frozen suspensions (9-11), and agitation with abrasives or glass beads (12-14). Several of these approaches have been used in commercially available devices. The most elaborate is the pressure cell disintegrator developed by Ribi, et al. (11,15), in which the cells are ruptured by forcing a liquid suspension at very high pressure through a small, gas-cooled orifice. The apparatus has the advantage of high capacity and temperature control, but it was considered too expensive for our current use. Devices which disrupt microbes by shaking at high frequency

with glass beads (e.g., the Mickle disintegrator (16)) have been most popular, because they are efficient, inexpensive, and simple to operate and do not fragment the walls excessively. These, however, usually treat rather small amounts of cells for our purposes and, unless operated in a cold room, are not readily adaptable to the maintenance of low temperature — a capability we considered essential to minimize the possibility of physical or chemical changes taking place in the cell walls during the rupturing process.

In spite of the obvious disadvantage of introducing a possible source of contamination, a method of cell disruption based on agitation with glass beads appeared most promising for our purposes. A simple procedure described by Lamanna and Mallette (17) permits rupturing relatively large quantities of microorganisms by stirring them in an aqueous suspension with glass beads in a commercial blender. In our earliest trials with this method, the temperature was not easily controllable, the rate of cell breakage was rather slow, and, during the lengthy stirring periods necessary, there was considerable glass-bead breakage and erosion of the stainless-steel blender blades to form collodial particles, which were troublesome to remove. These difficulties were partially overcome by stirring the suspension of cells and glass beads by an overhead stirrer at high speed in a stainless-steel cup surrounded with crushed ice. Since the geometry of the cup and stirrer is important for efficient cell rupture, a schematic drawing of the apparatus is shown in Fig. 1. The cup, with four internal baffles, is held in a cylindrical Lucite jacket, which can be filled with crushed ice. The stirrer blades, taken from a commercial Osterizer blender, are attached to the end of a stainless-stell shaft (Virtis Macro) driven by a 1/5-hp Precise Super-30 motor. The stirrer shaft and the top of the cooling jacket are fitted with complementary parts of an aerosol-spray trap (Virtis).

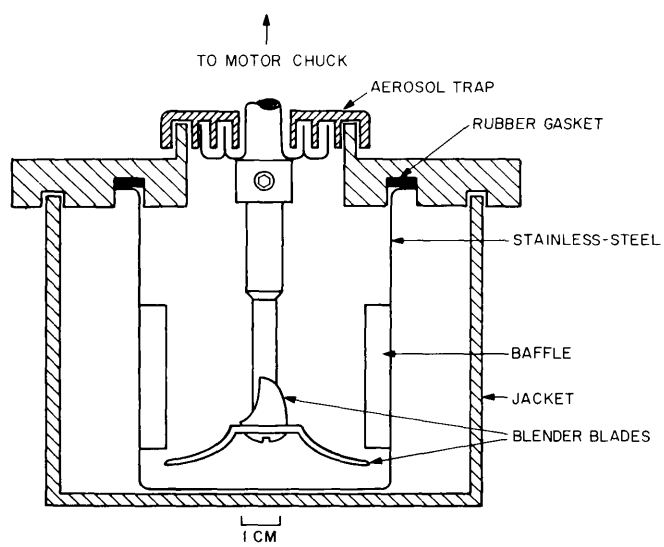

Fig. 1 - Apparatus for disruption of microbial cells by high-speed stirring with glass beads

To make a run, a precooled charge of 100 to 150 cc of glass beads and about 75 cc of precooled microbial suspension were introduced into the cup, which was packed in crushed ice in the securely anchored jacket. The motor and stirrer were lowered so that the lower blades rotated under the baffles in the cup but without touching the bottom. Runs of 3 to 8 min with a stirrer speed of about 3400 rpm (60 v) with 10-sec pauses

every minute to permit dissipation of heat were usually sufficient. Longer disintegration times than necessary to break most of the organisms were avoided because of excessive fragmentation of the cell walls and the generation of colloidal steel and glass. At speeds greater than about 3500 rpm, the stirrer shaft was likely to bend, giving spectacular but dangerous results. As a precautionary measure, the apparatus was always operated from behind a transparent safety shield.

During our search for a convenient and reliable method of rupturing microbial cell walls, a cell homogenizing device described by Merkenschlager, Schlossmann, and Kurz (18), hereafter abbreviated MSK, became commercially available (B. Braun, Melsungen, Germany, and Bronwill Scientific, Rochester, N.Y.). Although the apparatus was originally used for the extraction of intracellular enzymes, its application to cell-wall preparation was obvious (19). The apparent advantage which the MSK apparatus offers over high-speed stirring, namely, exclusion of erodable metal surfaces and provision for low-temperature control, made an evaluation attractive. In this apparatus a suspension of washed, intact cells and glass beads are placed in a 50 to 75 ml glass bottle, and the bottle inserted in a cylindrical steel chamber lined by rubber support strips. The chamber is mounted by bearings on a pin at one end. This pin is in turn attached eccentrically to the drive shaft. When the drive shaft is rotated, the bottle moves in a circular manner with a radius of about 6.5 mm but is prevented from actually rotating about its own axis by springs attached between the chamber and the frame of the apparatus. The apparatus may be run at speeds of 2000 and 4000 rpm.

Cooling of the shaken suspension is provided by expanding carbon dioxide flowing around the bottle. The apparatus is provided with a long steel capillary tube for leading the carbon dioxide from a siphon tube in a storage tank to the back of the chamber which holds the bottle. With use this capillary tube frequency broke during high-speed agitation because of metal fatigue. We have eliminated this difficulty by replacing the final 14 in. of the steel capillary with Teflon tubing (0.070 in. I.D.) and fixing the capillary about 1 in. from its end with the support provided. The Teflon tubing must fit tightly on the end of the steel capillary and be secured by wrapping a rubber band over it.

We have found the bottles provided with the MSK apparatus to be impractical because of their high cost and the tendency of the ground-glass stoppers to stick and break. We substituted 2-oz bottles (Aloe 17350) with screw caps. The Bakelite cap furnished was replaced with a polyethylene cap with a 1/16-in.-thick silicone rubber liner. The diameter of these bottles is slightly less than those supplied with the apparatus, and to insure a snug fit 2-in. lengths of 1-in.-diameter Gooch tubing were placed concentrically around the bottles. A preferable solution allowing better cooling of the bottle contents would be to increase the thickness of the rubber support strips in the apparatus. The bottles were discarded after a few runs when erosion of the interior surface became obvious.

A typical cell-rupture run was made as follows. The bottle containing glass beads was precooled to near 0°C in a refrigerator. The microbial suspension, similarly cooled, was added, and the bottle tightly capped. The chamber of the MSK apparatus was cooled by a flow of carbon dioxide for about 10 sec, and the bottle inserted and shaken at 4000 rpm for 15-sec intervals, followed by 5 sec at rest, but with a continuous flow of carbon dioxide. After the first minute, the cooling period was reduced to about 10 sec for each 20-sec shaking period. At the end of every minute of agitation, the bottle was removed and examined to be sure that the contents were cold but not frozen. Some experience is required for the successful control of temperature with the present cooling arrangement. At the end of the run the entire contents of the bottle were washed out with cold water or buffer, and the cell-wall isolation process was begun.

Evaluation of Abrasive Beads

Although many kinds of abrasive particles have been used for the mechanical destruction of microorganisms (12), small glass beads have been used almost exclusively for the preparation of cell walls since their introduction by King and Alexander (13) and Dawson (14). Their relative purity and ready availability in suitable size ranges are strong recommendations for their use. However, it cannot be assumed that such beads manufactured in large quantities primarily for metal polishing or highway-sign reflectors are likely to be entirely satisfactory for preparative biological work. Iron, for example, is commonly present both in a particulate form and as a surface contaminant. It is well known that glass itself releases alkali in the presence of water (20). Where large surface areas are exposed as in the case of the small glass beads usually used for cell disruption, this alkalinity can increase the pH of the media sufficiently to be potentially damaging to some cell-wall components (21). Even though the effect can be reduced by a preliminary leaching of the beads, the release of alkali from fresh glass surfaces resulting from bead breakage during shaking is unavoidable.

The glass beads which we evaluated were "Superbrite" (Minnesota Mining and Mfg. Co.), with average diameters given by the manufacturer of 0.1 mm, 0.2 mm, and 0.38 mm, and "Glasperlen" (B. Braun Apparatebau), with an average stated diameter of 0.17 to 0.18 mm. New beads were treated with concentrated hydrochloric acid until the green color (due mainly to iron) ceased to be released. Beads previously used for rupturing microorganisms were treated with concentrated nitric acid or a mixture of concentrated nitric and sulfuric acids. After acid treatment the beads were exhaustively washed. This was conveniently done by placing the beads in a large chromatographic column and slowly back-flushing with distilled water until the pH of the effluent was 5 or above. In this step broken beads and foreign particulate matter can be hydraulically separated and washed out the top of the column if the flow rate is suitably adjusted. The beads were finally washed with reagent-grade acetone or ethanol, transferred to a large evaporating dish, and dried in an oven. The method of dealing with the problem of alkali release from these beads during cell disruption will be described later.

The most effective bead diameter for disruption of most microbial cells in the MSK apparatus is between 0.1 mm and 0.2 mm. Beads with a 0.38-mm diameter produce somewhat less efficient rupture, fracture more readily, and, thereby, release more alkali to the suspension. Beads with a 0.1-mm diameter rupture at least as well as 0.2-mm beads, fracture less readily, and produce less alkali but are more troublesome to clean and handle. As a compromise, we used 0.2-mm and 0.170 to 0.18-mm diameter beads for most of our work.

It has been reported that 20 to 50 mesh crosslinked polystyrene beads were successfully used to rupture microorganisms in a continuous-flow shaking apparatus (22). Recently, others have apparently found such beads useful in batch operation (23,24). Anticipated advantages over glass beads are the absence of alkali release and reduced heat generation during shaking. Whether these beads introduce contamination to shaken suspensions is apparently not known. We have tried styrene-divinylbenzene copolymer beads (Dow Chemical Co.), 50 to 100 mesh, for disrupting *Bacillus megaterium* cells in the MSK apparatus. Cell rupture occurred, but there was much agglutination among cells, cell walls, and beads, which was not readily dispersable. This difficulty was not mentioned by the other investigators who have used such beads, but it was sufficiently serious to cause us to confine most of our work to glass beads.

Suspension Media

Microbial cells are usually disrupted in water or dilute aqueous buffers, but there seems no a priori reason to suppose that these media are necessarily the best for

cell-wall preparation. Media whose thermodynamic water activity has been lowered by the addition of suitable solutes, for example, could conceivably affect the extent or rate of rupture of microorganisms as a result of osmotic shrinkage of the whole cell or the dehydration of the cell wall. We examined this possibility by comparing runs in the MSK apparatus using *Micrococcus lysodeikticus* in solutions of sodium sulfate (15 and 23 wt-%), lithium bromide (60 wt-%), and sucrose (30 and 45 wt-%). Relative to the control suspension in 0.05M phosphate buffer, the rupture rates in sodium sulfate solutions were about the same, while those in sucrose and lithium bromide were significantly lower. We concluded that lowering the water activity of the suspending media offered no advantage in cell-wall preparation. Media of high viscosity were also unfavorable.

Organic liquids also have potential advantages as suspending media for cell disruption (25,26). It may be anticipated that rupture of the surface layers of microbial cells would be easier in organic media than in water by analogy with other hydrophilic, gel-like solids which are embrittled when water is replaced by alcohols, hydrocarbons, etc. Such media would, of course, be objectionable if the cell walls contained proteins or lipids which could be denatured or extracted. With cell walls composed predominantly of polysaccharide and peptidoglycan, however, organic liquids could be found which would not be likely to produce irreversible changes.

We used *Bacillus megaterium* in exploratory experiments with alcohol and pentane as the suspending media. In alcohol suspension, shaking for up to 5 min in the MSK apparatus did not produce a significant number of cell walls as judged by microscopic appearance. Shaken suspensions, subsequently washed and resuspended in water, appeared to have only a slightly reduced content of cytoplasmic constituents; granules were still present. Cells shaken in suspension with pentane (following several washes in alcohol) gave a similar result with the additional complication that much agglutination occurred between cells and beads. This behavior indicated that the cytoplasmic components coagulated irreversibly in organic media and acted as a sort of glue to hold the cell wall and cytoplasmic constituents together so that no substantial rupture or cytoplasmic loss could occur. A similar phenomenon was observed by Edebo (27) in the sonic treatment of microorganisms in the presence of protoplasmic precipitants, such as heavy metal cations. The approach was abandoned as a means of rupturing vegetative bacteria for cell-wall preparation. It did prove useful, however, for crushing bacterial spores.

Two additional digressions from the usual manner of rupturing microbial cells were tried. Frozen suspensions of *Bacillus megaterium* were shaken with glass beads in the MSK apparatus after preliminary crushing under liquid nitrogen. In spite of the fact that the bottle of suspension could be strongly cooled by a rapid flow of expanding carbon dioxide around it, extensive clumping occurred in the suspension owing probably to local heating, melting, and subsequent refreezing. Some cells were ruptured, but the approach did not appear promising. Frozen-dried preparations of *Bacillus megaterium* cells were mixed with glass beads and shaken in the dry state in the MSK apparatus. Extensive rupture of the cells occurred, but microscopic examination revealed extensive fragmentation of the cell walls. Dissipation of heat generated during shaking was also more difficult than in liquid suspensions. The method might be useful for special purposes, but it was not pursued further.

A most useful addition which should be made to the suspension media before cell disruption is an inhibitor of autolytic enzymes. The danger of degradative changes occurring in cell walls as a result of the action of enzymes associated with the wall or released into the medium during rupture is, as shown by Weidel, Frank, and Leutgeb (28) and others (29), a real one. Some investigators have added substances intended to inhibit these enzymes, but there was no assurance that an effective inhibition of cell-wall degradation actually occurred (30,31). Others have recommended the use of such agents as heat or high concentrations of surfactants (28). These agents are objectionable for our purposes because of the drastic irreversible changes likely to be produced in wall

structure and composition. Until suitable specific inhibitors have been found, the safest alternative seems to be to carry out the disruption and purification procedures rapidly and at low temperature (cf. Ref. 32).

Distilled water is a frequently used suspension medium for microbial disruption, but we found, in agreement with Kolb (21), that the alkali released by the beads caused the pH of an aqueous suspension to rise from 2 to 3 units during shaking. Consequently, we used phosphate buffers exclusively as the suspension medium. The concentration usually used was 0.05M, but recent checks in which low concentrations of microorganisms were used showed that a pH of 8 was occasionally exceeded. A buffer concentration of 0.1M would be a better choice for routine use, especially with beads 0.2 mm in diameter and larger.

ASSESSMENT OF THE EXTENT OF CELL RUPTURE

An obvious method of determining the extent of cell breakage is to measure the viability of the homogenized suspension. Ruptured cells will not form colonies when plated on agar; however, unruptured cells will not necessarily form colonies either. The deprivation of nutrients, changes in temperature, and the mechanical disturbances involved in harvesting, washing, centrifuging, etc., of organisms which were initially viable may damage them enough to make them nonviable (33,34). They may, nonetheless, contain cytoplasmic materials which are not an inherent part of the cell wall. As an absolute measure of cell-wall production, the method is thus reliable only with hardy organisms which do not die too readily under the normal conditions of manipulation. For viable counts, we used the drop method of Miles and Misra (35) for making surface colony counts on agar plates, with minor modifications as described in Ref. 36.

Phase microscopic examination of aqueous suspensions of the homogenate or of dried, stained preparations is a convenient way of determining the extent of rupture. While the shape of the empty cell walls usually resembles that of the intact cell, there is usually a very striking difference in the light adsorption and refractility, especially with Gram-positive organisms. The use of a basic cytoplasmic stain sometimes helps to enhance the difference in appearance between intact cells and cell walls. We have used acidic crystal violet according to the staining procedure of Lamanna and Mallette (37).

Less direct, but very convenient, are optical methods based on the extent of release of cytoplasmic material. A reduction in turbidity of the suspension occurs as cell rupture proceeds, because empty cell walls and dispersed cytoplasm generally scatter much less light than intact cells. The optical-density decrease in red light is much used to measure such a turbidity decrease. The extent of rupture can also be followed by measuring the amount of released cytoplasmic constituents remaining in solution after centrifuging out the particulate matter — cells and cell walls. Since nucleic acids absorb strongly at 260 mμ and are found exclusively and abundantly in the cytoplasm, an examination of the supernatant at this wavelength provides a convenient measure of the extent of rupture.

We have used these two optical methods extensively as relative measures of the degree of cell rupture during our evaluation of methods of rupturing microbial cells. The procedure usually used was to remove samples of a few tenths of a milliliter during and at the end of a run, transfer them to 1-ml test tubes to allow beads to settle out and bubbles to rise, and then with a micropipette remove a sample of the suspension for appropriate accurate dilution with water for turbidity measurements in a Klett colorimeter (red filter 66) or in a Spectronic-20 (Bausch and Lomb) spectrophotometer at 600 mμ. Subsequently, this suspension or a similarly prepared one was centrifuged for 10 to 15 min at about 10,000 g's, and the supernate was further diluted with water for optical-density measurement at 260 mμ in a Beckman DU spectrophotometer. As rough guides for making the appropriate dilutions, we used 1:40 for the turbidity measurement and

1:200 for the optical-density measurement at 260 mμ for a microbial suspension originally containing 30 mg/ml dry weight. Appropriate adjustments in dilution were made for other suspension concentrations.

ISOLATION OF CELL WALLS

Following the rupturing process, the cell walls must be separated from the unwanted components of the homogenate: the glass beads, small particles of glass and metal, unbroken cells, detached flagella, pili, and cytoplasmic constitutents. During the separation processes, it is important to proceed rapidly and at a low temperature to minimize the possibility of damage to the walls from autolytic enzymes. Even these precautions may not be sufficient to prevent degradation of the walls in some cases (28). Most of the purification procedure involved only simple methods. The glass beads were separated from the homogenate by filtration through a coarse glass frit and were repeatedly washed with cold 0.05M phosphate buffer (pH 7) until the filtrate appeared clear. The combined filtrate was then centrifuged in a refrigerated centrifuge (4°C) for 15 to 20 min at 10,000 g's. The sediment was washed by resuspension in phosphate buffer, recentrifuged, washed in 1M sodium chloride to assist in desorbing cytoplasmic constituents from the walls, and then washed again in dilute buffer. The sediment after the last centrifugation, consisting mainly of cell walls, unbroken cells, and particles of glass and metal (if high-speed stirring was used), was then resuspended in a small amount of buffer for further purification.

The conventional method of separating cell walls from intact cells and other debris in the washed homogenate is differential centrifugation. Its usefulness depends on the fact that in the usual aqueous suspending media the walls sediment more slowly than the intact cells and particulate debris. The walls can be scraped off the top of the pellet after centrifugal deposition, and if the process is repeated several times, a satisfactory cell-wall preparation can be obtained. The method is time consuming and wasteful of material.

An improvement on separation by conventional centrifugation can be effected, if the sedimentation is stopped after most of the intact cells and heavy debris have settled to the bottom of the centrifuge tube but while the cell walls are still suspended. To provide a hydrodynamically stable medium which can be subjected to the usual handling without convective mixing, a homogeneous solution of a solute, such as sucrose or glycerol, or, preferably, a preformed density gradient of the solute can be used in the centrifuge tube (38-41). The cell homogenate is deposited on the surface of the solution and spun in a swinging-bucket rotor. If the concentration of the solution is suitably chosen, an effective fractionation of cell walls can be made in many cases.

A still more attractive alternative where a high-speed centrifuge is available is the use of zonal centrifugation in density gradients so adjusted that each particulate species in the homogenate can sediment to, or close to, its buoyant density level. The method has been extremely useful for isolating intracellular constituents and promises to become even more popular with the advent of high-capacity zonal centrifuge rotors (42). Our evaluations of this approach have been made with conventional swinging-bucket rotors in tubes containing preformed gradients.

Among the more useful nonelectrolytes which we used in preparing density gradients for cell-wall isolation were sucrose, Ficoll (Pharmacia trade name for polysucrose), and Renografin (Squibb trade name for N,N-diacety-3,5-diamino-2,4,6-triiodobenzoate) (43) and among the electrolytes used were sodium bromide, potassium formate, sodium perchlorate, and potassium tartrate. The more commonly used cesium and rubidium halides and sulfates form sufficiently dense solutions for this purpose (44), but they were considered too expensive for routine isolation of large quantities of cell walls. Stock solutions of the electrolytes were filtered before use (HA, Millipore).

Approximately linear density gradients were formed by a continuous mixing procedure using two concentrations of the solute whose densities corresponded to the extremes desired in the density gradient (45). The more concentrated solution was pumped by a peristaltic pump (Harvard Apparatus Co., Model 600-1200) through silicone or gum rubber tubing at a fixed rate into a measured volume of the more dilute solution with continuous mechanical mixing. Simultaneously, the mixture was pumped from the dilute-solution container into the bottom of the centrifuge tube at double the rate of the concentrated-solution input. These rates were conveniently fixed by using two parallel rubber tubes in the pump leading from the mixing compartment to the centrifuge tube and a single rubber tube of the same size on the concentrated solution input. The solution entering the centrifuge tube thus increased continuously in density, and the less dense solution was pushed toward the top of the tube. A suitable range of density from the bottom to the top of the tube was usually about 0.1 density unit (g/cc) chosen (after an exploratory run on a steeper gradient) so that the buoyant density of the walls was approximately in the middle of the tube.

A suspension of the washed sedimentable material from the cell-rupturing operation was placed on top of the gradient and centrifuged at 20,000 g's in the Sorvall swinging-bucket head (Sorvall HB4) for 2 hr or more or in the Spinco rotor 25.1 at 40,000 g's for 1 hr. The band or bands of interest were removed by pipette or by siphoning off the contents from the bottom. One or more additional density-gradient runs were sometimes run on the isolated cell-wall fraction to obtain still cleaner separations. Excessive loading of the gradients can lead to unsatisfactory fractionations.

The final cell-wall fractions were then washed with water or 0.05M phosphate buffer until free of the density-gradient solute and examined for purity.

ASSESSMENT OF CELL-WALL PURITY

A simple and sensitive way of judging the purity of the final cell-wall fraction is by inspection of wet mounts with the phase microscope. Inorganic particles, intact cells, or cells containing granules or considerable cytoplasmic material are easily seen. Our preparations were always subjected to such inspection. With some cell-wall preparations, especially those from Gram-negative organisms, judgment by this method is difficult, because opacities are not uniform from one cell wall to another. In these cases examination with the electron microscope yielded further information. For this purpose, water-washed wall preparations were sprayed or placed dropwise onto 400-mesh carbon-coated grids, allowed to dry, and shadowed at an angle of about 1:3 with platinum. Examination was made in the Hitachi HU-11 electron microscope usually at 75 kv.

It is quite possible that contamination of cell walls could exist without being apparent by light or electron microscopy, e.g., by uniform adsorption over the wall surfaces of constituents from the cytoplasm. While no general solution to the problem of distinguishing contamination from true cell-wall constituents is available, it is possible to carry out analyses for some of the most likely foreign materials. Nucleic acids, since they are always present in relatively large amounts in the cell and are known not to be constituents of cell walls, are one such class of materials. They absorb markedly at 260 mμ, and an examination of the cell-wall preparation over a range of wavelengths in this region of the spectrum offers a convenient method of detecting their presence. Cell-wall suspensions cannot be examined directly in the spectrophotometer, however, since our studies and those of others (46) show that they scatter sufficient light to obliterate adsorption peaks at 260 mμ, even though significant amounts of nucleic acid are present. Although corrections for light scattering can be made, it appeared simpler to solubilize the nucleic acid by acid extraction and then examine the extract spectrophotometrically after centrifuging down the particulate matter. Our procedure was to treat wall suspensions (Klett reading about 100) for 20 min at 100°C with sufficient concentrated perchloric

acid to give a 0.5M solution. The solutions were centrifuged 15 min at 10,000 g's; 4 ml of the supernate were neutralized with 3M sodium hydroxide, adjusted to pH 7 with 0.5 ml of 0.5M phosphate buffer, and diluted with water to a total of 5 to 10 ml. Ultraviolet optical absorption measurements were made from 220 to 320 mμ using a water blank treated in the same way as the cell-wall suspensions. The adsorption curves were examined for peaks at 260 mμ.

RESULTS

Microorganisms

Four species of bacteria and one yeast were used for most of the evaluation of methods of cell-wall preparation. *Micrococcus lysodeikticus* (ATCC 4698) was cultured in Penassay broth (Difco) with 10 g of glucose added per liter; *Escherichia coli* (K-12) was cultured in trypticase soy broth (Difco); *Bacillus megaterium* (ATCC 13632) and *Bacillus subtilis* (var. *niger*) were cultured in a medium of casamino acids (Difco), yeast extract, and salts given by Edebo, Hedin, Holm, and Zacharias (Medium 3) (47). *Saccharomyces cerevisiae* was cultured in a malt-extract medium. Cultures were grown at 37°C in an apparatus described by Echols (48) or occasionally in 1-liter amounts in Fernbach flasks on a rotary shaker. Cells were harvested at about the end of the logarithmic phase of growth (usually 12 to 20 hr for bacteria and 66 hr for yeast) and washed three times in 0.05M phosphate buffer (pH 7.0). The cells were resuspended in this buffer after the final wash, and suspension concentrations were determined on an aliquot dried to constant weight at 105°C (allowing for the weight of the included buffer salts).

Rates of Rupture

Figure 2 shows the results of typical runs with the high-speed stirring apparatus. Two suspension concentrations of *Serratia marcescens*—57 mg/ml and 28-1/2 mg/ml—were used with 0.2-mm-diameter glass beads. An additional run at a concentration of 57 mg/ml using 0.1-mm-diameter glass beads was also included. The rate of rupture was followed by measurement of absorbance at 260 mμ of suitable diluted samples of the suspension supernate after centrifugation to remove particulate matter. Turbidity measurements could not be meaningfully used because of the significant contribution to the reading by the colloidal glass and steel generated by stirring. The results with the two bead sizes are essentially the same, and there is no apparent difference in the course of breakage with a twofold dilution of the suspension, since the curve for the dilute suspension is lower than the more concentrated one by the amount of the dilution factor, i.e., 50%. Most of the cell rupture appears to be complete in the first 2 min of stirring. Similar results were obtained in this apparatus with *Bacillus megaterium*.

To establish optimum conditions for cell rupture in the MSK apparatus, preliminary runs were made with *Bacillus subtilis* vegetative cells using variable amounts of beads and volumes and concentrations of cell suspensions. Figure 3 shows the effect of varying the suspension volume, with fixed concentration of organisms and amount of glass beads, on the rate of cell rupture. The turbidity of the diluted suspension (freed of glass beads) was used as a measure of the extent of rupture. The highest rate of rupture is obtained with 12 ml of suspension, although the differences in the three runs are not great. Suspension volumes as low as 8 ml gave rupture rates comparable to the 12-ml run. With still smaller volumes the control of temperature becomes more difficult, probably because of the reduced fluidity of the suspension.

Since the ratio of 12 ml of suspension to 33 g of beads gives good results, a second series of runs was made maintaining this ratio but changing the total volume. The results are shown in Fig. 4. There is no change in the rupture rate except at the highest

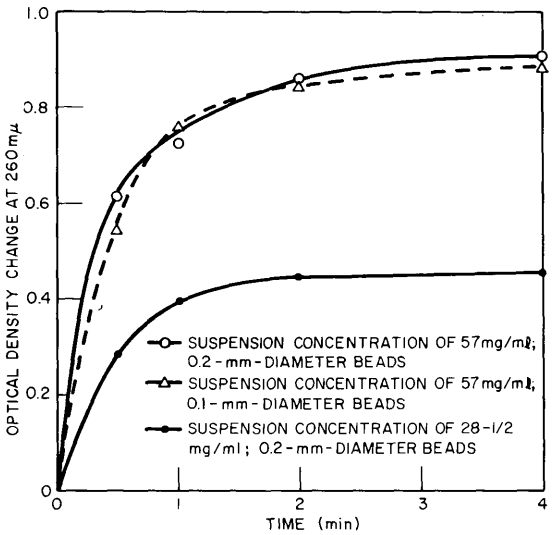

Fig. 2 - The effect of varying the bead diameter and concentration of *Serratia marcescens* cells on the rate of rupture by high-speed stirring with glass beads. The beads weigh 270 g, and the volume of the suspension is 87.5 ml.

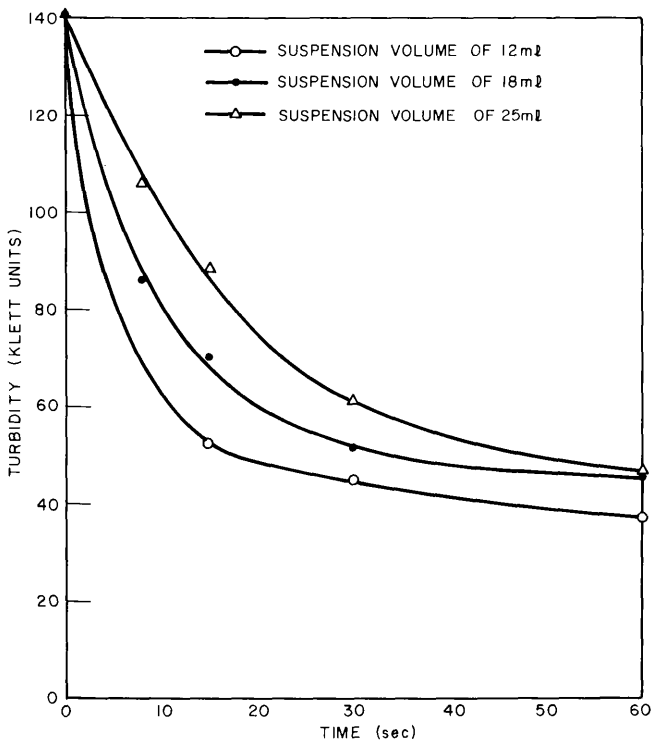

Fig. 3 - The effect of a variable suspension volume on the rate of rupture of *Bacillus subtilis* cells in the MSK apparatus. The beads have a 0.2-mm diameter and weigh 33 g; the suspension concentration is 15 mg/ml.

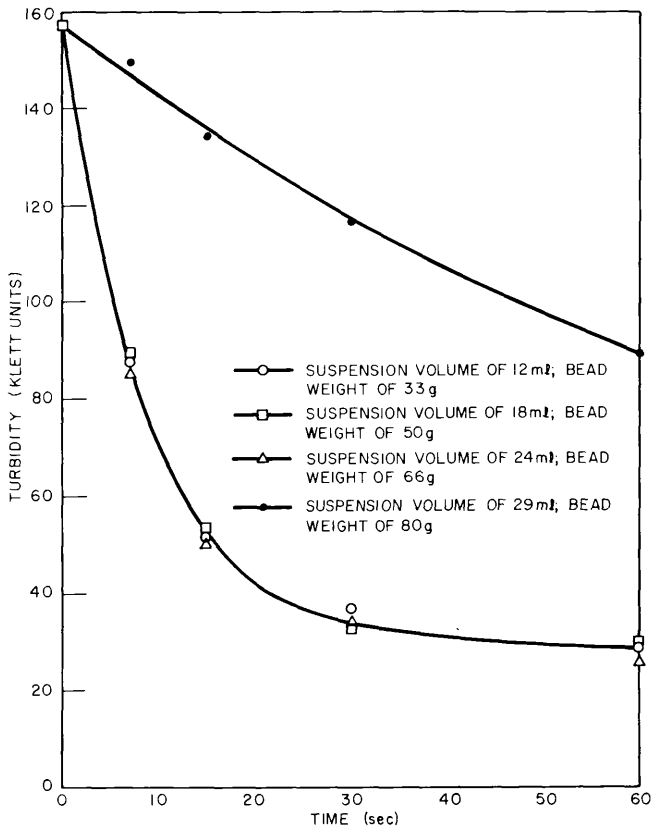

Fig. 4 - The effect of a variable total volume, with a fixed ratio of suspension volume to bead weight, on the rate of rupture of *Bacillus subtilis* cells in the MSK apparatus. The beads have a 0.2-mm diameter, and the suspension is 15 mg/ml.

volume, where it is greatly reduced. This volume fills the bottle to within about 1.0 ml of capacity. Thus, in accord with earlier observations of Rodgers and Hughes, using a different shaking device (49), it appears that an appreciable air space is necessary for efficient rupture of microorganisms by this type of mechanical method. The sharp optimum volume observed by these authors was not observed by us, however.

Figure 5 shows the effect of varying the concentration of organisms, using a volume of suspension and weight of beads known to be in the optimum range on the rate of rupture. Rupture rates are highest at suspension concentrations of 15 to 45 mg/ml. A significant reduction in efficiency occurs at the highest concentration of 90 mg/ml. Microscopic examination of the suspensions shaken at this concentration showed a greater percentage of cells only partially freed of their cytoplasmic contents compared to suspensions in the optimum range of concentration. These results are likely to be due largely to an increased viscosity and a greater tendency to foam. The apparent rate of rupture of the 5-mg/ml concentration is low, because colloidal glass particles from fractured beads contribute significantly to the turbidity. The rupture rate is high when judged by microscopic appearance.

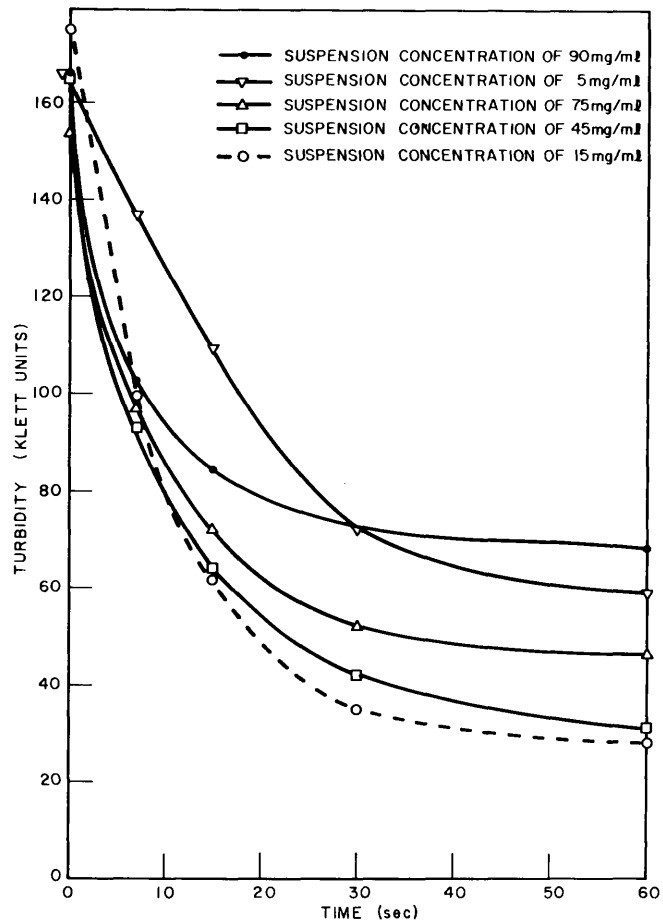

Fig. 5 - The effect of a variable suspension concentration on the rate of rupture of *Bacillus subtilis* cells in the MSK apparatus. The beads have a 0.2-mm diameter and weigh 50 g; the volume of the suspension is 18 ml.

To assess the general applicability of the MSK apparatus for microbial cell rupture, a variety of organisms have been tried along with different methods of measuring the extent of rupture. Typical results are presented in Figs. 6 to 10.

Figure 6 shows the results with *Micrococcus lysodeikticus* using two different suspension concentrations. Samples removed at different shaking times were diluted sufficiently so that the turbidity and supernate absorbance curves for the different concentrations can be compared directly. Rupture seems to be essentially complete in less than 3 min, and there is no significant difference in rupture efficiency over the range of suspension concentrations used.

Figure 7 shows a run with *Escherichia coli* (K-12) using viability as well as turbidity of the suspension and absorbance at 260 mμ of the supernate as criteria of rupture progress. All show similar courses and indicate essentially complete breakage after 2 min.

Figure 8 shows the progress of rupture of *Bacillus megaterium* in the MSK apparatus using the percentage of cells stained by crystal violet as a criterion as well as turbidity and 260 mμ absorbance. Breakage is again complete at the end of about 2 min.

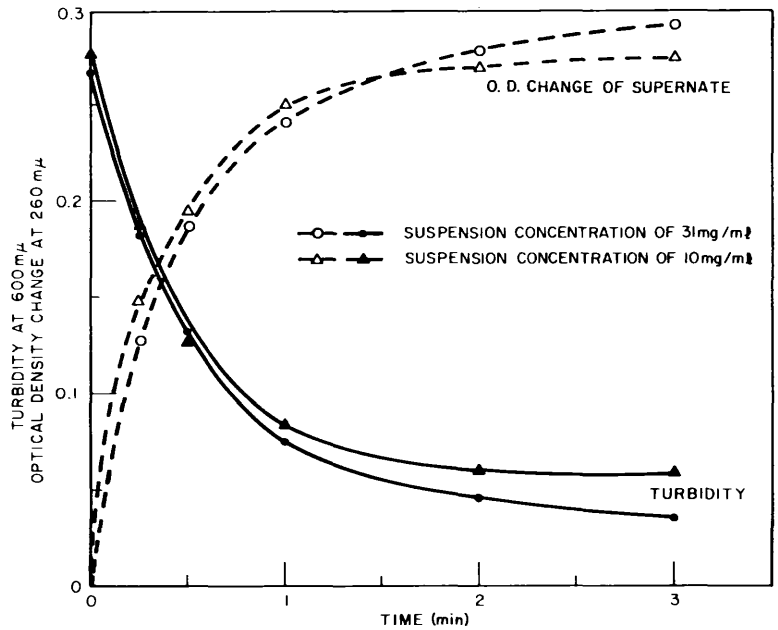

Fig. 6 - The rate of rupture of *Micrococuss lycodeikticus* cells in the MSK apparatus as measured by the reduction in turbidity and change in optical density of supernate at 260 mμ, using different suspension concentrations. The beads have a 0.2-mm diameter and weigh 41 g; the volume of the suspension is 25 ml.

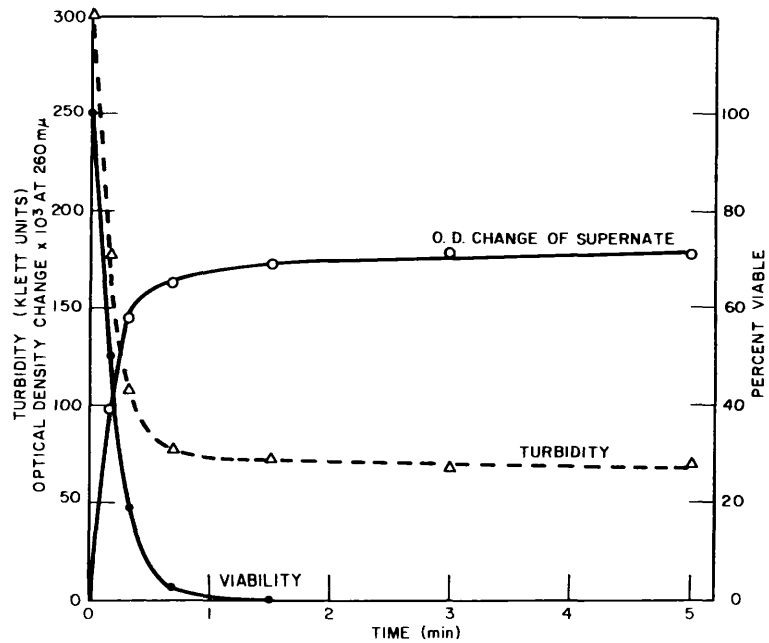

Fig. 7 - The rate of rupture of *Escherichia coli* cells in the MSK apparatus as measured by the reduction in turbidity, viability, and change in optical density of supernate at 260 mμ. The beads have a 0.2-mm diameter and weigh 46 g; the volume of the suspension is 25 ml and its concentration is 16 mg/ml.

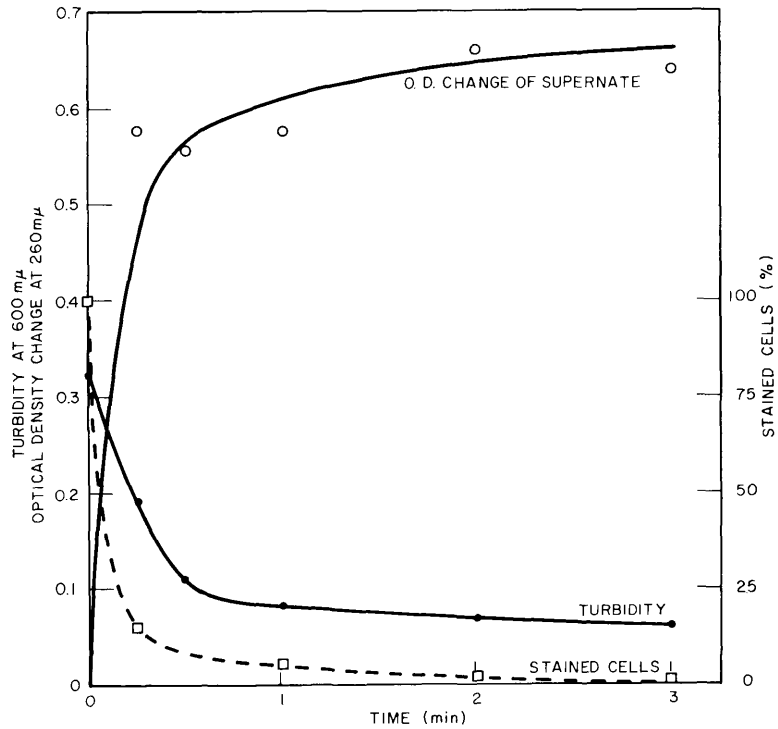

Fig. 8 - The rate of rupture of *Bacillus megaterium* cells in the MSK apparatus as measured by the reduction in turbidity, percentage of stained cells, and change in optical density of supernate at 260 mμ. The beads have a 0.2-mm diameter and weigh 54 g; the volume of the suspension is 25 ml and its concentration is 49 mg/ml.

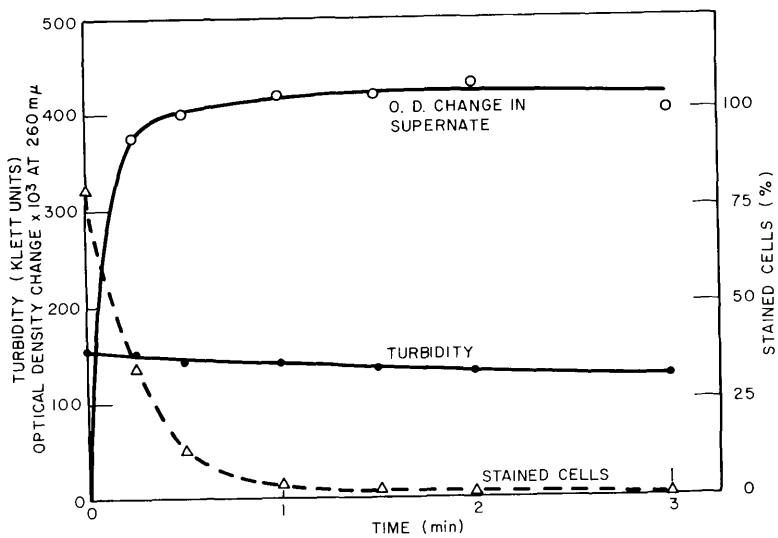

Fig. 9 - The rate of rupture of *Saccharomyces cerevisiae* cells in the MSK apparatus as measured by the reduction of turbidity, percentage of stained cells, and change of optical density of supernate at 260 mμ. The beads have a 0.2-mm diameter and weigh 46 g; the volume of the suspension is 25 ml.

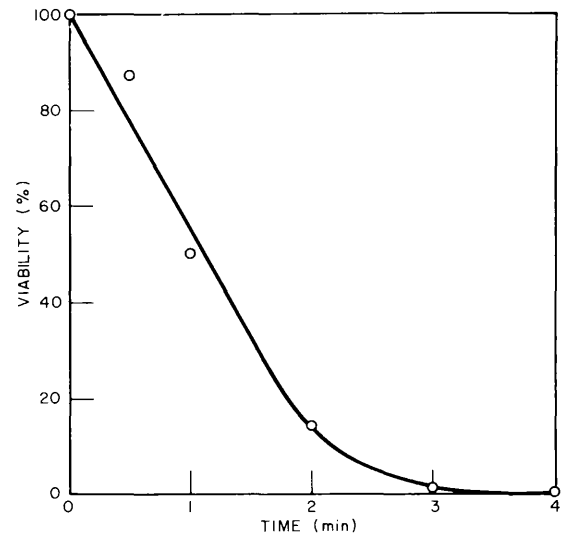

Fig. 10 - The rate of rupture of spores of *Bacillus subtilis* as measured by the reduction in viability in an ethanol medium. The beads have a 0.2-mm diameter and weigh 46 g; the volume of the suspension is 25 ml.

Figure 9 shows results obtained with yeast, an organism which is considered difficult to rupture by other methods. While the curves for stained cells and for material absorbing at 260 mμ indicate rapid breakage, the turbidity values change very little. It is probable that the large size and thickness of these walls cause them to scatter light almost as much as intact cells do.

Although the product of the breakage of bacterial spores cannot be classified as cell walls, it is appropriate to give the result of one such experiment to illustrate the effectiveness and range of applicability of the method. Figure 10 shows the rapid loss in viability with time of shaking in the MSK apparatus of *Bacillus subtilis* var. *niger* spores suspended in absolute alcohol. Microscopic examination revealed concomitant loss in spore refractility. It was demonstrated that exposure of intact spores of *Bacillus subtilis* to alcohol did not in itself cause any significant loss in viability.

Isolation of Cell Walls

Preliminary work on the isolation by density-gradient centrifugation of cell walls from intact cells, particles of glass, and other debris present in the suspension after cell rupture showed that no one solute was satisfactory for all kinds of cell walls. Electrolytes, such as sodium bromide, produced agglutination and little or no fractionation with *Eschericha coli* (strains K-12 and Crookes) and yeast. Sucrose gave good separations with *Escherichia coli*, and Renografin was satisfactory for yeast. With *Bacillus megaterium*, on the other hand, no satisfactory separation of cell walls from cells with included granules could be made on sucrose gradients, whereas good fractionation was possible with sodium bromide. Sodium bromide was also better than sucrose for purifying *Sacrina lutea* and *Micrococcus lysodeikticus* cell walls. Sodium bromide is not suitable for *Bacillus subtilis* var. *niger* cell walls, since their buoyant density exceeds that of the saturated solution. Potassium tartrate, however, gives an excellent fractionation.

Ficoll, a high-molecular-weight polysucrose polymer, offers, as a density-gradient solute, the potential advantage of being less damaging to biological materials than low-molecular-weight solutes. The latter necessarily have greater dehydrating effects because of the higher osmolar concentration and lower water activities which their solutions possess. Buoyant densities of cell walls were lower in Ficoll than in any of the

other solutes used for density gradients. Sharp separations of pure cell walls were usually not obtained, however, and the high viscosity of the gradient solutions makes them difficult to work with.

Table 1 shows the buoyant densities of selected cell walls in the solutes which we have found advantageous for their purification. These can be taken only as general guides for fractionation of cell walls, because cell-wall density may vary somewhat with different growth conditions for the particular organism; different strains of an organism can also be expected to have different cell-wall densities. The conditions for cleanest separation will in general have to be established for each particular kind of cell wall. A study of the factors influencing the buoyant density of cell walls in solutions of different solutes has been made (2) and will be presented in a subsequent report.

Table 1
The Buoyant Densities of Microbial Cell Walls after Centrifugation in Density Gradients of Various Solutes

Cell Walls	Density-Gradient Solute	Buoyant Density (g/ml)
Bacillus subtilis (var. *niger*)	Potassium tartrate	1.34
Bacillus megaterium	Sodium bromide	1.50
Micrococcus lysodeikticus	Sodium bromide	1.43
Sarcina lutea	Sodium bromide	1.44
Escherichia coli (K-12)	Sucrose	1.25
Serratia marcescens	Sucrose	1.23
Saccharomyces cerevisae	Renografin	1.31

Assessment of Cell-Wall Purity

The results of tests for the purity of several cell-wall preparations using the ultraviolet absorption spectra of hot perchloric acid extracts are shown in Figs. 11 and 12. No materials with absorption peaks at 260 mμ are present in any of the Gram-positive walls (Fig. 11). With Gram-negative walls, exemplified here by *Escherichia coli* (Fig. 12), the results of this analysis are less certain. A broad peak with a maximum at about 275 mμ is present, which suggests the presence of proteinaceous material in the extract. This is not surprising, since Gram-negative cell walls are known to contain proteins (1). Absorbance peaks due to protein could, however, obscure a small peak at 260 mμ. The situation is further exemplified by the absorbance curves of extracts of cell walls to which about 5% (on a number basis) of washed intact cells have been deliberately added and of the same amount of intact cells alone (Fig. 12). The extracts of intact cells have a broad peak at 260 mμ, and the presence of cell walls in the mixture shifts the observed peak to longer wavelengths. Thus, the purified cell-wall preparation does not appear grossly contaminated with nucleic acids, but its presence in significant amounts cannot be excluded on the basis of these data. A more specific analysis for nucleic acid would be required to decide this.

Figures 14 to 18 show the electron micrographs of shadowed preparations of typical purified microbial cell walls. The marked difference in appearance between these

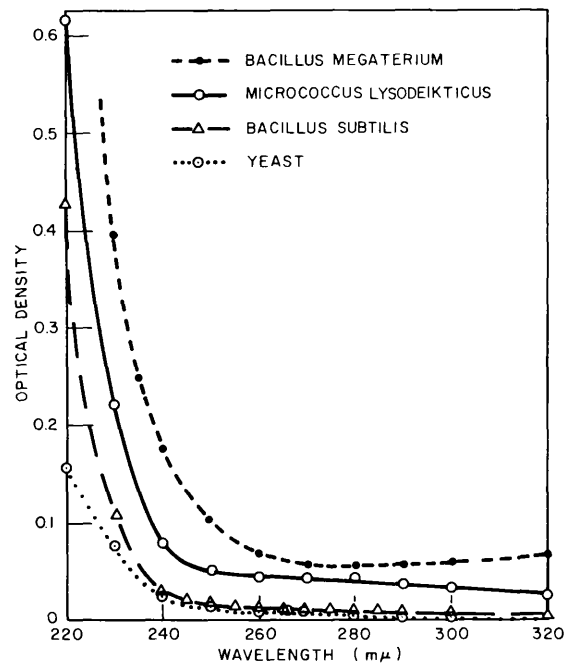

Fig. 11 - The ultraviolet absorbance of hot perchloric acid extracts of purified cell walls from gram-positive organisms

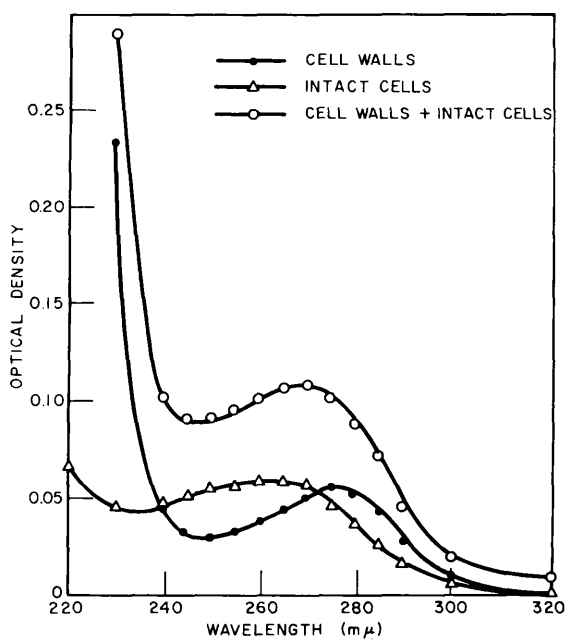

Fig. 12 - The ultraviolet absorvance of hot perchloric acid extracts of cell walls and intact cells of a gram-negative organism, *Escherichia coli*. The amounts of cell walls used for extraction alone and in the mixture with intact cells were the same. The amounts of intact cells used for extractions alone and in the mixture were also the same but numbered only about 5% of the cell walls.

preparations and an intact cell can be seen by comparison with Fig. 13. The cell walls from Gram-positive organisms (Figs. 14-16 and Fig. 18) all appear to be empty bags free of electron-dense cytoplasmic inclusions. The thickened bands visible in some *Micrococcus lysodeikticus* cell walls (Fig. 15) correspond to the areas of new cell-wall formation at the place where cell division would occur. The cell walls from a Gram-negative organism, *Escherichia coli*, (Fig. 17) apparently have irregular deposits of electron-dense material on the surface or inside. This is in agreement with the results of others (1) and supports the evidence from light-microscope examination and from ultraviolet absorption of hot perchloric acid extracts that Gram-positive organisms yield cleaner walls than do the Gram-negatives.

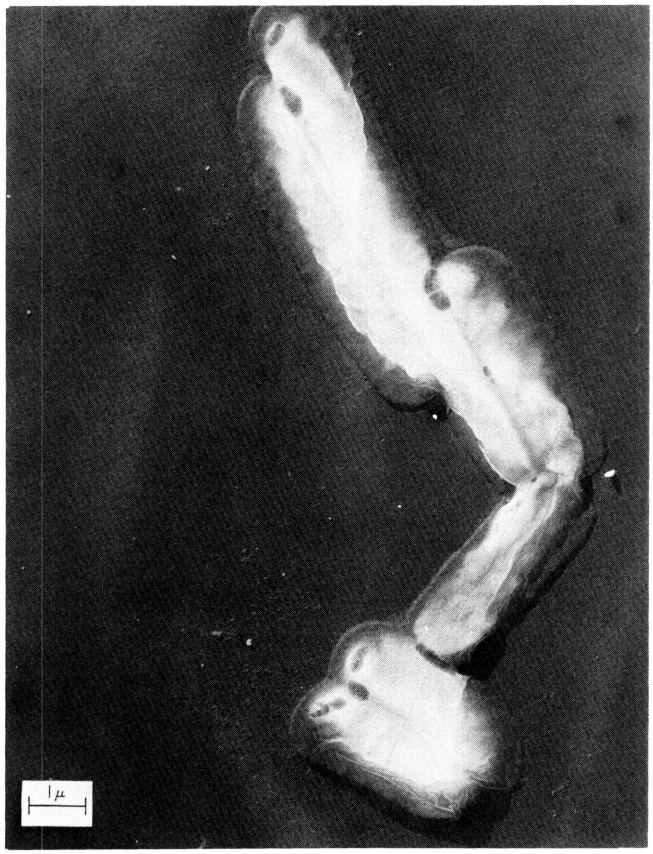

Fig. 13 - Intact cells of *Bacillus megaterium*

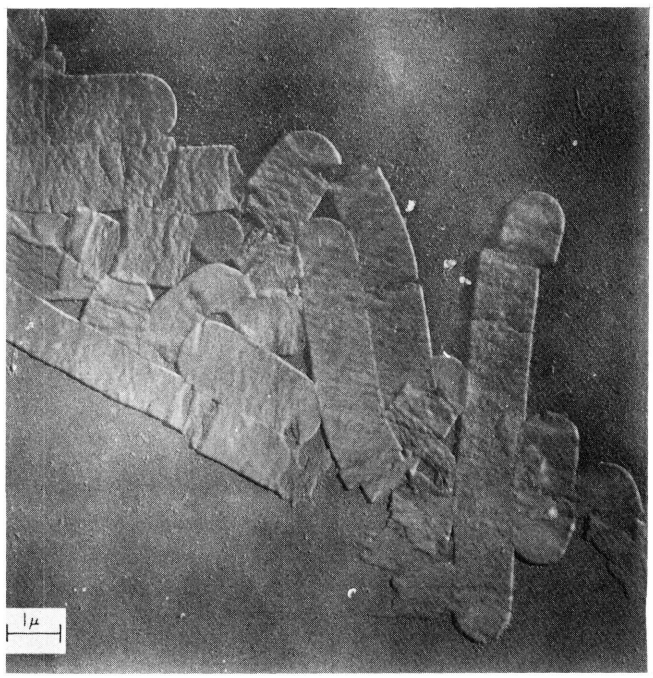

Fig. 14 - Cell walls of *Bacillus megaterium*

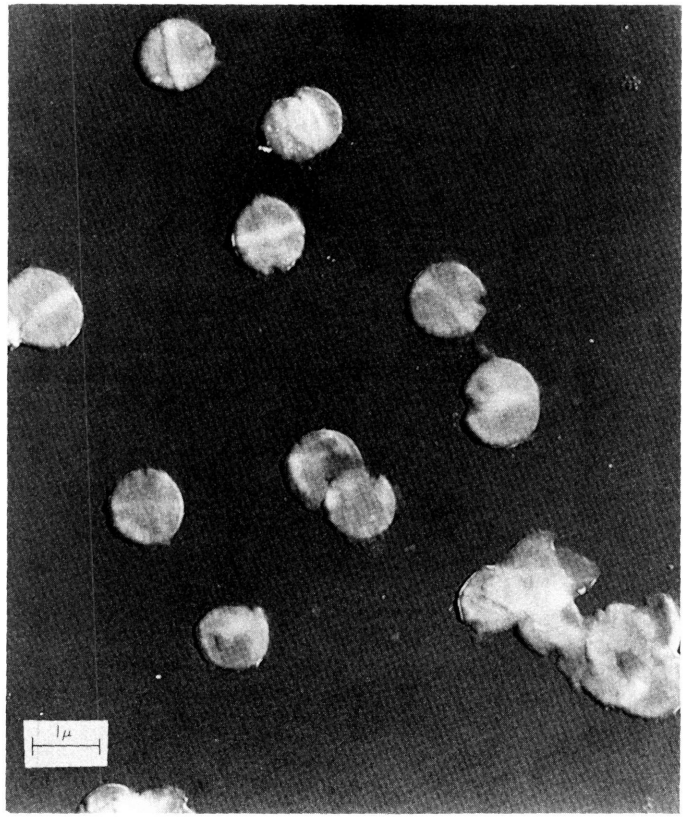

Fig. 15 - Cell walls of *Micrococcus lysodeikticus*

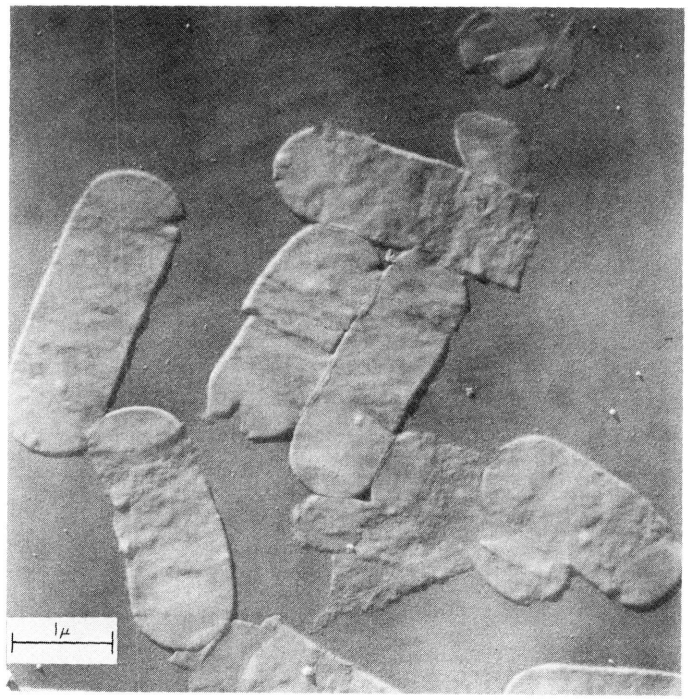

Fig. 16 - Cell walls of *Bacillus subtilis*

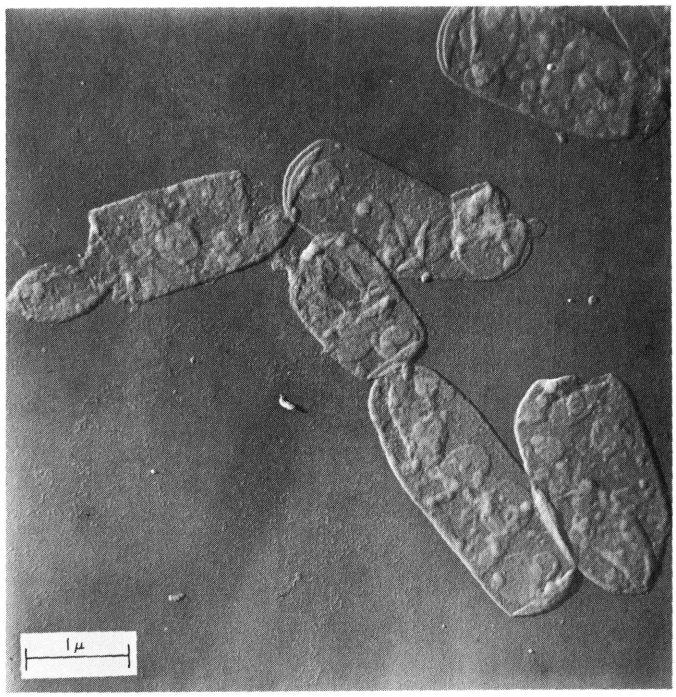

Fig. 17 - Cell walls of *Escherichia coli*

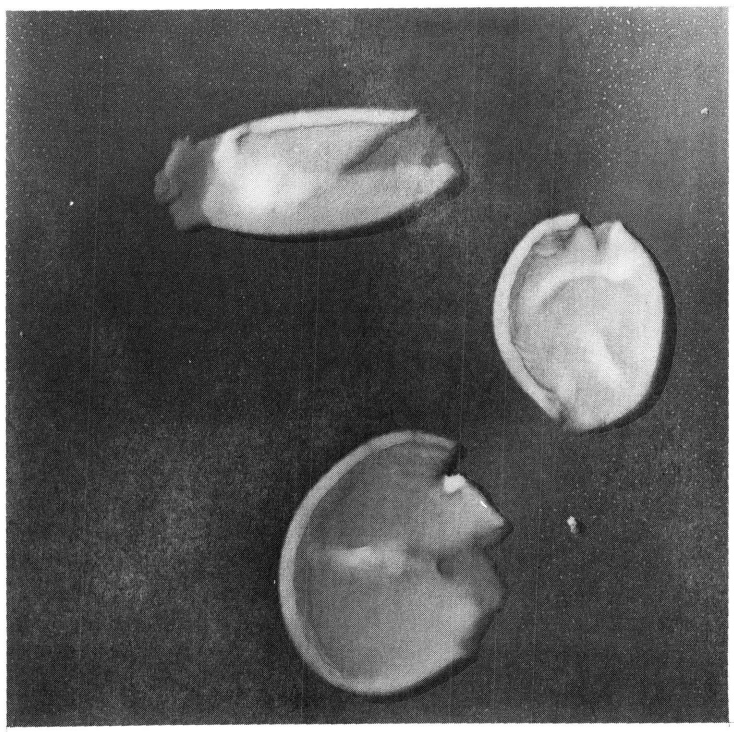

Fig. 18 - Cell walls of *Saccharomyces cerevisiae*

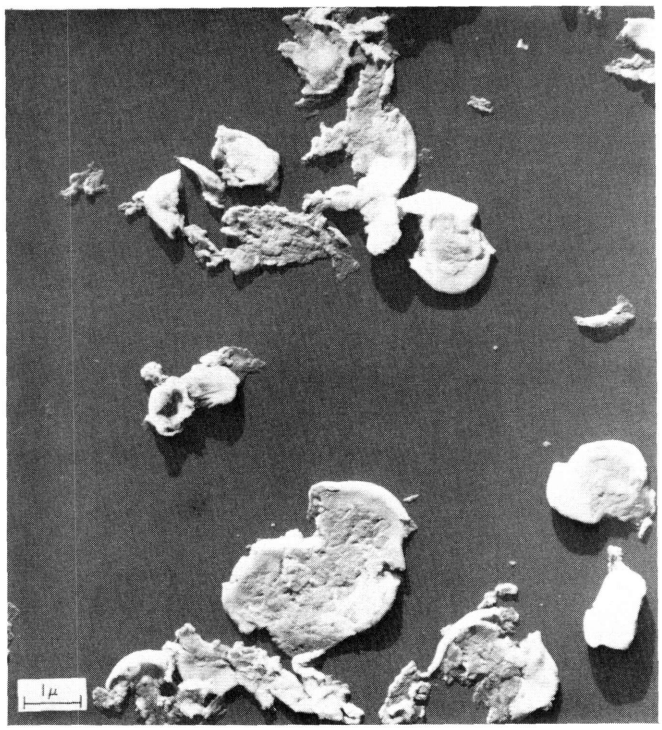

Fig. 19 - Spores of *Bacillus subtilis* ruptured in ethanol. For comparison a relatively undamaged spore is shown in the lower right corner.

Figure 19 shows the extensive breakage and the brittle nature of the fractures occurring when *Bacillus subtilis* spores are ruptured in ethanol.

DISCUSSION

The sequence of operations involved in a typical microbial cell-wall isolation is outlined in Fig. 20.

The high-speed stirring apparatus provides an effective, simple, and inexpensive means of rupturing gram amounts of microorganisms in high yield. The principal disadvantage arises from the relatively large amount of colloidal glass and steel generated. Removal of these particulate contaminants can sometimes be troublesome, especially when there is a tendency for the cell walls to associate with them. The method employed for maintaining low temperature is not entirely adequate. The use of subzero freezing mixtures instead of crushed ice around the containing vessel would probably be an improvement. The apparatus would not be suitable for rupturing pathogenic organisms without design changes which would eliminate the possibility of escape of aerosols from the stirred suspension.

The MSK apparatus offers several advantages over high-speed stirring. No metal is in contact with the microbial suspension, and much less colloidal material is formed during the rupturing process; the possibility of aerosol escape is also eliminated. The capacity per run is less than in the high-speed stirring apparatus; however, the breakage

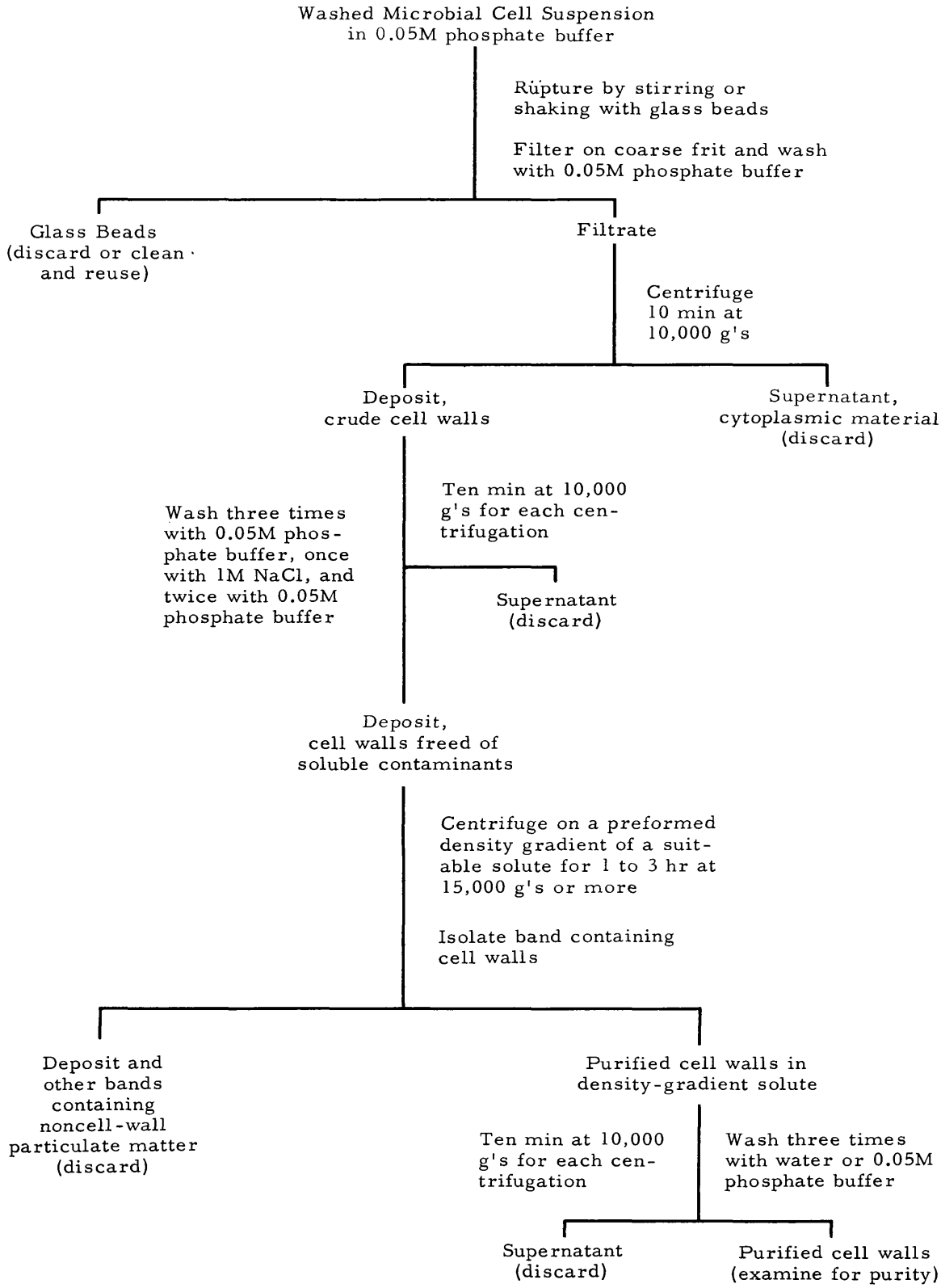

Fig. 20 - The general scheme for the preparation and isolation of microbial cell walls

rate is rapid enough that large amounts of cells can be ruptured in multiple batches in a short time. The apparatus could be readily adapted to accommodate bottles with twice the capacity of those used here (23), but this extra weight might well produce sufficient imbalance during shaking to shorten the life of the bearings. Low temperatures are possible in the MSK equipment; however, the method of controlling the temperature is not ideal. Maintenance of the equipment subsequent to the described simple changes which were made in the bottles and in the capillary leading the carbon dioxide coolant to the shaking chamber has not been a problem.

It is apparent from the results of the experiments with the MSK apparatus carried out to establish the conditions for optimum performance (Figs. 2-4) that good rupture efficiency occurs in a wide range of conditions. If the volume of suspension does not exceed 80 to 85% of the capacity of the bottle and the concentration of organisms is less than about 60 to 70 mg/ml, nearly complete breakage can be obtained in less than 2 min at 4000 rpm. These volume and concentration limits will be lower for some organisms which produce copious foam or have a highly viscous cytoplasm.

Figures 5 to 10 show that efficient rupture can be obtained with a variety of organisms. For vegetative bacteria the shaking time required for 90% rupture (estimated from turbidity measurements) varies from about 25 sec for *Escherichia coli* to about 65 sec for *Micrococcus lysodeikticus*. Baker's yeast also falls within this range, but *Bacillus subtilis* spores in ethyl alcohol require a little more than 2 min shaking for a 90% loss in viability. These rupture rates are comparable to the highest reported in the literature (44,30,50) and are far higher than most. Compared to the 25 sec required here for 90% rupture of *Escherichia coli*, King and Alexander (13) required 2.1 hr and Furness (51) 3-1/2 min. The slower rupture rates obtained by these earlier workers is undoubtedly due to the lower frequency which their shaking equipment permitted (300 to 700 cpm). In cases where the rupture rate has been determined as a function of frequency of shaking with a given piece of equipment, the rupture rate rises rapidly above a frequency of about 2000 cpm (18,50). While the geometry of the shaken vessel and the amplitude and mode of oscillation can also be expected to affect the rupture rate (49,51), these factors appear to be of minor importance. A systematic investigation of all the variables involved in the mechanical rupture of microbes by glass beads remains to be made.

It might be anticipated from the shape of the various curves that an exponential relationship exists between the degree of rupture of microorganisms and the time of shaking with glass beads. This has indeed been shown to be the case by earlier investigators using other types of shaking equipment. Curran and Evans (12) and King and Alexander (13) showed that the viable count of spores or vegetative forms of bacteria, shaken with abrasives or glass beads, dropped with time according to a logarithmic law. Cooper (30) established that a similar relationship existed between the turbidity change of a *Staphylococcus aureus* suspension and the time of shaking. We have replotted the data of Fig. 7 to see whether similar relationships exist between our measurements of degree of rupture and the time of shaking in the MSK apparatus. From the results, shown in Fig. 21, it is clear that a logarithmic relationship holds between viability, turbidity, optical density of released cytoplasmic substances absorbing at 260 mμ, and the time of shaking, at least up to the final stages of rupture. Death rates in this instance thus show the same behavior as those commonly produced by other lethal agents, such as heat and chemicals (34).

Some authors have pointed out the apparent existence of a small fraction of bacterial cells which have an abnormally high resistance to rupture by shaking with glass beads (30,51). This fraction, if real, is small (0.1% or less), and for this reason an investigation of the phenomenon was considered not pertinent for our main purposes.

Among the methods used to assess the degree of cell rupture there seems to be good general agreement. Figure 21 serves to illustrate the agreement obtainable between

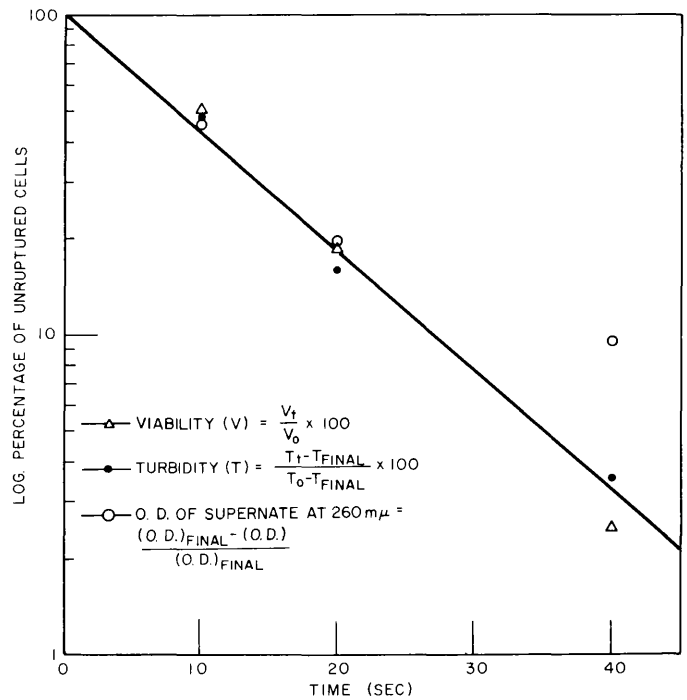

Fig. 21 - Logarithmic plots of the data from Fig. 7 on the rate of rupture of *Escherichia coli*

turbidity, viability, and optical density of 260-mμ absorbing substances. Within experimental error all points lie on the same curve until the final stages of rupture. All methods are equally reliable measures of the rupture rate in this instance. Numerous exceptions to this agreement exist in other cases, however. Viability measurements sometimes indicate higher rupture rates than the other methods for reasons already discussed (51). Turbidity measurements may indicate a lower than actual rupture rate when the presence of colloidal glass or steel contributes substantially to the reading as in the case with high-speed stirring and in the MSK apparatus with low concentrations of microbial suspension. In the rupture of yeast (Fig. 9), it is apparent that turbidity is not a sensitive method of measuring the degree of rupture. If the possibility of such exceptions are kept in mind, however, turbidity measurement still offers the most rapid means of making determinations of the extent of rupture during a run.

Centrifugation in density gradients of various solutes has proven a convenient and efficient method of separating cell walls from unwanted material. Admittedly one or more exploratory centrifugations must be carried out to establish the most favorable solute and density range for the walls of a particular species of microorganism. Once the conditions are found, however, the method provides a faster, easier, less wasteful separation than differential centrifugation.

In the course of making density-gradient fractionations of cell walls, we have observed that in many cases not all of the walls have the same buoyant density. With *Bacillus megaterium* in a sodium bromide gradient, for example, most of the walls after centrifugation are found in a band extending from a density of 1.49 to 1.51 g/cc. However, walls identical in appearance by light and electron microscopy can also be found in small amounts in bands both above and below this range of density. The presence of such walls with atypical densities was not due to a nonhomogeneous inoculum nor does it

depend on the age of the culture. A similar situation exists with the walls of *Micrococcus lysodeikticus*. It would be interesting to know how the chemical and physical structure of walls with different densities differ from one another. Investigation of the problem may pose a difficult separation problem, however, because granules, cell walls with visible inclusions, or other debris are usually found at the same density levels as the walls with abnormal densities (but not necessarily in direct association with them).

The results of the tests for contamination of cell-wall preparations of Gram-positive organisms using ultraviolet analysis of acid extracts (Fig. 11) and light and electron microscopy (Figs. 14-16 and Fig. 18) indicate a high degree of purity. Cell walls of Gram-negative organisms (Figs. 12 and 17) are less satisfactory. Treatment of these walls with enzymes or chemical extractants can be expected to result in a cleaner appearance, but there is an attendant risk of changing the inherent character of the wall substance (1). We propose instead to introduce such treatments only if a particular study seems to demand it. At that time the possibility of alterations occurring in the feature of the walls under examination will have to be evaluated.

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13. ABSTRACT

A biophysical characterization of microbial cell walls can be greatly facilitated by the availability of walls free from other cell constituents. Isolated cell walls for this purpose are best prepared by rupturing the microbial cell by mechanical means, removing soluble cytoplasmic constituents by washing, and subsequently separating the walls from unbroken cells and particulate debris by centrifugation. In this investigation methods were explored for the efficient rupture, without undue fragmentation, of typical microorganisms using violent agitation with small glass beads effected by high-speed stirring or by shaking in a commercial cell homogenizer. The extent of rupture of the microorganisms was measured by viability loss, microscopic appearance, turbidity, and the ultraviolet adsorption of released cytoplasmic constituents. Clean fractionations of cell walls with minimum losses were accomplished by centrifugation on preformed density gradients of solutions of selected salts and nonelectrolytes. The purity of the final cell-wall product was assessed by light and electron microscopy and by the ultraviolet light adsorption of acid extracts. Autolytic degradation of the walls was minimized by maintaining low temperatures during all stages of preparation. The cell walls of Gram-positive organisms met all of the criteria of purity; those from Gram-negative organisms were less homogeneous.

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