LETTER TO THE EDITOR

Comments on "Erythrocyte and Ghost Cytoplasmic Resistivity and Voltage-Dependent Apparent Size"

Dear Sirs,

Akeson and Mel (1983) recently reported on the determination of the cytoplasmic resistivity of human red blood cells and ghost cells by electronic cell sizing (resistive pulse spectroscopy). The method is based on the experimental finding of Zimmermann et al. (1973, 1974) that the voltage signal generated by a cell during its passage through an electric field changes once the breakdown voltage of the membrane is exceeded. As pointed out by Zimmermann et al. (1976*b*, 1980*a*,*c*), the internal resistivity of cells can be calculated from the ratio of the slopes of the curves obtained by plotting the signal against the electric field strength before and beyond the critical field strength resulting in breakdown.

In the context of their experiments, Akeson and Mel reported on a new phenomenon in the electronic sizing of red blood cell

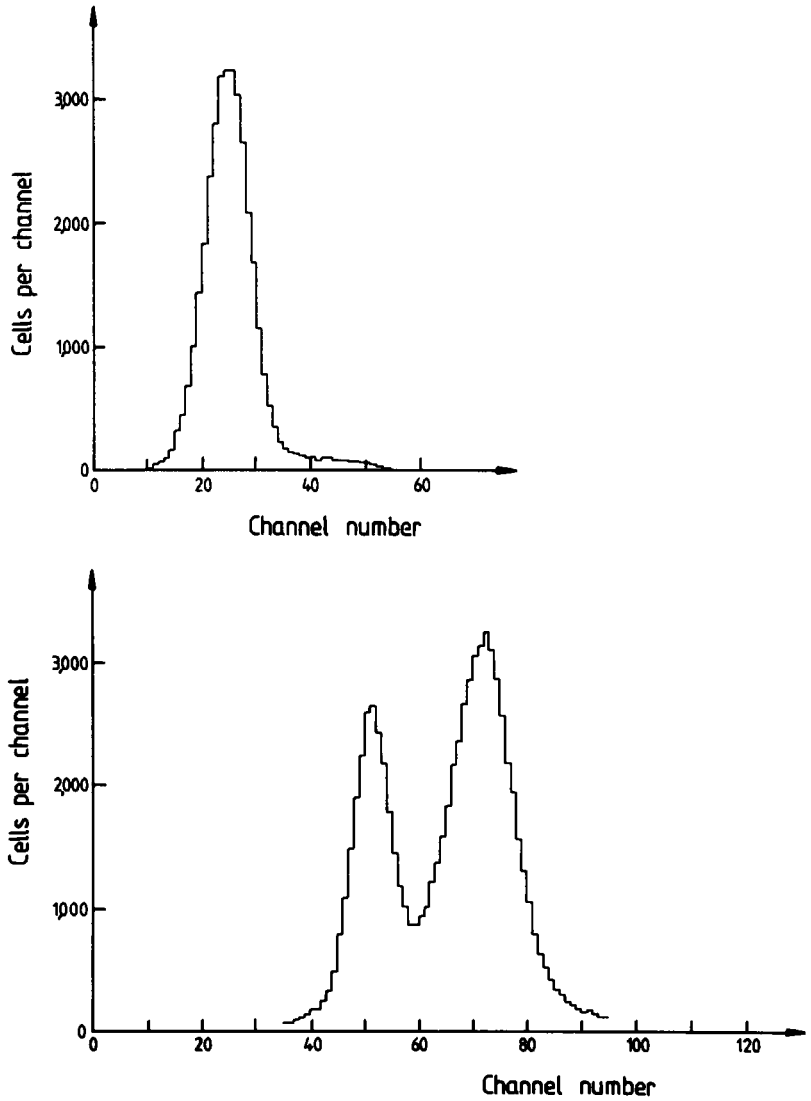

FIGURE 1 Size distribution of partly hemolysed human erythrocytes measured with a particle volume analyser with hydrodynamic focusing and a diameter and length of 60 μm for the measurement orifice (AEG-Telefunken, Ulm, West Germany). The cells were incubated in a 0.475% NaCl solution (specific resistance 129 Ω cm). (*a*) Size distribution at an electrical current of 0.2 mA through the measurement orifice, corresponding to a field strength of 0.91 kV/cm. (*b*) Size distribution at an electrical current of 0.9 mA through the measurement orifice, corresponding to a field strength of 4.11 kV/cm. This field strength is sufficient to induce electrical breakdown in the membrane. The distribution exhibits two separate peaks which are determined only by different conductivities in the cells.

ghosts, whereby the interaction with sufficiently supercritical electric fields rendered the ghosts completely transparent to the sizing process, so that the ghosts effectively "disappeared." The authors explained the occurrence of the "disappearing ghosts" by the assumption that the ghost cells underwent a second hemolysis in the high-field range, and that this event led to a complete equilibration between the cells and the medium. According to Akeson and Mel, such systems—being transparent to the sensing process—thus appeared to have vanished. In this communication we would like to demonstrate that the occurrence of "disappearing ghosts" must be attributed to an experimental artifact, and that the interpretation of this effect was carried out on the basis of erroneous assumptions.

A hydrodynamically focusing particle analyzer was used to determine the critical current and the critical electric field strength, respectively, resulting in electrical breakdown of the cell membrane (Zimmermann et al., 1973). This was achieved by measuring the size distribution of red blood cells and ghost cells as a function of the electrical field strength in the measurement orifice. This method is based on the finding that the size distribution is apparently underestimated when electrical breakdown of the cell membrane occurs. In our experiments the diameter of the orifice was 60 μm.

In the first set of experiments, human red blood cells were incubated in hypotonic (0.475%) NaCl solution. At this concentration, some of the spherical red blood cells undergo hemolysis. Abrupt hemolysis is only observed below 0.35% NaCl. The aim was to lyse only some of the red blood cells in order to obtain ghost cells in addition to intact cells under reproducible conditions. The formation of ghost cells was monitored under a phase-contrast microscope.

Fig. 1 *a* shows the size distribution of this mixed population (ghost cells and swollen erythrocytes) incubated in a 0.475% NaCl solution. The resistance of the medium was found to be R = 129 Ω cm. The field strength in the measurement orifice was 0.91 kV/cm, and thus well below the critical field strength required for the breakdown of the cell membranes (~2.4 kV/cm). At this field strength, the distribution of the mixed population is normal. However, at high, supracritical field strength in the measurement orifice, the distribution splits into two peaks (Fig. 1 *b*). Initially, when the breakdown voltage has just been exceeded, there is merely a spread in the distribution (not shown). Towards higher field strengths, however, there are always two distinctly separate peaks, such as those shown in Fig. 1 *b*, for a field strength of 4.11 kV/cm. The spread in the distribution as a function of field strength can be explained in terms of a dependence of the critical electrical field strength required for breakdown on the radius of the cells (see integrated Laplace equation and Zimmermann et al., 1976 *b*). In Fig. 2 the peak channel of the distribution (or distributions) (partly shown in Fig. 1) is plotted as a function of the current through the measurement orifice. Above a certain current intensity (0.5 mA), the signals split up. One straight line continues with a reduced slope, while the other displays a slightly negative slope. In other words, the signal decreases with increasing current through the orifice. From the slopes of the two straight lines after electrical breakdown, and the slope prior to electrical breakdown, it is possible to calculate the intracellular resistivities of the two populations. For cells from the population with the greater mean size after breakdown (Fig. 1 *b*, peak at higher channel numbers), the intracellular resistivity is calculated to be 190 Ω cm, whereas for cells from the population with the smaller mean size after electrical breakdown (Fig. 1 *b*, peak at lower channel numbers), the value is found to be 118 Ω cm. The cells found to have an intracellular resistivity of 190 Ω cm are intact, swollen red blood cells which, as a result of their hemoglobin content, have a higher internal resistivity than the external medium (Pauly and Schwan, 1966). The cell population with the intracellular resistivity of 118 Ω cm corresponds to the ghost cells. The internal resistivity is lower (and thus the conductivity higher) than that of the external medium (129 Ω cm).

This finding is easily explained by the measurement of the resistivity of the supernatant of the cell suspension after hemoly-

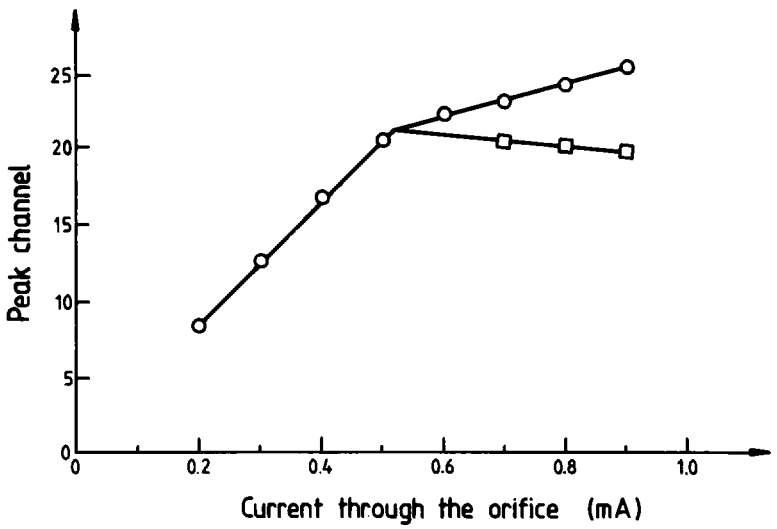

FIGURE 2 Peak channel of the size distribution of partly hemolysed erythrocytes as a function of electrical current through the measurement orifice. For conditions see Fig. 1. Up to 0.5 mA, the peak channel is proportional to the current, but above 0.5 mA the size distribution splits up into a distribution with a reduced increase in the channel number and a distribution with regressive channel numbers, i.e. for this distribution the signal height is reduced with increasing current. The separation of the original size distribution into two size distributions is caused by cells with different internal conductivities.

sis. The hemolysing red blood cells increase the conductivity of the hypotonic medium by their electrolyte content. The resistivity of the medium therefore dropped to 120 Ω cm. After equilibration with the medium, the internal resistivity of the ghost cells must have the same resistivity as the medium. The internal resistivity of the ghost cells with 118 Ω cm corresponds well with the resistivity of the hemolysing medium with 120 Ω cm.

The following conclusion can be drawn from this experiment: The two populations, erythrocytes and ghost cells, have the same mean volume (130 μm), so that they cannot be distinguished when the size distribution is determined by electronic sizing at low field strength (or currents). Only when the electrical breakdown voltage, which—as is readily evident—must be 1 V for both populations, is exceeded by means of the external electrical field, the two populations can be distinguished by their different internal conductivities. Whether and to what extent a second peak occurs at field strengths exceeding the critical field strength, depends on the conditions of preparation (see below) and on the resealing conditions of the ghost cells, since both these variables influence the internal conductivity (Zimmermann et al., 1976 *a,c*). We believe that Akeson and Mel overlooked one of the two peaks at high electrical field strengths in their measurements. This assumption is further supported by the experiment described below.

For this second set of experiments (Fig. 3 *a,b*), ghost cells were obtained by electrical hemolysis of human red blood cells in isotonic electrolyte solution (composition: 105 mM KCl, 20 mM NaCl, 4 mM $MgCl_2$, 7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, and

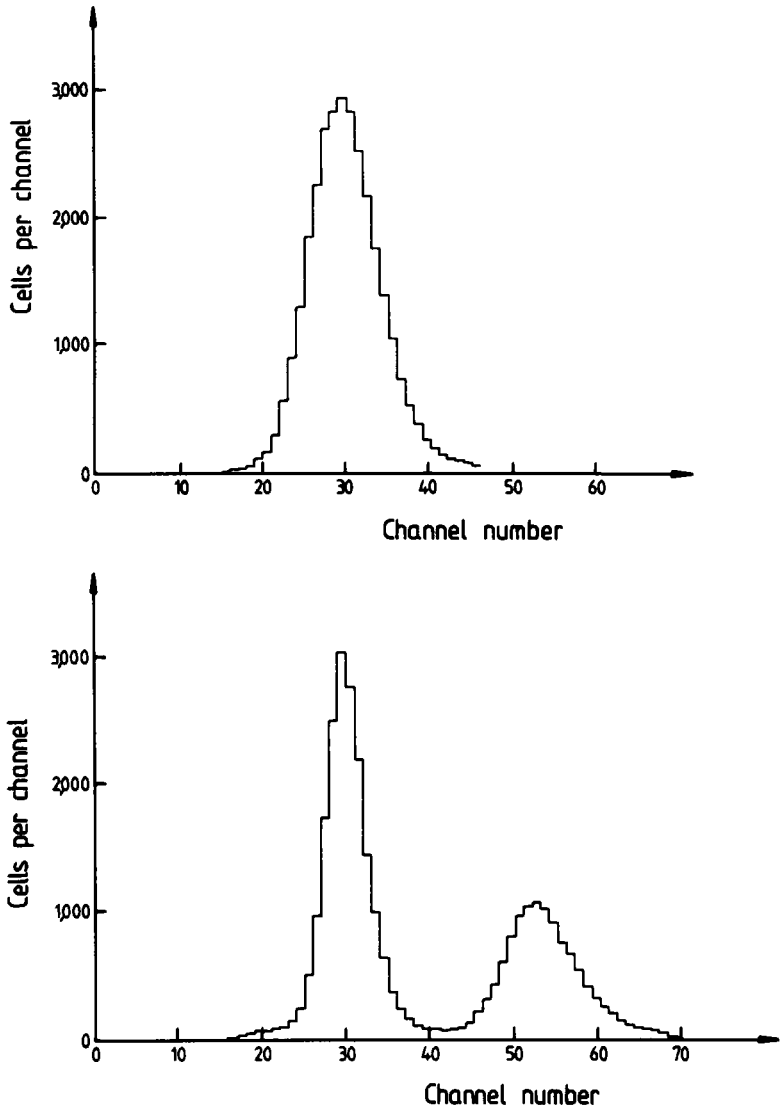

FIGURE 3 Size distribution of human ghost cells measured with a particle volume analyser with hydrodynamic focusing and a diameter and length of 60 μm for the measurement orifice. The cells were incubated in a 0.95% NaCl solution containing 130 mM benzyl alcohol. (*a*) Size distribution at an electrical current of 0.2 mA through the meansurement orifice, corresponding to a field strength of 0.44 kV/cm. (*b*) Size distribution at an electrical current of 0.9 mA through measurement orifice, corresponding to a field strength of 1.96 kV/cm. This field strength is sufficient to induce electrical breakdown in the membrane. The distribution exhibits two separate peaks. The peak at the lower channel numbers contains cells with a higher internal conductivity. Their proportion is 60%. The peak at the higher channel numbers contains cells with a lower internal conductivity. Their proportion is 40%.

10 mM glucose, pH 7.2; field conditions: 40 μs, 12 kV/cm; Zimmermann et al., 1976*a*). The size distribution of the resulting mixed population was measured in a 0.95% NaCl medium containing 130 mM benzyl alcohol. Benzyl alcohol reduces the electrical breakdown voltage (Pilwat et al., 1975; see also for an interpretation of this effect Dimitrov, 1984) and, in turn, the critical electrical field strength. As in the first example, only a single, normal distribution is observed at low field strengths in the orifice of the particle analyzer (0.44 kV/cm). (Fig. 3 *a*). At supracritical field strengths (in this case at 1.96 kV/cm because of the presence of benzyl alcohol), there are again two separate ghost cell distributions (Fig. 3 *b*). In Fig. 3 *b* the peak at lower channel numbers (smaller cells) represents ~60% of the cells, the peak at higher channel numbers, 40%. Under these conditions of hemolysis, the mean sizes and electrical breakdown voltages are also indentical for the two populations, so that they can only be distinguished by their different internal conductivities beyond the electrical breakdown voltage. Electrically produced ghost cells, whose distribution is measured in the absence of benzyl alcohol, do not split up even at high field strengths. As mentioned above, this indicates that the ghost cell population is homogenous with respect to both the breakdown voltage and the internal conductivity. The addition of benzyl alcohol, on the other hand, apparently leads to cell membrane leakiness in some of the cells, so that their internal conductivity changes. A new population is created. Since the generation of a second population with a different conductivity by the addition of benzyl alcohol is extremely reproducible, this method was used in preference to osmotic lysis methods in our experiments. With osmotic abrupt hemolysis (100 mOsm) as used by Akeson and Mel, up to three peaks may be produced at supracritical field strengths in a less-reproducible fashion, indicating the presence of ghost cells with variably permeable membranes (see also Bodemann and Passow, 1972). With the aid of their apparatus, Akeson and Mel investigated the relative number of ghosts (counted per second) as a function of exposure time to 100 mOsm phosphate buffer solution over a period of ~5 min, i.e. during lysis or shortly after lysis. During lysis in particular, there are always mixed populations present. These consist partly of nonhemolysed erythrocytes, and partly of ghost cells with different hemoglobin contents, and hence with different internal conductivities. Using a window discriminator, the authors apparently recorded only one distribution with their measuring apparatus, i.e. they overlooked the second (or third) distribution. For this reason, they came to the erroneous conclusion that the count rate of the cell suspension is reduced by "disappearing ghosts."

Their explanation that the ghost population splits up before entering the measurement orifice, namely into one proportion that remains unchanged and another that undergoes a second hemolysis with concomitant exchange with the external medium, so that the cells can no longer be recognized in the "sensing process", also appears wrong to us. Among other things, their argument is based on the assumption that the critical field strength for the electrical breakdown of the cells is already reached in front of the measurement orifice. In the case of short measurement orifices, such as those used by Akeson and Mel and by ourselves, this is certainly true, because part of the field protrudes out of the orifice (up to 50% and more) (see Zimmermann et al., 1976*b*, Kachel, 1979). When long measurement orifices are used, as illustrated in Fig. 4, it can be shown that ghost cells that have already undergone breakdown still continue

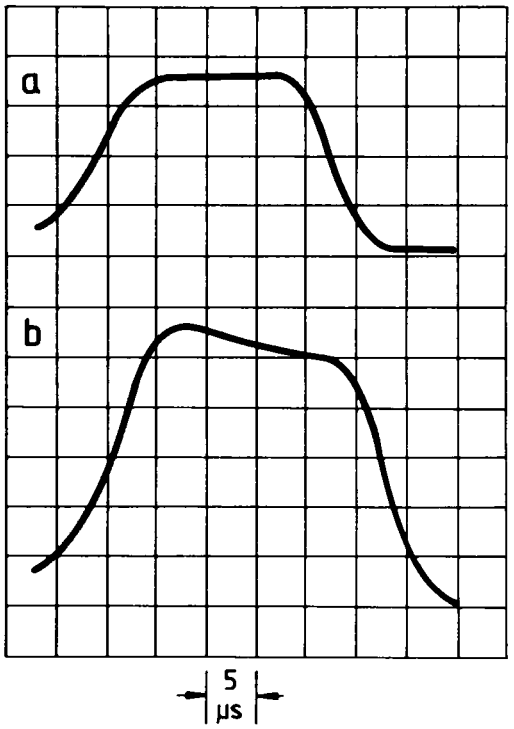

FIGURE 4 Resistance pulses produced by spherical ghost cells passing through an orifice of 44 μm in diameter and 200 μm in length. (*a*) Pulse before electrical breakdown. (*b*) Pulse after electrical breakdown. After electrical breakdown the resistance pulse slightly decreases, probably due to the increasing permeability of the membrane, which changes the shape of the cells electrolyte space.

to elicit a signal, although this does decrease slightly during the measurement period of 20 μs in such long capillaries (Fig. 4 *b*). This decrease is easily explained in terms of permeability changes in the membrane and field-dependent dissociation processes in the presence of high electrical fields (Coster and Zimmermann, 1975, Zimmermann et al., 1980*b*).

In general, the following statement therefore applies: When a cell approaches the field of the measurement orifice, it is recognized as a result of the associated resistance change in the sensing zone of the measurement orifice, independent of the influence of the electrical field on the membrane. At high field strengths, where the ghost membrane becomes electrically conducting, only the internal conductivity of the cell continues to contribute to the signal. When the intracellular conductivity of the ghost cells becomes completely identical with that of the external solution, the cells do not become transparent and apparently disappear, as postulated by Akeson and Mel, but rather the signal is no longer augmented, i.e. the signal height is constant, as is approximately the case in Fig. 2. These deliberations are in total agreement with the theory of Jeltsch and Zimmermann (1979) (see Fig. 1), but this was not correctly interpreted by Akeson and Mel in their work. Jeltsch and Zimmermann (1979) demonstrated that the integrated Laplace equation for the induced membrane potential is no longer valid after electrical breakdown. However, this has nothing to do with the determination of the internal conductivity in cells which membranes that have been made permeable by electrical means.

Finally, we would like to point out that our experiments and

considerations cast doubt on only part of Akeson and Mel's work. The great value of their work undoubtedly lies in the proof and confirmation of our previously published findings that dielectric breakdown measurements could represent a valuable tool in measuring the internal conductivity of cells (Zimmermann et al., 1980*a*). This could be of great importance for clinical diagnostics, and only practice will show which experimental arrangements are most suitable.

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