TRANSCELLULAR ION FLOW IN *ESCHERICHIA COLI* B AND ELECTRICAL SIZING OF BACTERIAS

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ABSTRACT Dielectric breakdown of cell membranes and, in response, transcellular ion flows were measured in *Escherichia coli* B 163 and B 525 using a Coulter counter as the detector with a hydrodynamic jet focusing close to the orifice of the counter. Plotting the relative pulse height for compensated amplification of a certain size of the cells against increasing detector current, a rather sharp bend within the linear function was found, which did not occur when measuring fixed cells or polystyrene latex. The start current for transcellular ion flow causing the change of the slope is different for the potassium-deficient mutant B 525 in comparison with the wild-type B 163, indicating a change in the membrane structure of B 525 by mutation and demonstrating the sensitivity of the method for studying slight changes in membrane structure in general. The theoretical size distributions for two current values in the range of transcellular ion flow were constructed from the true size distribution at low detector currents, assuming an idealized sharp changeover of the bacterial conductivity from zero to one-third of the electrolyte conductivity.

INTRODUCTION

The Coulter counter (Coulter Electronics, Inc., Fine Particles Div., Hialeah, Fla.) has been used frequently in the counting and sizing of particles in conducting fluids. The function of the counter is based on the current constriction in the flow system of the conducting medium in which nonconducting particles are suspended. Relatively nonconducting particles such as microorganisms or blood cells, cause an increase in the resistance of the current constriction during their passage through the orifice (1). For a first approximation the height of the resulting current or voltage pulse is proportional to the volume of the cell, therefore the cell size distribution can be obtained by linear amplification and subsequent pulse height analysis. The advantages of this method for cell sizing are that cells can be counted at rates of about 5,000 cells/s, living cells suspended in adequate electrolyte solutions can be analyzed over a wide range of suspension concentrations, and the volume sensitivity of the detector offers higher accuracy for analysis of particle populations with small variations in diameter (which is true of many cell populations). The size distribution

obtained by this volume-sensitive detector is less dependent on particle shape than that obtained by optical methods. However, for accurate cell sizing using a Coulter detector, some disadvantages and sources of inaccuracy must be considered. The resolution of the detector is low because the nonuniform current density at different locations within a cylindrical hole causes a dependence of the pulse height on the path of the individual particle passing the orifice (2, 3).

The orifice diameter restricts the size range to be analyzed. The lower limit is determined by the electronic noise level and the upper limit is given by invalidation of the linear relationship between volume and pulse height as well as by blocking the orifice.

The resulting signal-to-noise ratio depends not only on the electronic noise level but also on a noise background introduced by the particles which lowers the sensitivity of the detector for analysis of small particles in the presence of large particles in the same suspension. This effect results from those particles moving outside the hole but still close enough to the orifice so that their movement takes place in a region of a small but recognizable current density gradient.

Furthermore, distortion of the distribution can be due to the dependence of the output pulse amplitudes on the shape of the particles and their orientation during their passage through the orifice. The properties of the suspension medium can be changed by electrolysis. Another difficulty that can arise is contamination of the orifice resulting in a reduction of the effective volume of the detector orifice and an increase of pulse height for the same particles. Another important source of error in respect to the actual size distribution can occur by dielectric breakdown, caused by high voltage gradients across the cells in the orifice. By exceeding a certain electric field strength within the orifice, transcellular ion flow will occur, resulting in a change of conductivity of the particle during analysis. On the other hand, when the disadvantages and sources of errors mentioned above are absent the accurate measurement of the electric field strength or the current strength, at which the dielectric breakdown occurs, will permit a conclusion to be reached regarding the specific membrane properties of the investigated cells.

In order to study the dielectric breakdown which is dependent on the membrane composition and on the influence of the surrounding medium, a method of measurement must be developed which excludes the most restrictive disadvantages of the Coulter detector, due to the inhomogeneous current density within the orifice cross section and the additional noise introduced by particles. To overcome these disadvantages the particles should be injected only into the center of the orifice and those particles outside the hole should be kept out of the current density field. A detector system which gives a reasonable solution to these problems was developed very recently and employed for sizing and counting of blood cells. The function of this detector system is outlined briefly to provide the background of the following measurements with bacterias which were obtained using this improved detector (4, 5).

METHOD

Fig. 1 shows a schematic diagram illustrating the principle of operation of the new detector system. A jet capillary tube, constricted at both ends, is mounted centrally and close to the orifice. The diameter of the top constriction outlet is approximately equal to the diameter of the orifice, i.e., 20 μm which is necessary for sizing and counting of bacteria.

The distance between the capillary outlet and the orifice input is also within this range. A particle suspension is introduced into the jet capillary tube, which is about 3–5 cm long and encloses a defined volume. Particle-free electrolyte surrounds the jet and is fed into the container connected to the output of the orifice. If low pressure is applied to this container, particle-free electrolyte as well as particle suspension is sucked through the orifice, and hydrodynamic focusing of the suspension stream into the orifice occurs as indicated in Fig. 1. A focusing cone is formed within the distance between the jet and the orifice, decreasing the

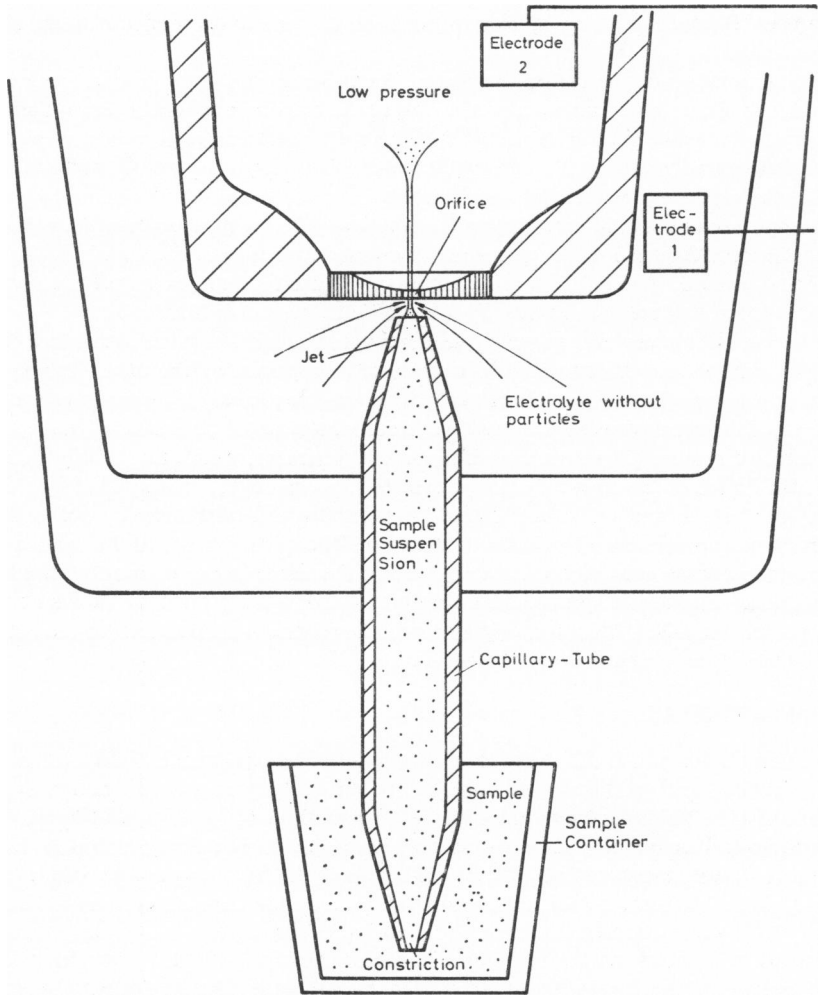

FIGURE 1 A schematic diagram of the detector.

diameter of the suspension stream by a factor of 10 or more. Therefore, all particles travel along a path at the center axis of the orifice. This detection principle improves upon the features of the Coulter detector for particle sizing in the following respects and allows studies of dielectric breakdown.

(*a*) Resolution is greatly increased. No skewness occurs. Latex spheres of 2 μm ϕ (ϕ = diameter) have been analyzed within 2% standard deviation which is in agreement with the producers specification (Serva, Heidelberg, Germany). Better test particles are not available at present. The noise background introduced by the particles is nearly eliminated. Only particles within the focusing cone contribute additional noise.

(*b*) Nonspherical particles are oriented with their long axis in the moving direction. Random orientation is eliminated. However, the dependence of the pulse amplitudes on the shape of the cells remains (shape factors for spheres is 1.5, for long cylinders 1.0).

(*c*) Furthermore, when employing this detector system, the cells are not in contact with the current-carrying electrolyte apart from a very short time interval of about 100 μs before measurement. Products built up by electrolysis have no time to exert effects on the shape or volume of cells.

(*d*) Contamination of the orifice is avoided. Particles do not come into contact with the wall of the cylindrical hole. Blocking occurs only at the bottom input of the jet capillary where the constriction is made smaller. This has the advantage that blocking can be removed easily.

The sample container of Fig. 1 is inserted into a mechanically operated lift which is actuated outside of the shielding chamber of the detector.

The sample container is lifted to dip in the capillary tube for measurement into the sample fluid; it is lowered for termination of measurement and exchange of containers. If the sample container is lowered, the surface tension at the constricted entrance cuts off the suspension flow, because the low pressure at the top is not able to overcome this tension.

By inserting a container with particle-free electrolyte this tension at the constricted entrance disappears and the suspension will flow again and be replaced by the pure electrolyte. The pulse count rate will drop to zero. In this way it is possible to analyze a known volume of the suspension, if the measurement was initiated before insertion of pure electrolyte.

The detector is supplied with a constant current generator, which can be adjusted within 0.1–1.5 mA. Pulses of the 20 μm ϕ detector are 10–15 μs in length, preamplified by a current-sensitive input stage and main amplified with a band-width restriction by active filters of 1.5 μs rise time and subsequent base line restoration. The output pulses of this main amplifier are fed to the analogue to digital converter using the linear ramp method with 100 MHz clock frequency and special procedure to inhibit false triggering on noise pulses at the first smooth rise of the pulses. Memory, display, and data representation technique are equivalent to those generally used in pulse height analysis.

MATERIAL

E. coli, strains B 163 and B 525, were used throughout the experiments. Both strains require histidine, leucine, and methionine for growth. B 163 is the precursor of the mutant B 525 being defective in potassium retention (6, 7). The method of growing and harvesting the organisms, as well as the incubation conditions, have previously been described in detail (7).

The bacteria were incubated at 20°C in a medium of the following composition: (referred to 1 liter) Tris, 110 mmol; HCl, 90 mmol; $MgSO_4$, 1 mmol; KCl, 12 mmol; Na_2HPO_4, 40 mmol; NaH_2PO_4, 20 mmol; glucose, 1%, The pH value of the incubation medium was 7.2, the time of incubation about 30 min. To remove foreign particles from the incubation medium before incubation of the bacteria the media were filtered through washed 0.1 μm Millipore filters (Millipore Corp., Bedford, Mass.). The suspension density was

about 0.06% to reduce the probability of coincident passage of two or more cells through the orifice.

RESULTS AND DISCUSSION

Fig. 2 shows the size distribution of 0.79 and 1.01 μm ϕ latex particles with the 20 μm ϕ and 25 μm long detector orifice used in the improved detector for the first time. The detector current was adjusted to 1.2 mA. The detector noise was equivalent to about 0.1 μm^3 particle volume which corresponded to a particle diameter of 0.6 μm. Fig. 3 shows the size distribution of cells of *E. coli* B 163 and B 525 in comparison with 1.01 μm ϕ latex particles measured with a low detector current of 0.5 mA. The resolution of the detector was sufficiently high to give a true representation o fthe bacteria size distribution. The mean spherical volume of the two strains is slightly different.

Transcellular ion flow and, therefore, dielectric breakdown of the bacterial membranes was discovered by increasing the detector current. Plotting the channel number (e.g., of the right side at half-pulse height) of the bacterial cell pulses against increasing detector current a rather sharp bend within the expected linear function was found which did not occur when measuring polystyrene latex particles or bacterial cells fixed with glutardialdehyde (Fig. 4). Following the function of the living cells of B 525 with increasing detector current the proportional section ended about 0.7 mA and changed over within a region of 0.05 mA into a linear section of decreased slope. This phenomenon was also observed by sizing and counting of red blood cells. These observations can be explained by an ion break through the cell membrane which occurs if a certain electric voltage difference across the cell length has been exceeded (8).

This was proved by measuring the transcellular start current for different orifice dimensions in the case of red blood cells. A 40 μm ϕ and 40 μm long orifice produced

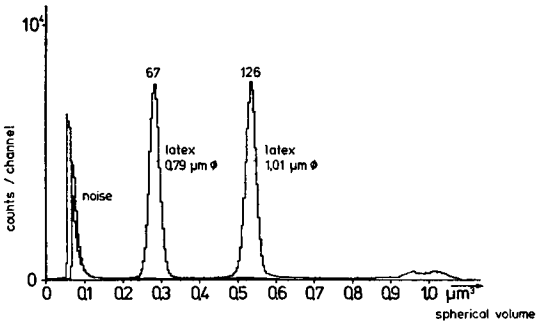

FIGURE 2

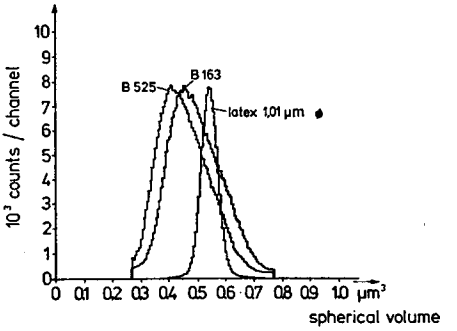

FIGURE 3

FIGURE 2 Latex size distribution using the 20 μm ϕ and 25 μm long detector orifice at 1.2 mA detector current.

FIGURE 3 *E. coli* B 163 and B 525 size distribution measured with the detector orifice of Fig. 2 at 0.5 mA detector current.

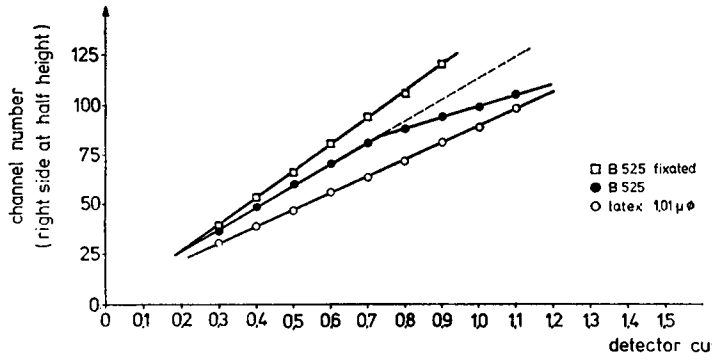

FIGURE 4 Transcellular ion flow through cells of B 525. The channel number of the right side at half-pulse height of the distribution is plotted against increasing detector current, showing in comparison the dependence of the pulse height of fixed cells of B 525 and latex (1.01 μm ϕ) read in the same way on the detector current.

a starting current of about 0.4 mA, a 70 μm ϕ and 70 μm long orifice produced a starting current of about 0.7 mA which was due to the different electric field strength within these orifices.

The decreased slope of the linear section observed after the transcellular start current corresponded to the difference of conductivity between electrolyte and cell plasma. This hypothesis was proved by measuring ghost cells and membrane vesicles prepared from B 163, resulting in a horizontal course after the start of transcellular flow.

Fig. 5 shows the deformation of the true size distribution of *E. coli* B 163 for different detector currents by another method demonstrating more significantly and accurately the existence of dielectric breakdown of the membrane. The distribution curves were presented with compensated gain, so that the increase of pulse height with increasing detector current was compensated by decreasing the amplification before pulse height analysis. The compensation was evaluated by analysis of latex particles so that their size distribution for all different current values were congruent as expected considering the linear relationship of Fig. 4. In addition, the results of Fig. 5 will always show the same number of 200,000 bacteria for each size distribution.

While the size distribution for 0.4 and 0.5 mA detector currents were equivalent, because the field strength was still too small for membrane breakdown, the first change for the biggest cells could be recognized at 0.6 and 0.7 mA detector currents. The pulse heights for these large cells were underestimated by transcellular ion flow through their conductive cytoplasma, while small bacteria remained still unaffected. Further increase of current also led to underevaluation of smaller bacterial cells. (The maximum current was limited to 1.1 mA by maximum voltage of the current source). Similar changes of the distribution curves dependent on the detector current were found in the case of B 525.

Fig. 6 shows the function of relative pulse height for compensated amplification of a certain size of cells of *E. coli* B 163 and B 525 against detector current. A rather large size of 0.7 μm^3 was chosen because these bacteria entered the region of transcellular ion flow long before the current limit was reached. According to Fig. 6 the wild-type B 163 shows a lower starting current (0.54 mA) for transcellular flow in comparison with the potassium-deficient mutant (0.6 mA), corresponding apparently to a different membrane behavior against voltage breakdown.

This result might be interesting considering the results obtained by measurements of the regulation of the internal potassium concentration by sugars in the mutant in comparison with the wild-type (9). The experiments have been interpreted on the basis of a change of a membrane component being involved in potassium transport. We expect that further measurements with this method could give more insight in the different membrane structure of this mutant.

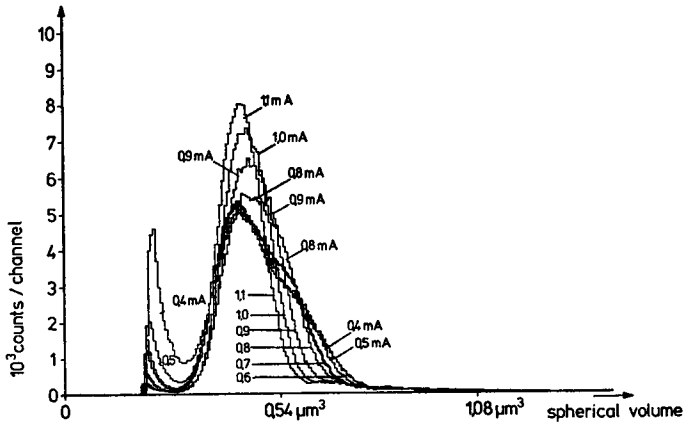

FIGURE 5 The dependence of the size distribution of cells of B 163 upon the detector current measured by compensated amplification.

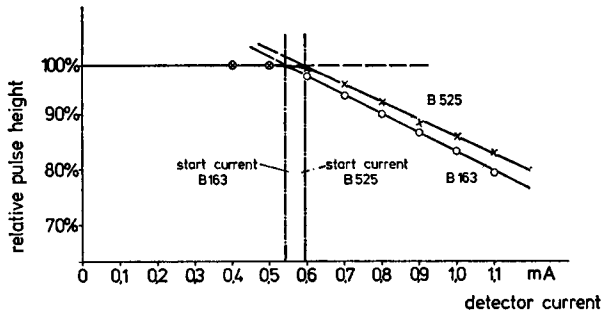

FIGURE 6 Transcellular ion flow through cells of B 163 and B 525. The relative pulse height for compensated amplification is plotted against the detector current in the case of a cell volume of 0.7 μm^3.

The data presented here on these two strains should only demonstrate the sensitivity of the method of dielectric breakdown of the membrane for studying slight changes in membrane structure.

The slightly different slope of the linear section of Fig. 6 observed after running through the starting current for transcellular flow was due to a different conductivity of the cytoplasma caused probably by the different internal potassium concentration (9).

Assuming a sharp changeover into the transcellular ion flow as seen in Figs. 4 and 6 the theoretical size distribution for two current values was derived from the 0.4 mA size distribution of cells of B 163 in the Fig. 5. The construction method was derived from the fact that the incremental change of pulse height above the starting current is only a certain fraction of that change below the starting current. According to Fig. 6 one can assume an idealized sharp changeover of the bacteria conductivity from zero to one-third of the electrolyte conductivity, i.e., this fraction will be two-thirds. Therefore, the number of cells belonging to an incremental pulse height change of three units or the area under the pulse height distribution for three units of pulse height change with respect to the original size distribution without transcellular ion flow (0.4 or 0.5 mA) can now be converted into an incremental change of two units of pulse height for the same number of cells or the same area. The construction starts at the cell size where the sharp changeover is assumed for that specific orifice current. The area under the first three units of increasing pulse height is calculated and converted into a rectangular area of the same size but only for two units of increased pulse height. The resulting theoretical distribution is a step curve, the resolution of which depends on the size of the pulse height units.

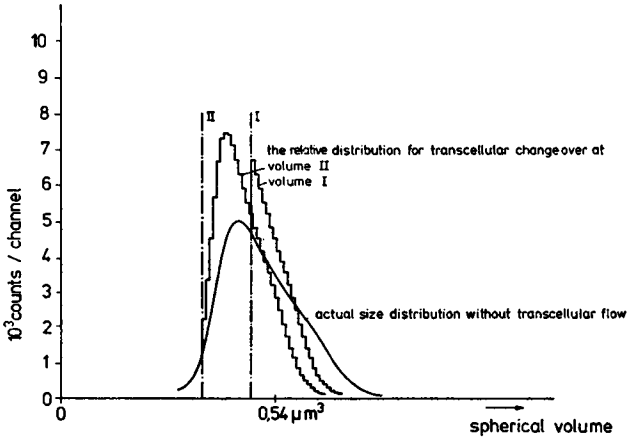

FIGURE 7 The theoretical size distribution for two different current values derived from the 0.4 mA size distribution of cells of B 163 in Fig. 5, assuming an idealized sharp changeover of the bacteria conductivity from zero to one-third of the electrolyte conductivity. The two different size values are indicated as I and II, corresponding to the actual size distribution for about 0.8 and 1.0 mA detector current in Fig. 5.

Fig. 7 shows how well this idealized assumption agrees with the actual measurements using two different size values indicated as volumes I and II in Fig. 7 which correspond to the actual size distributions for about 0.8 and 1.0 mA detector current in Fig. 5.

The jump of the theoretical distribution at cell volume I, where the sharp changeover was assumed, and the slight peak shift of the curve constructed from the changeover at volume II, can be explained by a relative smooth bend from zero into cytoplasma conductivity of the bacteria, which has also been observed for red blood cells.

To summarize, we hope that the method described above will open a new field for studying membrane composition of bacterial cells by dielectric breakdown of the membrane. The existence of transcellular ion flow was proved and it was demonstrated that this parameter depends very sensitively on changes in membrane structures. Apart from the sizing and counting of the cells it is furthermore possible to draw simultaneously conclusions in respect to the conductivities of the cytoplasma.

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