

Fast Intracellular pH Determination in Single Cells by Flow-Cytometry

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The maintenance of the intracellular pH within narrow limits is an important requirement for the correct function of cellular metabolism. The cytoplasmatic pH is close to neutral [1] while the intralysosomal pH is acidic [2]. During anaerobic metabolism the intracellular pH drops to acidic values, a condition which is frequent in tumor cells [3]. It is of considerable interest to know the mechanisms of intracellular pH regulation in such cells in order to understand the underlying disorders. The lower pH in tumor cells could also be of value for the identification of tumor cells during early tumor growth and metastasis formation.

A variety of methods for intracellular pH measurements [1, 4, 5] have been described in the past. These methods are, however, not suitable for fast pH determinations within single cells in suspension.

We have developed a new method for fast intracellular pH determination in single cells by flow cytometry using the dye 2,3-dicyano-hydroquinone (DCH). DCH shows a pH-dependent shift of the wavelength of fluorescence emission, in contrast to most fluorescent pH indicators [6] included fluorescein which have the fluorescence emission at a constant wavelength but of pH-dependent intensity. A sequential dual-wavelength excitation is necessary for reliable intracellular pH determinations with such dyes. This makes it even with flow cytometers difficult to measure the intracellular pH in single cells [5]. With DCH it is possible to determine the intracellular pH of single cells by flow cytometry in a one-step measurement using a one-wavelength excitation (300–400 nm) and a simultaneous measurement of the fluorescence emission at two different wavelengths (420–440 nm (F1) and 500–580 nm (F2)). The ratio of both fluorescences is a measure for the intracellular pH.

DCH is not taken up by living or dead cells. The cells appear as black dots under the fluorescence microscope. Using, however, esters of DCH as 1,4-diacetoxy-2,3-dicyano-benzol (ADB), 1,4-dibutyroxy-2,3-dicyano-benzol (BDB) or the N-protected alanyl derivative, 1,4-(tert-butyloxy-carbonyl-l-alanyl-oxy)-2,3-dicyano-benzol (AlaDB), living cells become fluorescent within a few minutes. The esters cross the cell membrane easily but do not enrich in cell membranes or lipid droplets. DCH is liberated intracellularly from the esters by esterases. DCH is apparently not toxic for the cells during a 15 to 20 min incubation. It also does not seem to interact to a great extent with protein since the wavelength of the maximum of the fluorescence emission spectrum is not shifted in a measurable way when DCH is incubated with 10 mg/ml bovine serum albumin. The major part of DCH is found in the cytoplasm and a minor part (around 10%) within the cell organelles of rat liver if the organ is perfused with 20 µg/ml ADB solutions in saline prior to homogenization in isotonic sucrose and 1-h centrifugation at 100000 g to separate the cytoplasmic from the cell organelle fraction.

The flow cytometric measurements were performed with a Fluvo-Metricell flow cytometer which was developed earlier in our laboratory [7]. The instrument is capable of simultaneously measuring the electrical cell volume and two fluorescences. Data acquisition and display were accomplished by FORTRAN IV computer programs [8, 9]. Single-cell suspensions were prepared immediately after removal of the tissues from the organism by syringing and stored between 0 and 4 °C until the incubation with ADB in order to minimize pH changes. The intracellular pH remained constant for several hours under these conditions. Cells were suspended in saline solution, weakly buffered with 5 mM Tris/

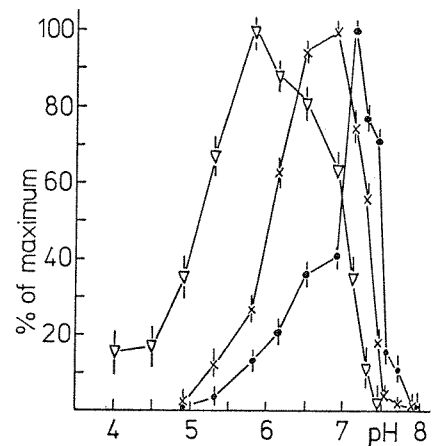


Fig. 1. pH distribution curves of cells from rat bone marrow (●—●), mouse Ehrlich ascites tumor (×—×) and human ovary cancer (▽—▽). Each curve represents the measurement of 1 to 5 · 10⁴ single cells

HCl to pH 7.35. ADB was added to the cell suspension from a stock solution containing 1 mg/ml ADB in dimethyl-sulfoxide to obtain a final ADB concentration of 10–20 µg/ml in the cell suspension. The suspension was incubated 5–15 min at room temperature prior to the measurement. Two parameter histograms (F1 vs. F2) were collected in a multichannel analyzer and stored on magnetic tape for further evaluation. Three parameter data were directly collected on tape in on-line list mode [8]. They were further evaluated as F1 vs. F2 histograms or as cell volume vs. F1/F2 ratio as indicator of the intracellular pH. A one-parameter pH distribution curve (Fig. 1) was calculated from the F1 vs. F2 histograms. The intracellular pH measurement can be calibrated by suspending the cells in buffers of different pH, containing ADB and H⁺ ionophores as nigericin, X-537A or channel formers as gramicidin D. We have obtained calibra-

tion curves between pH 5.5 and 8.5 by calculating the F1/F2 ratios of the accumulated intracellular DCH at the respective pH values.

It is of interest that the intracellular pH is not the same for all cells of a suspension of rat bone marrow cells. The pH distribution curve (Fig. 1) shows that, although most of the cells are located quite narrowly around pH 7.2, a significant number of cells have a lower intracellular pH around pH 6.9. By preparative separation of the cells according to cell volume in an elutriator centrifuge and by simultaneous cell volume and fluorescence measurement in the flow cytometer it was demonstrated that these cells belong to the blastoid compartment which contains myeloblasts, erythroblasts and granulocytes. Ehrlich ascites tumor cells were acidic with a curve maximum at pH 6.9. Cells of a human ovary carcinoma were even more acid with a broad pH distribution curve and a maximum at pH 5.9. It is important that all the cells which accumulate DCH are live cells. Dead cells suspended in ADB remain unstained. Therefore, ADB similarly as

fluorescein-diacetate can be used as a vital stain. Additionally one obtains by such dyes information on the enzyme kinetics in living cells [11] during the phase of intracellular accumulation of the fluorescent reaction product. A plateau of intracellular fluorescence is reached after 10–15 min incubation at room temperature. The height of this plateau is informative with regard to the steady state between DCH formation by hydrolysis and DCH efflux from individual cells. ADB and similarly BDB and AlaDB in addition to their usefulness for pH determination provide several other important information on the metabolic state of cells without further measurements.

The intracellular pH as determined by the ADB method represents probably not only the cytoplasmic pH since ADB penetrates to some degree into cell organelles. A way to obtain information on the pH in sub-compartments of the cell could be to synthesize substrates which are only cleaved by compartment-specific enzymes.

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