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Received July 26, 1965. P.S.E.B.M., 1965, v120.

## Rapid Changes in Bacteria Following Introduction into Hypertonic Media. (30591)

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When the osmotic environment of Gram-negative bacteria is changed the bacteria respond by losing or gaining water in hypertonic or hypotonic solutions respectively(1). In solutions of non-permeating solutes the bacteria remain in a dehydrated condition and, if the concentration of the solute is great enough, plasmolysis occurs and the protoplast shrinks to a small volume and breaks away from the cell wall. Solutes which can pass freely across the cell membrane allow osmotic equilibrium to be established across the membrane, and the cell volume at equilibrium will be approximately equal to the volume of the cell before it was introduced into the solution of the permeating solute. A convenient technique for study of the changes which bacteria undergo on immersion in various osmotic environments is measurement of the changes in the light scattering ability of suspensions of the bacteria(2,3). However, a limitation to the methods so far used has been the inability to follow the rapid changes in light scattering which occur immediately after bacteria are introduced into a new osmotic environment. From a knowledge of the kinetics of the osmotic dehydration of bacteria obtained from such a technique and rates of entry of permeating substances, estimates of the hydraulic permeability and other pertinent information about the permeability properties of the bacteria might be made. By modifying the rapid mixing stopped-flow apparatus designed by Gibson and Milnes(4) the changes in the intensity of light scattered at 90° to an incident beam can be followed approximately 2.0 to 3.0 milliseconds after a suspension of Gram-negative bacteria is rapidly and efficiently introduced into a new osmotic environment.

*Description of apparatus.* The modification made to the Gibson-Milnes apparatus is illustrated in Fig. 1; the remainder of the apparatus is exactly as in the original design. The mode of operation is the same as described by Gibson and Milnes(4) except that a suspension of bacteria is mixed with a solution of the solute under investigation by pulling the arm of the hydraulic actuator. A parallel beam of light is directed through the mixed bacterial suspension approximately 5 mm from the second mixer. This distance represents a time of 2.0 to 3.0 milliseconds at the flow rates obtained in the apparatus. A small lens, placed at its focal distance from the illuminated suspension, collects light laterally scattered from the incident beam and directs this light onto the cathode of an end-window photomultiplier. The signal from the photomultiplier is fed into a cathode follower and then into an oscilloscope. The sensitivity of the photomultiplier is varied as required by using different load resistors and the time constant of the circuit is kept at 5 milliseconds by the insertion of an appropriate capacitor in parallel with the load resistor. The oscilloscope trace is triggered by the operation of a microswitch, actuated by the arm

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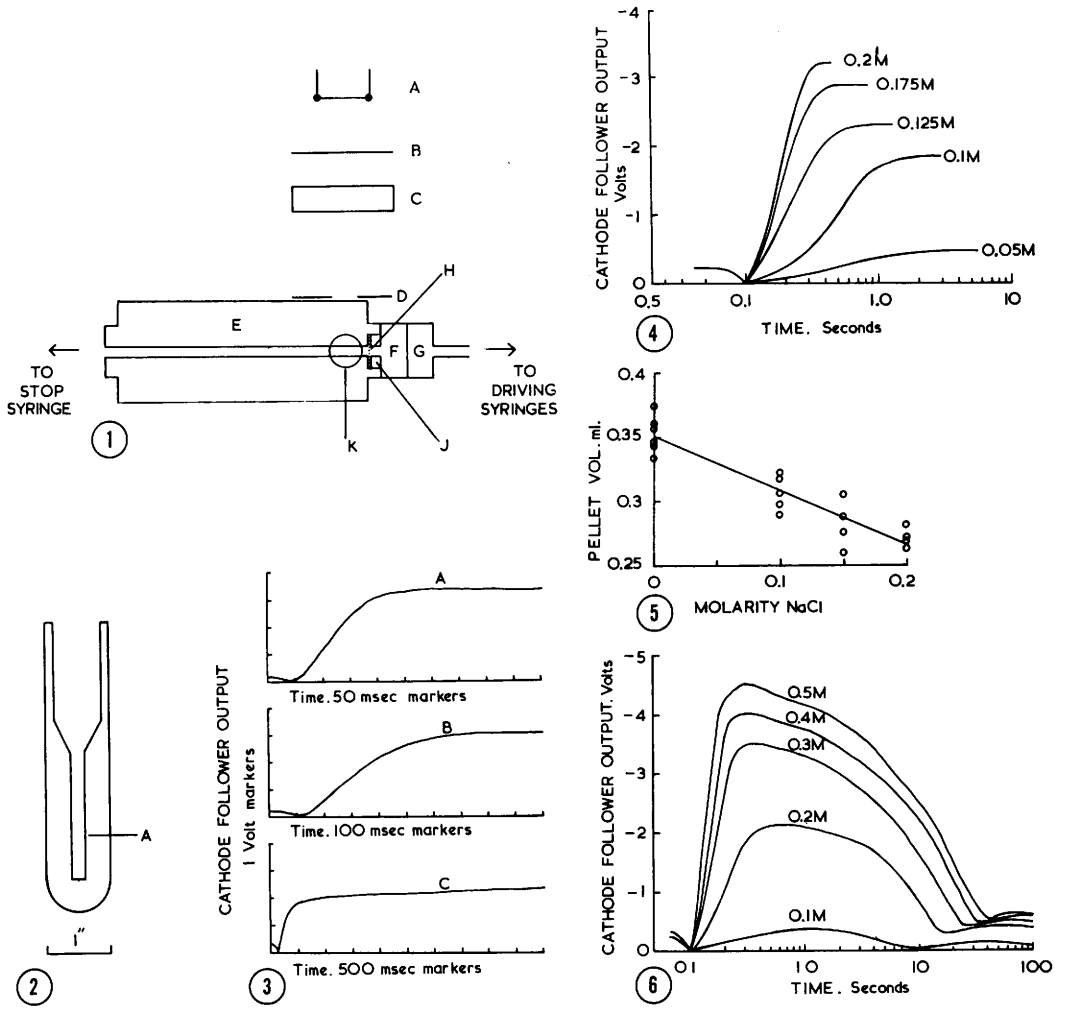


FIG. 1. Side elevation of modifications made to the Gibson-Milnes stopped flow apparatus. The parts shown replace the observation cell and lamp housing in the published apparatus. A. Ribbon filament lamp; Mazda type F/16; B. Heat filter; Chance type HA1; C. Cylindrical lens; D. Slit 5 mm by 1.5 mm; E. Observation chamber made from a Perspex block, 65 mm by 12.5 mm. A hole, 2 mm in diameter, is drilled through the center; F. and G. Mixing chambers of the same design as those in the Gibson-Milnes apparatus; H. Disc of stainless steel mesh, 120 holes to the linear inch, held in place by an annulus of Perspex, J, which is pressed into the end of the Perspex block; K. Lens of short focal length, approximately 1.5 cm, directing scattered light onto the cathode of an end-window photomultiplier (not shown).

FIG. 2. Cytocrit tube. The tube is made from Perspex. The diameter of the narrow portion, A, is approximately 0.2 inch and is drilled slowly to avoid overheating the plastic. The bottom of the tube is made flat. The portion A is calibrated in ml and the calibration marks are made around the circumference of the tube so that parallax errors are eliminated. The tubes, if carefully fabricated to avoid overheating of the plastic and consequent crazing, are strong enough to withstand repeated centrifugation at 5000 rpm in a centrifuge head of 6 inches radius.

FIG. 3. Tracings made from photographs of oscilloscope traces showing the changes in light scattering which occur when an aqueous suspension of *S. marcescens* is mixed with sodium chloride solutions containing M/75 sodium phosphate buffer to give a pH of 7.0. The change in light scattering due to an osmotic gradient of 0.2 M sodium chloride is shown in curve A, a gradient of 0.15 M in curve B and a gradient of 0.1 M in curve C. In this Figure and in Fig. 4 and 6 an increase in light scattering is shown by an upward movement of the trace.

FIG. 4. A series of oscilloscope traces as shown in Fig. 3 plotted with a logarithmic time

which pushes the driving syringes, a few milliseconds before flow is stopped. To avoid the effects of hydraulic pressure on the bacteria which may occur when a stopping syringe is used, the liquid flow is stopped by allowing the driving block to encounter a stop. The tortuosity of the path of the liquid through the apparatus allows the liquid column to stop rapidly. The oscilloscope trace is recorded with a standard 35 mm camera attachment.

When a slow moving trace is used to observe changes in light scattering, triggering is unnecessary and mixing is carried out a short time after the trace has started. Extended observations are conveniently made by allowing the oscilloscope trace to run repetitively while recording each pass on successive frames with the camera attachment.

An important feature of the modification is the inclusion of a stainless steel mesh after the mixers. The mesh produces turbulence and destroys the spiral motion imparted to the flowing liquid by the tangential jets in the mixers. Without this mesh, rod-shaped bacteria aligned in the spiral flow and light scattering changes occurring as the bacteria became randomly oriented obscured the changes produced by the new osmotic environment.

*Bacterial suspensions. Serratia marcescens* (ATCC 14041, strain 8UK) was cultured in trypticase soy broth at 31°C in a shake flask for 18 hours. The cells were concentrated by centrifugation and washed twice with glass distilled water before suspending in glass distilled water to give a suspension containing approximately $2 \times 10^{10}$ cells/ml.

*Measurement of bacterial cell volumes.* This was conveniently carried out by a modification of the thick suspension technique of Conway and Downey(5). The use of Blue Dextran 2000, (molecular weight, 2,000,000, Pharmacia Fine Chemicals Inc., New York), which could be easily determined spectrophotometrically owing to its strong absorption at 650 mμ, eliminated problems associated with the interference of leakage products in chemical determination of the high molecular weight dextrans usually used in the technique.

Equal volumes of a thick suspension of water-washed *S. marcescens* containing approximately 10% by volume of bacteria, were delivered into calibrated cytocrit tubes. The cytocrit tubes are made to the design shown in Fig. 2. The volume of bacteria in each tube was approximately 0.3 ml. Eight cytocrit tubes were used for each set of determinations and this allowed 3 determinations in duplicate using different concentrations of the solute being investigated and one pair of volume determinations in water. After sedimenting the bacteria by centrifugation the supernatant was removed and the bacteria were resuspended in water or the various osmotic environments being studied. The tubes were again centrifuged and the supernatant was removed from each tube so that an equal volume of bacteria and supernatant remained in the tube. A convenient volume was 0.6 ml. To each tube was then added 0.4 ml of a 0.1% solution of Blue Dextran in either water or the solution being investigated. The bacteria and the supernatant liquid were then carefully mixed using a stainless steel wire or thin glass rod. After recentrifugation a sample of each supernatant was withdrawn for determination of the Blue Dextran content. Optical density measurements were made on suitably diluted samples in 1 cm cuvettes using a Beckman DB spectrophotometer. The volume of the bacterial cells was then calculated from the measured dilution of the Blue Dextran by the accessible water volume.

scale. Each sodium chloride solution marked is the osmotic gradient to which the cells of *S. marcescens* had been submitted. Zero time has been arbitrarily taken as the time at which minimum light scattering occurs and, to avoid the complication of zero on a logarithmic scale, 0.1 second has been added to each time value on the oscilloscope trace. The origin of the ordinate has been arbitrarily made zero volts at the minimum level of light scattering.

FIG. 5. The volume of pellets of *S. marcescens* in various sodium chloride concentrations. Eight determinations were made for each concentration of sodium chloride in 4 separate experiments. Coincident values are shown as one circle. The line has no theoretical significance.

FIG. 6. Light scattering changes produced on mixing an aqueous suspension of *S. marcescens* with various solutions of urea. Presentation of the traces is the same as in Fig. 4.

*Results.* The changes in intensity of scattered light produced when *S. marcescens* in aqueous suspension is introduced into various concentrations of sodium chloride are shown in Fig. 3. The flow was stopped a few milliseconds after the start of the trace. The level of light scattering at the start of the trace will be approximately the level expected from an aqueous suspension of the bacteria since only a few milliseconds before the onset of osmotic modifications have elapsed after the bacteria have been mixed with the solution of sodium chloride, and osmotic modification of the bacteria is then negligible. A slight decrease in light scattering then occurs which is roughly constant for each concentration of sodium chloride. The reason for the decrease is obscure but if bacteria, previously suspended in 0.05 M sodium chloride, are mixed with higher concentrations of sodium chloride, the decrease is not observed. After the decrease, the intensity of scattered light increases at a rate characteristic of the osmotic gradient into which the bacteria have been introduced, to reach a final value dependent on the concentration of sodium chloride. Extended observations show that the maximum is maintained except in concentrations of sodium chloride below 0.1 M when a very slow decrease has been observed. The light scattering changes are more conveniently represented on a graph with a logarithmic time scale as shown in Fig. 4.

Measurement of the volume of *S. marcescens* in solutions of sodium chloride up to 0.2 M shows that the cells decrease in volume uniformly with sodium chloride concentration (Fig. 5). In 0.2 M sodium chloride the cells have approximately a 24% smaller volume than they have in water.

Light scattering changes produced when *S. marcescens* in aqueous suspensions is introduced into solutions containing various molarities of a permeating substance, urea, are shown in Fig. 6. The initial decrease in light scattering observed on mixing the bacteria with sodium chloride solutions is also observed on mixing with urea solutions. After the decrease the intensity of scattered light increases to a maximum related to the concentration of urea, then the intensity decreases smoothly to a minimum. The cells have not quite reached equilibrium with the new environment as a further slight increase in light scattering occurs which is incompletely shown in the Figure.

Pellet volume determinations show that cells of *S. marcescens* have the same volume in urea solutions as they have in water.

*Discussion.* The geometrical arrangement of the detection system of the apparatus enables light produced by multiple scattering in the bacterial suspension to be collected from a large solid angle at right angles to an incident beam of white light. The increase in intensity of scattered light observed when the bacteria are introduced into the hypertonic sodium chloride solutions is due to the change in size, shape and refractive index of the bacteria. When the bacteria are mixed with solutions of urea they undergo an initial osmotic dehydration which produces an increase in light scattering and then, as urea enters the cell, osmotic equilibrium is regained as the cell swells with re-entry of water. The light scattering intensity decreases as the cells swell. The time taken for the dehydration and entry of urea, approximately 40 seconds in 0.5 M solution, is shorter than the values obtained by Elo(6) for *Bacterium coli* by microscopic observation of the course of deplasmolysis of the bacteria after addition of urea solutions to a suspension of bacteria plasmolysed by sucrose solutions. Hill(7) obtained a value of 41 seconds for the time taken for urea to permeate into cells of *Bacterium fischeri* by determining the time at which luminescence of the bacteria was extinguished after immersion in a 1.0 M solution of urea.

The technique could be used for many studies of bacterial permeability but it will not always be possible to relate changes in light scattering by bacterial suspensions to the entry of permeating substances, since leakage of intracellular materials will produce changes in the refractive index of the cell and thus may cause light scattering changes similar to those shown for the entry of urea into *S. marcescens*. Cell volume determinations give an indication of the be-

havior of the cell in the various osmotic environments but determination of the actual leakage and the concentrations of the substrates before and after permeation would give more complete information.

*Summary.* The rapid osmotic dehydration of *S. marcescens* produced when an aqueous suspension of the organisms was rapidly and efficiently mixed with solutions of sodium chloride was followed from 2.0-3.0 milliseconds after mixing by the change in light scattering of the suspension. After a small decrease the light scattering rose to a maximum which depended on the concentration of sodium chloride. The increase in light scattering was associated with the shrinking of the bacteria during osmotic dehydration. When the bacteria were introduced into a solution of a permeating solute, urea, there was an initial increase in the light scattering followed by a decrease to a value almost equal to that produced by the suspension in water. These changes are probably due to the initial shrinking of the bacteria due to osmotic dehydration followed by swelling to the original volume as urea and water enter the cells.

I wish to acknowledge the technical assistance of Mr. John F. Blackmer and the advice and criticism of Dr. John B. Bateman.

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Received July 26, 1965. P.S.E.B.M., 1965, v120.

## Comparative Effectiveness of Two Culture Techniques for Isolation of Rubella Virus.* (30592)

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Initial techniques for isolation of rubella virus in cell culture were based upon demonstration of viral interference in primary cultures of African green monkey kidney (AGMK)(1) and recognition of cytopathogenic effects (CPE) as well as of viral interference in primary human amnion cells (PHA)(2). This paper reports a comparative study of the sensitivity of these two types of cell culture for the primary isolation of rubella virus. Additionally, isolations were simultaneously attempted by cultivation of human placental and fetal tissues, and by inoculating extracts prepared from portions of these tissues into cultures of PHA cells.

* Supported in part by a research grant (AI-01023) from Nat. Inst. of Allergy & Infect. Dis., Nat. Inst. Health, U.S.P.H.S., and by grants from Parke Davis and Co., United Cerebral Palsy Research and Educational Foundation, Inc.

*Materials and methods.* Twenty-four specimens collected from patients with clinical rubella were tested. These included 5 throat washings† and 19 products of conception. Specimens were processed as previously described(3). Products of conception were ground by hand (10-20% wt per volume of nutrient medium) and centrifuged; portions of the supernatant were used as inocula. Isolations were attempted either immediately after collection or after storage of materials at −55 to −65°C for periods of up to 8 years. Volumes of 0.2 ml were routinely sampled for virus and employed in the subculture procedures.

PHA cultures were prepared in this laboratory and maintained with bovine amniotic

† Certain specimens kindly supplied by Dr. Samuel D. Bell, Jr., Dept. of Microbiology, Harvard School of Public Health.