Mass Spectrometric Analysis of Drug-induced Changes in Na⁺ and K⁺ Contents of Single Bacterial Cells

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Time-dependent changes in the intracellular Na⁺/K⁺ ratio of *Escherichia coli*, induced by the nitrofur derivative HN32 [2,4-diamino-6-(5-nitrofuryl-2)-5-ethylpyrimidine], were measured by laser-induced mass spectrometry of single bacterial cells. The results show good agreement with data on viable cell and total cell counts, release of ATP and ¹⁴CO₂ production demonstrating that the single cell analysis of intracellular sodium and potassium concentrations may supply reliable information on cell viability and, furthermore, offer additional information not available from established gross methods.

INTRODUCTION

The laser microprobe mass analysis (LAMMA[®], Leybold-Heraeus, Köln, F.R.G.), a new microanalytical technique, which is based on the laser-induced ionization and subsequent mass spectrometric analysis of very small volumes (~1 µm³) is a promising method in fields of research related to microbiology and bacteriology by supplying information on single bacterial cells (Seydel & Lindner, 1981). At present, however, statements on the molecular level can be made only on the basis of mass fingerprints (Seydel & Heinen, 1980), because (mainly due to the lack of information on the fragmentation mechanisms involved in the laser-biological sample interaction) it is not possible to identify reliably peaks other than those of some elements. These include the cations sodium, potassium, magnesium and calcium. These elements are well-known for their regulatory role in cell function (e.g. Glynn *et al.*, 1974). The sodium-potassium ratio should be a particularly sensitive indicator of cell viability. The principle intention behind the application of the single cell mass analysis to the measurement of sodium and potassium ratio (or content) of bacterial cells was to test the possibility of utilizing this method for therapy control in leprosy (Seydel *et al.*, 1982). Leprosy is an infectious disease caused by a mycobacterial species, *Mycobacterium leprae*. The organism cannot be cultivated *in vitro*, giving rise to severe problems in developing new drugs for treatment of the disease. Up to now therapy control was performed via the mouse foot pad test (Shephard, 1960), a time consuming and not always reliable technique. Therefore, it should be of great advantage if information on the influence of a therapy on the infection could be obtained from a very limited number of bacteria isolated from biopsies.

After initial encouraging results on the effects of various treatments of bacterial species on their mass fingerprints, or even the sodium-potassium ratio (Seydel *et al.*, 1982), the aim of the present investigation was to verify these first results on a broader basis and to compare the findings from single cell mass analysis with those from more established techniques such as viable cell counting, ATP levels and ¹⁴CO₂ production from [¹⁴C]glucose metabolism. These investigations were performed using *Escherichia coli* K12 and the drug HN32, a nitrofur derivative which was shown to be a very powerful antibacterial agent (Seydel & Wempe, 1980) although its specific mechanism of action is not yet known. Only recently it has been shown by Lopez & Fortnagel (1981) that another nitrofur derivative, nitrofurantoin, has a broad antimicrobial activity and affects the ATP and GTP contents of different bacteria.

METHODS

Organism and growth conditions. *Escherichia coli* K12 strain W 3110 was used as a test organism, and maintained as agar slant cultures. The culture broth was dextrose/salts/Casamino acids (Difco, vitamin free) and has been described by Anton (1960). To obtain more pronounced changes in the intracellular cation concentration upon drug interaction, the medium was slightly modified by replacing the K salts by Na salts. A 10-fold dilution of an overnight culture in 400 ml modified Anton medium (giving approximately 10^7 cells ml^{-1}) was allowed to grow for 1 h. From this two 400 ml cultures were inoculated in 500 ml flasks at a starting cell density of about 2×10^4 cells ml^{-1} . To the exponentially growing cultures, the nitrofurantoin derivative HN32 [2,4-diamino-6-(5-nitrofuryl-2)-5-ethylpyrimidine] was added (20 μM ; approximately fivefold the MIC) at a density of 10^7 cells ml^{-1} and samples were taken at time intervals.

Total count method. Samples of the experimental cultures were diluted with a particle-free solution (membrane-filtered, 0.15 μm pore size) containing 0.85% (w/v) NaCl and 0.2% (w/v) formaldehyde, so that a count of 1000 to 30 000 organisms was obtained. Diluted samples were counted with a Coulter counter, model 'ZB', equipped with a 30 μm orifice.

Viable count method. Samples were diluted to obtain 10 to 100 colonies per plate then 1 ml of the appropriate dilution was mixed with 15 ml agar (Standard I Nutrient Agar, Merck) and poured into Petri dishes. Plates were incubated for 48 h and colonies were determined in triplicate.

$^{14}\text{CO}_2$ measurement. Continuous radiometric detection of the production of $^{14}\text{CO}_2$ from [^{14}C]glucose was performed in a methane flow counter. For this, the culture flasks (500 ml) containing 50 μCi (1.85 MBq) [^{14}C]glucose (Amersham Buchler, Braunschweig, F.R.G.) were sealed with a rubber stopper penetrated by three needles, two of which descended into the culture medium and led sterile air through it (2 l h^{-1}) and allowed small samples to be taken for other measurements. The third needle descended only into the air space above the culture and withdrew the $^{14}\text{CO}_2$ -containing air into the counter. During growth, the cultures were gently shaken periodically.

ATP measurements. For the measurement of bacterial ATP an enzymic reaction based on the firefly bioluminescence was used [for reviews, see Lundin, 1981; McElroy & De Luca, 1981]. Test kits (Lumac System, Basel, Switzerland) were used as directed by the manufacturers. Light emission was monitored using a specially adapted liquid scintillation counter giving a detection limit of 5 pg ATP in a sample volume of 100 μl . For each measuring point two 100 μl samples were prepared, one from the original cell suspension, the other from the cell-free suspension obtained by membrane filtration (1 μm pore size). In this way, the percentage of total ATP released from the bacteria was calculated and corrected for bacterial interference. Each value was determined in triplicate.

Laser microprobe mass analysis (LAMMA[®]). The LAMMA instrument has been described in detail elsewhere (Heinen *et al.*, 1980). Briefly, it represents a combination of a laser microscope with a time-of-flight (TOF) mass spectrometer. The microscope serves for the observation of the specimen to be investigated as well as for focusing an UV pulse laser beam ($\lambda = 265$ nm, pulse duration 30 ns, power 10^8 – 10^{10} W cm^{-2}) on to the specimen (spot size ~ 1 μm diam.). The effect of the laser beam is to form positive and negative atomic and molecular ions which are detected alternately by the TOF analyser (mass resolution up to 1000, detection limit up to 10^{-20} g for sodium and potassium in an analysed volume of 1 μm^3).

For LAMMA analysis the bacteria had to be washed quickly and thoroughly as follows to avoid ion dislocation. The bacteria were obtained by centrifugation at 2000 g for 10 min, and resuspended in distilled water at 20 °C. After mixing they were again centrifuged and the organisms were resuspended in distilled water. A drop of the final suspension was mounted on a Formvar-coated Cu grid (as used for electron microscopy), and excess fluid was drained off with tissue. In this way a widespread distribution of the bacteria was achieved allowing the laser vaporization of one single cell at a time. From each sample, 30 single cells were analysed and the Na^+/K^+ ratios were averaged. All ratios are based on the ^{41}K isotope which has an isotopic abundance of 6.7%, ^{39}K representing 93.3%.

Chemicals. The nitrofurantoin derivative 2,4-diamino-6-(5-nitrofuryl-2)-5-ethylpyrimidine (HN32) was a generous gift of Dr K. Gutschke from Nordmark Werke, Uetersen, F.R.G.

RESULTS AND DISCUSSION

Two typical positive ion mass spectra of single untreated (top) and treated (bottom) cells are shown in Fig. 1. The treated sample was taken 3 h after HN32 application. The peaks at m/e values of 23, 24, 25, 26, 39, 40, 41 can be attributed to ^{23}Na , ^{24}Mg , ^{25}Mg , ^{26}Mg , ^{39}K , ^{40}Ca and ^{41}K , respectively. The peaks at $m/e > 70$ have a pattern which was also found for other bacterial species. There is some evidence that this pattern might originate from cell wall components.

The drastic shift in Na^+/K^+ ratio of *E. coli* cells induced by treatment with HN32 is shown in

Fig. 1. Positive ion LAMMA spectra of single *E. coli* cells at maximum inhibition induced by HN32 (bottom) and of the corresponding control (top).

Fig. 1. Furthermore, an influence of the drug on the pattern of the mass peaks at $m/e > 70$ becomes obvious but cannot be interpreted at this time.

The different techniques used for monitoring the effect of the drug HN32 on cell growth kinetics are compared in Fig. 2. In each case, the values for the untreated controls are compared with those for the treated cultures. The increase in the cation ratio (Fig. 2a) correlates well with the decrease in viability (Fig. 2d).

The ATP measurements show considerable scatter due to the low cell number at the beginning of the control experiment and of the treated sample during the entire experiment. Nevertheless, it is clear that the ATP release shows a delayed response of 2 to 3 h after drug addition (Fig. 2e). Total ATP content, however, i.e. the sum of the ATP in bacteria and filtrates, increased in parallel with total cell numbers and $^{14}\text{CO}_2$ production, in both control and treated culture (results not shown). A reliable interpretation of this delayed ATP release cannot be given at the moment. Further studies are required to determine whether the permeability of the bacterial membranes for ATP or ATP hydrolysis and the release of ATP influence the observed behaviour.

The distribution of the Na^+/K^+ ratio within one sample was very narrow for the untreated control as well as for the samples with the highest shift in this ratio (7.5 h) and at 25 h, whereas the distribution was quite broad for samples taken shortly after drug application (5.5 h, 6.5 h) and in the state of 'recovery' (11.5 h, 12.5 h). This might be due to the fact that not all of the

Fig. 2. Time dependence of various responses of *E. coli* to treatment with HN32. (a) Cation ratio ($^{23}\text{Na}^+ / ^{41}\text{K}^+$) averaged over 30 single cells for each measuring point; (b) total cell number from Coulter counter analysis; (c) $^{14}\text{CO}_2$ production from ^{14}C glucose metabolism; (d) viable cell number from plate counts; (e) extracellular ATP from bioluminescence. The closed circles show values for the untreated control and the open circles the values for the treated samples. The drug was added at the time shown by the vertical dashed line.

individual bacteria are affected in the same way by the drug because the manifestation of the impairment might be time dependent due to different physiological states of the cells. After longer periods of drug treatment, most of the cells, but not all, are impaired and the viable cells grow to a noticeable number again (Fig. 2b) with restoration of the normal Na^+ / K^+ ratio (Fig.

2a). A similar effect of shifting the Na^+/K^+ ratio was observed with other drugs (cephalosporins, aminoglycosides etc.) and in another culture medium (Standard I Nutrient Agar). A drug-dependent shift in the Na^+/K^+ ratio was also found at drug concentrations far below the MIC.

In future investigations, the distribution of cation concentrations within cell populations will be our major concern. From this, new information on interactions at the cellular level might be expected. A prerequisite for this, however, will be the development of appropriate computer software. Another important task would be the attribution of the higher mass peaks ($m/e > 70$; see Fig. 1) to well defined molecules or molecular fragments of certain cell components. By increasing the laser power, more complex mass spectra can be achieved due to a more intensified fragmentation and the generation of mass peaks at higher m/e values, a fact which might be helpful for this task.

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