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Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry

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A.S. KAPRELYANTS AND D.B. KELL. 1992. The fluorescent dye rhodamine 123 (Rh 123) is concentrated by microbial cells in an uncoupler-sensitive fashion. Steady-state fluorescence measurements with *Micrococcus luteus* indicated that provided the added dye concentration is below approximately 1 mmol/l, uptake is fully uncoupler-sensitive and is not accompanied by significant self-quenching of the fluorescence of accumulated dye molecules. 'Viable' and 'non-viable' cells are easily and quantitatively distinguished in a flow cytometer by the extent to which they accumulate the dye. The viability of a very slowly growing chemostat culture of *Mic. luteus* is apparently only about 40–50%, as judged by plate counts, but most of the 'non-viable' cells can be resuscitated by incubation of the culture in nutrient medium before plating. The extent to which individual cells accumulate rhodamine 123 can be rapidly assessed by flow cytometry, and reflects the three distinguishable physiological states exhibited by the culture ('non-viable', 'viable' and 'non-viable-but-resuscitable'). Gram-negative bacteria do not accumulate rhodamine 123 significantly because their outer membrane is not permeable to it; a simple treatment overcomes this. Flow cytometry using rhodamine 123 should prove of general utility for the rapid assessment of microbial viability and vitality.

INTRODUCTION

It is usually considered that the ability of a microbial cell to reproduce itself on a nutrient agar plate constitutes the benchmark method for determining how many living cells may be contained in a sample of interest (Postgate 1976). Nowadays, however, many so-called 'rapid' methods have been developed to allow a speedier assessment of the 'viable' microbial load in a sample (e.g. Harris & Kell 1985; Adams & Hope 1989). Of routine microbiological methods, only direct counting of cells (see Norris & Swain 1971; Quesnel 1971; Pettipher 1983; Harris & Kell 1985; Fry, 1990; Hall *et al.* 1990; Herbert 1990), usually stained with an appropriate fluorochrome and counted under an epi-fluorescent microscope, can give a rapid answer to the question of how many 'viable' cells may exist in a sample. In exponentially growing cultures there is usually a good correlation between viable counts and (say) acridine orange-based direct counts, but in natural environments these direct counts can exceed the viable counts by several orders of magnitude (Roszak & Colwell 1987).

It is also widely recognized that, especially in nature, the distinction between life and non-life is not absolute; many cells may exist in moribund (Postgate 1967) or 'dormant' (Stevenson 1978) forms or states which will not form colonies on nutrient media (i.e. are 'non-culturable') but which will yet give a 'viable' direct count (see Postgate 1976; Poindexter 1981; Morita 1982; Mason *et al.* 1986; Kjellberg *et al.* 1987; Roszak & Colwell 1987; Morita 1988; Matin *et al.* 1989; Gottschal 1990). Such cells have been referred to as 'pseudosenescent' (Postgate 1976) or 'somnicells' (Roszak & Colwell 1987; Barcina *et al.* 1990). In some cases, cells that have undergone some kind of stress are, or may be, considered 'injured', and may be resuscitated by pre-incubation in nutrient media before plating out (e.g. Ray 1979; McFeters 1990). Amongst brewers, the term 'vitality' is used to refer to those properties of a culture of otherwise 'viable' cells which reflect its capacity rapidly to metabolize after transfer from a nutrient-poor to a nutrient-rich environment. In this paper we use the term 'viable' to refer to a cell which can form a colony on an agar plate when plated (at appropriate dilutions) directly from the

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sample of interest, 'vital' to refer to one which can do so only after resuscitation, and 'non-viable' to refer to a cell which cannot do so under any tested condition.

Metabolizing bacteria, like the mitochondria of eukaryotes, have the ability to concentrate lipophilic organic cations from the external medium by passage across their cytoplasmic membranes into the cytoplasm. More than 10 years ago Konings & Veldkemp (1980) proposed that the (uncoupler-sensitive) extent of this ability would reflect the energetic status *in vivo*. Most commentators assume that the uptake of such lipophilic cations is electrophoretic, being driven by a transmembrane potential (negative inside) generated by respiration or by ATP hydrolysis. However, since the uptake of these cations is normally accompanied by the extrusion of a similar number of protons, and is thus effectively electroneutral, this assumption is probably incorrect (Tedeschi 1980; Kell 1988). Indeed, Chen's group have recently shown (Smiley *et al.*, 1991) that the uptake of a related lipophilic cation within *individual* mitochondria is spatially heterogeneous, and thus cannot be driven simply by a delocalized membrane potential.

Chen and his colleagues (Johnson *et al.*, 1980; Chen *et al.* 1982; reviewed by Chen 1988), and subsequently many others (e.g. Emaus *et al.* 1986; Ronot *et al.* 1986; Petit *et al.* 1989; Skowronek *et al.* 1990), have shown that the polar, water-soluble, cationic, fluorescent dye rhodamine 123 is highly concentrated by eukaryotic mitochondria in an energy-dependent fashion (in that the accumulation is reversed by uncouplers), and Matsuyama (1984) indicated that the same dye could be used to stain living bacteria in an uncoupler-sensitive manner, the extent of uptake being assessed qualitatively by light microscopy.

In a typical flow cytometer (Melamed *et al.* 1979; Shapiro 1988; Kell *et al.* 1991), individual particles pass through an illumination zone and appropriate detectors, gated electronically, measure the magnitude of a pulse representing the extent of light scattered or, with optical filters, the magnitude of the particle's fluorescence. The magnitudes of these pulses are sorted electronically into 'bins' or 'channels', permitting the display of histograms of the number of cells with a certain property *vs* channel number.

In view of the above, it occurred to us that it might be possible to distinguish 'non-viable', 'viable' and 'non-viable-but-resuscitable' cells in terms of the extent to which they would take up rhodamine 123 (Rh 123). Indeed Shapiro (1990) has recently outlined the use of flow cytometry with the fluorescent dye hexamethylindodicarbocyanine for assessing microbial viability in a way analogous to this. The purpose of the present paper, therefore, is to describe experiments which we have carried out, mainly with *Micrococcus luteus*, which show that the extent of uptake of Rh 123 does depend on the energetic status of the cells of interest, and that one can indeed effectively and rapidly distinguish 'viable', 'vital' and 'dead' cells from each other by flow cytometry of cells stained with this dye.

MATERIALS AND METHODS

Organism

Micrococcus luteus (syn. *Mic. lysodeikticus*) Fleming strain 2665 was maintained on Nutrient Agar (Difco). In one experiment, *Escherichia coli* K12 strain C6 from the laboratory collection was also used (Davey *et al.* 1990).

Media

The growth medium ('lactate minimal medium') consisted of (g/l): NH_4Cl, 4; KH_2PO_4, 1·4; biotin, 0·005; L-methionine, 0·02; thiamine, 0·04; inosine, 1; $MgSO_4$ 0·07; $CuSO_4$, 0·000024; $MnCl_2$, 0·0005; $FeSO_4$, 0·001; Na_2MoO_4, 0·000025; $ZnSO_4$, 0·00005; and lithium L-lactate, 10. The pH was adjusted to 7·5 with NaOH before autoclaving (121°C for 25 min). For C-limited continuous culture, the medium was the same except that the L-lactate concentration was reduced to 1 g/l.

Both *Mic. luteus* and *E. coli* were also grown aerobically in a ('rich') medium containing 1.3% (w/v) Nutrient Broth E (Lab M).

Culture apparatus

Aerobic batch culture was carried out in 100 ml of medium contained in 500 ml Erlenmeyer flasks, which were incubated on an orbital shaker (New Brunswick Scientific, Hatfield, Herts, UK) at 30°C and rotated at 500 rev/min. Continuous culture was performed in an LH Fermentation (Maidenhead, Berks, UK) 500 series fermentor with a working volume of 500 ml, essentially as previously described (Dixon *et al.* 1988) except that air was sparged via the impellor at a rate of 800 ml/min. The temperature was controlled at 30°C. The dilution rate was set with a Pharmacia (Bromma, Sweden) Model P1 peristaltic pump that, to ensure effectively pulse-free operation, was connected to a tube which passed below the surface of the broth. No problems with grow-back were encountered. For the relevant experiments the dilution rate was 0·01/h and at least five volume changes were effected before measurements were taken.

Fluorimetry

A Perkin-Elmer 204-A fluorimeter (Clarke *et al.* 1982) was used with the excitation and emission wavelengths of 480

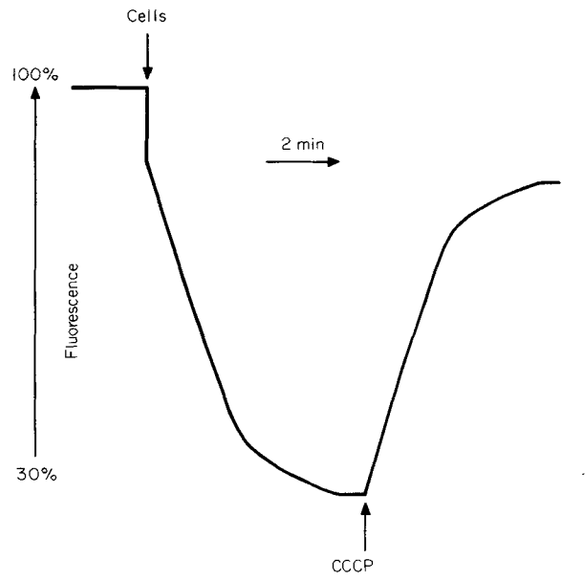

Fig. 1 Uptake and self-quenching of Rh 123 fluorescence by cells of *Micrococcus luteus*. Fluorimetric measurements were carried out as described in Materials and Methods, using excitation and emission wavelengths of 480 and 520 nm, in 3 ml of a medium containing 100 mmol/l potassium phosphate, pH 7·4, 1% lithium L-lactate and 3·5 μmol/l Rh 123. At the first arrow, cells taken directly from a batch culture (lactate minimal medium) in late-logarithmic phase were added to a final concentration of $3 \cdot 10^8$/ml. At the second arrow carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) in ethanol was added to a final concentration of 15 μmol/l. The addition of a similar volume of ethanol alone had no effect

and 520 nm respectively (see Fig. 1) and a slit width of 10 nm.

Flow cytometry

Flow cytometric experiments were carried out essentially as described by Davey *et al.* (1990) using a Skatron Argus 100 instrument (Skatron Ltd, Newmarket, Suffolk, UK) with a sample flow rate of 0·5–1·0 μl/min and a sheath fluid pressure of 1·5 kPa/cm^2. This instrument (Boye *et al.* 1983; Steen 1990) contains low-angle (<15°) and large-angle light-scattering detectors, plus a variety of fluorescence detectors selectable by appropriate filters. In our experiments we show results that were obtained solely from low-angle light-scattering (LS1) and a fluorescence detector designed for use with fluorescein isothiocyanate. The optical characteristics of the relevant filter are: excitation 470–495 nm, band stop 510 nm, emission 520–550 nm. Measurements were carried out according to the manufacturer's instructions, except that an additional bacteriological filter (Anotop 25, pore size 0·1 μm; Anotec Separations Ltd, Banbury, Oxon, UK) was placed in the sheath-fluid line. The instrument was run under the control of a Viglen (London) IIHDE microcomputer (IBM-PC-AT-compatible, 80286 processor, EGA screen), with software supplied by the manufacturer. This provides data files (in non-list-mode) in a standard format (Murphy & Chused 1984). The photomultiplier voltages were normally set at 500 V and 650 V for the light-scattering and fluorescence channels, respectively, and all measurements were gated by the light-scattering channel. Cells were carefully dispersed before measurement by repeated passage through a 0·4 mm needle to avoid clumping artefacts.

Monodisperse (C.V. < 2%) latex particles were obtained from Sigma or from Dyno Particles A/S, 160, Lillestrøm, Norway.

Cell viability measurements

Standard pour plates consisting of 1·3% Nutrient Broth E (Lab M) solidified with 1·5% purified agar (Lab M) were used. Cell dilutions were made at room temperature in quadruplicate with the lactate minimal medium described above. Cells were carefully dispersed by repeated passage through a 0·4 mm needle to avoid clumping artefacts. Plates were incubated at 30°C for 3 d and counted manually. (Although a rich medium was used for these plates we found that there was no difference in viabilities when judged on plates consisting of lactate minimal medium solidified with agar.)

Total cell counts

Unstained cells were counted with a phase-contrast microscope and an improved Neubauer counting chamber.

Epi-fluorescence microscopy

A Standard RA microscope (Carl Zeiss) was used, with an excitation filter passing light of wavelengths 450–490 nm and an emission filter passing light of 510 nm and greater.

Chemicals and biochemicals

Rhodamine 123 was obtained from Sigma. Other chemicals were of analytical grade and were obtained from Sigma or

BDH. Water was singly distilled in an all-glass apparatus, except that used in the sheath fluid of the flow cytometer which was further purified in a Millipore Milli-Q apparatus.

RESULTS

Rhodamine accumulation by *Micrococcus luteus* measured by steady-state fluorescence

The interaction of Rh 123 with respiring cells was studied first. Figure 1 shows a typical trace of the fluorescence of a Rh 123 solution: after the addition of cells there was a rapid decrease in fluorescence (representing probe binding) followed by a slow and further decrease in fluorescence due to accumulation of the probe by the cells with concomitant self-quenching of fluorescence. The addition of the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) caused efflux of the probe and a return of the total fluorescence to a level close to the value representing probe which had bound to, but not been accumulated by, the cells. (This also showed, as did control experiments lacking cells, that CCCP does not itself quench the fluorescence of Rh 123.) Similar results were obtained when, instead of adding uncoupler, the cells were allowed to become anaerobic.

To show that the decrease in fluorescence of Rh 123 was a consequence of its accumulation from the external aqueous phase, cells and Rh 123 were mixed in different ratios under the same conditions as those of Fig. 1; the cells and Rh 123 were incubated aerobically for 15 min and then the cells were rapidly centrifuged and the concentration of Rh 123 in the supernatant fluid determined fluorimetrically, that accumulated being calculated by difference. Figure 2 shows the relationship between the fraction of Rh 123 accumulated by the cells in the presence and absence of 15 μmol/l CCCP as a function of the concentration of Rh 123. It is evident that only at the lowest concentrations of Rh 123 do cells exhibt no energy-independent binding, and have an uptake that is fully sensitive to CCCP. As the internal volume of *Mic. luteus* is known to correspond to 0·5 $\mu l/10^8$ cells, it may be calculated that when the added concentration of Rh 123 was 0·87 μmol/l the accumulation ratio ($[Rh\ 123]_{in}/[Rh\ 123]_{out}$) was some 3000:1. Obviously this represents an enormous enhancement of the potential fluorescence within a cell.

The results of the dependence of the steady-state fluorescence of Rh 123 in the presence of cells of *Mic. luteus*, under conditions of maximum accumulation of the probe, and as a function of the concentration of the dye (Fig. 3), show that, as expected, the extent of self-quenching of the

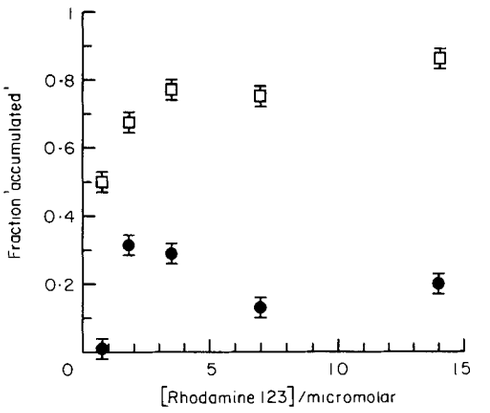

Fig. 2 Energy-dependent and energy-independent accumulation and binding of Rh 123 to cells of *Micrococcus luteus*. Experiments were performed as described in the text and in the legend to Fig. 1. □, Accumulation at the steady state; ●, accumulation after the addition of 15 μmol/l carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). Symbols represent mean ± S.E.M.

fluorescence of accumulated probe molecules decreases as the added (and hence the intracellular) concentration of the dye is decreased. Thus, if one wishes to avoid problems of an unknown degree of self-quenching when seeking quanti-

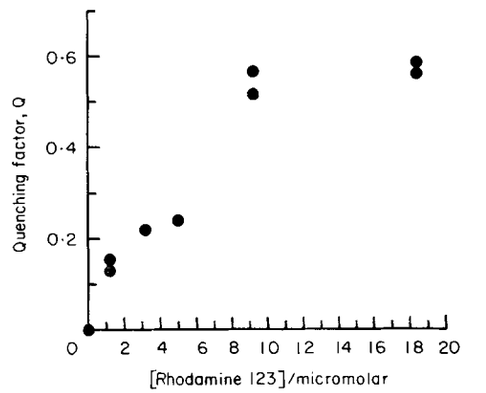

Fig. 3 Effect of Rh 123 concentration on the extent of self-quenching during accumulation by *Micrococcus luteus*. Measurements were made fluorimetrically, exactly as described in the legend to Fig. 1 except that the concentration of Rh 123 added was varied as indicated. The quenching factor Q is given as $(F_{CCCP} - F_{SS})/F_{CCCP}$, where F_{SS} is the fluorescence when cells had accumulated Rh 123 to a steady-state level and F_{CCCP} is the fluorescence after the addition of 15 μmol/l carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) to the cells had caused the probe to efflux to its maximum possible extent

tatively to measure the extent of dye accumulation fluorimetrically, the concentration of dye used should be as small as possible. The results indicated in Figs 2 and 3 thus show that added dye concentrations of less than 1 μmol/l exhibit both a negligible energy-independent accumulation and a negligible self-quenching of accumulated dye molecules. It is also noteworthy (Fig. 3) that the quenching parameter Q does not adopt negative values, indicating that Rh 123 is not accumulated in a region of the cell (e.g. a membrane) that causes it to increase its fluorescence quantum yield significantly. Given the highly polar nature of this dye (Chen 1988) this is not in fact surprising. Under these conditions of low dye concentration, then, the fluorescence of a cell stained with Rh 123 will depend only on the

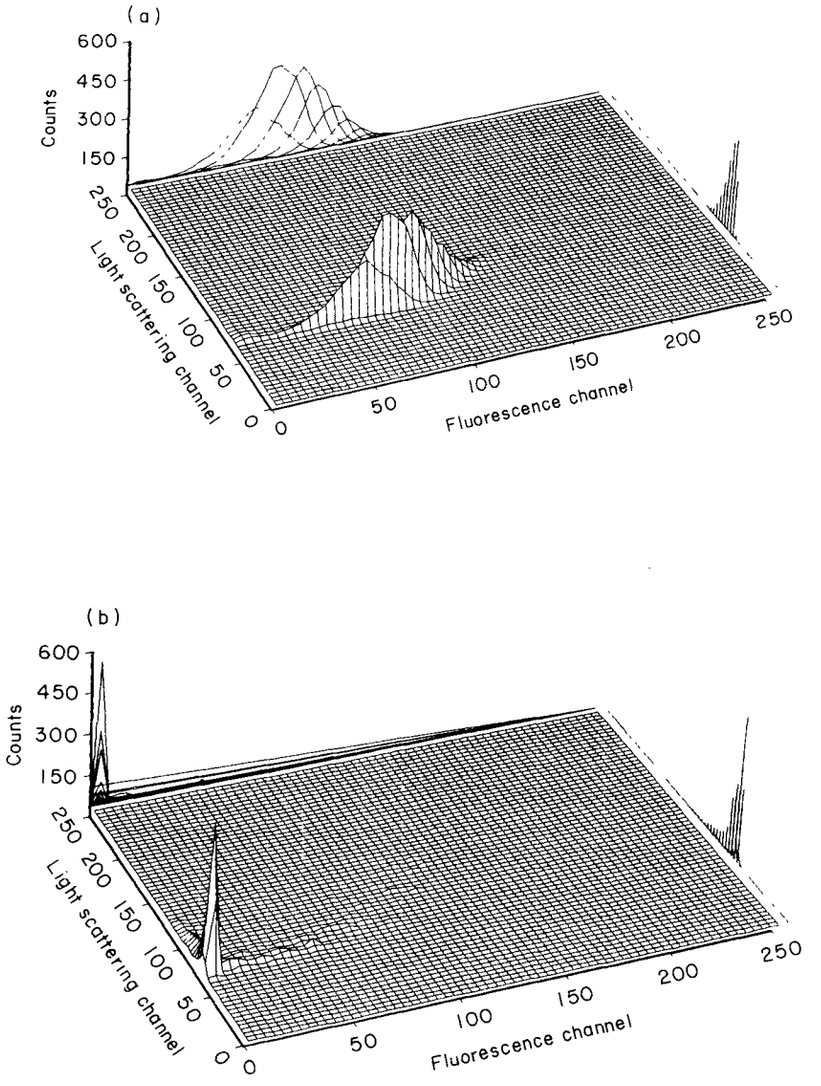

Fig. 4 Flow cytometric estimation of light scattering and rhodamine accumulation by cells of *Micrococcus luteus*. Fifty microlitres of a logarithmic batch culture of cells (O.D. $\frac{680}{1\,cm} = 4{\cdot}2$) in rich medium were added to 1 ml of lactate medium, and dispersed by repeated passage through a 0·4 mm needle to give a final cell concentration of approximately 10^8/ml. Rhodamine 123 was added to a final concentration of 0·26 μmol/l and the cells were incubated at room temperature for 10 min. Flow cytometry was carried out as described in Materials and Methods, and the total number of cells counted (usually in the range 2000–8000) were normalized to 10 000. The photomultiplier tube voltages were 500 V and 650 V for the light-scattering and fluorescence channels, respectively, and the amplification was logarithmic. In (b), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was added to a final concentration of 15 μmol/l and the cells further incubated for 5 min, before flow cytometric measurements

cell size and the concentration of dye accumulated internally.

Flow cytometry of cells stained with rhodamine 123

Figure 4 shows a typical plot of the low-angle light scattering and the fluorescence of *Mic. luteus* cells grown in batch culture and stained with Rh 123, in the absence (Fig. 4a) and presence (Fig. 4b) of 15 μmol/l CCCP. Larger channel numbers represent increased extents of low-angle light scattering and rhodamine fluorescence (accumulation). It is evident (Fig. 4a) that, as expected, there was a tendency for larger cells to have larger extents of fluorescence, although it is interesting to note that there was a significant heterogeneity in the extent to which a given cell (size) will accumulate Rh 123. The addition of what is nearly a saturating concentration of CCCP caused the efflux of accumulated Rh 123, but did not significantly affect the light scattered by the cells (Fig. 4b). That there is still an apparent fluorescence of CCCP-treated cells (i.e. not all fluorescence appears in the lowest channel) is due to two factors: (i) there is a leakage of scattered light into the 'fluorescence' results, representing the peak below channel 19 when the cells are not themselves fluorescent, as may be judged from control experiments (not shown) in which Rh 123 was not added; and (ii) it is very difficult to add sufficient CCCP to cause the efflux of every single molecule of Rh 123, i.e. to de-energize the cells completely. In the absence of CCCP, there was no evidence that the culture contained a significant fraction of 'non-viable' cells (i.e. it appeared that there was but a single population), and indeed cell viability measurements by plate counts (not shown) confirmed that the viability, within experimental error, was indeed 100%. It is also worth pointing out that the concentration of Rh 123 used (0·26 μmol/l) gave no decrease in cell viability when added to the agar medium used (results not shown), even though visual examination indicated a substantial accumulation of the dye by the colonies.

Similar experiments (not shown) in which the concentration of Rh 123 was varied indicated, as expected from the results shown in Fig. 3, that the lowest concentrations of dye (i.e. below 1 μmol/l) caused the greatest differences in the peak channel number before and after the addition of 15 μmol/l CCCP. Of course the total fluorescence signal also decreased as the concentration of dye decreased, and the concentration chosen (0·26 μmol/l) represented the optimum.

Figure 5 shows the outcome of a number of experiments similar to those of Fig. 4, in which both the CCCP concentration to which the cells were exposed and the photomultiplier tube (PMT) voltage of the fluorescence channel were

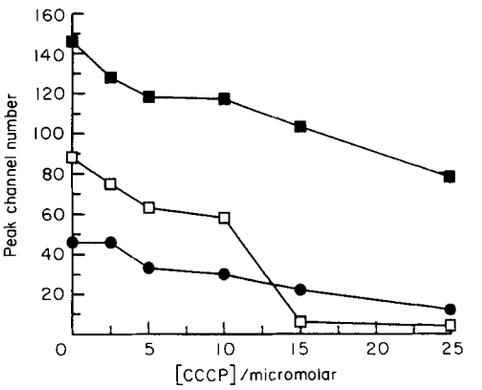

Fig. 5 Effect of the concentration of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) on the cytometric behaviour of *Micrococcus luteus* cells stained with Rh 123. Experiments were performed as described in the legend to Fig. 4, except that the concentration of CCCP was varied as indicated. ■, Photomultiplier tube (PMT) voltage 750 V, logarithmic amplification; □, PMT voltage 650 V, logarithmic amplification; ●, PMT voltage 700 V, linear amplification (× 16). The peak channels referred to are those of the fluorescence detector

varied. It may be observed (i) that the peak channel number for Rh 123 fluorescence depends significantly upon the PMT voltage, (ii) that it is possible to titrate a population of cells and gradually to de-energize it in a manner that is manifest as a decreasing extent of Rh 123 accumulation, 'full' deenergization serving to indicate the channel number corresponding to no (negligible) accumulative uptake of Rh 123, and (iii) logarithmic amplification allows a much better discrimination of the extent of cell energization than does linear amplification.

As it was clear that the extent of fluorescence of a cell stained with Rh 123 depended strongly on its level of membrane energization, it was of interest to study the relationship between cell viability and the accumulation of Rh 123. To this end, we starved a sample of cells, grown to stationary phase in batch culture in lactate minimal medium, and kept in the culture medium at 4°C for 1 month until their viability, as judged by plate counts, had decreased to $7 \pm 2\%$. They were stained in the usual way and studied by flow cytometry. The results are shown in Fig. 6a. A similar sample, taken from the mid-exponential phase of a batch culture (lactate minimal medium) was stained in the same way (Fig. 6b). It is clear that most of the starved, non-viable cells exhibit a negligible accumulation of Rh 123 compared with that of the viable cells, although the percentage of cells exhibiting fluorescence in a channel number greater than 19 is in this case 22% (Fig. 6a). Populations of these two types of cells were then mixed to give a

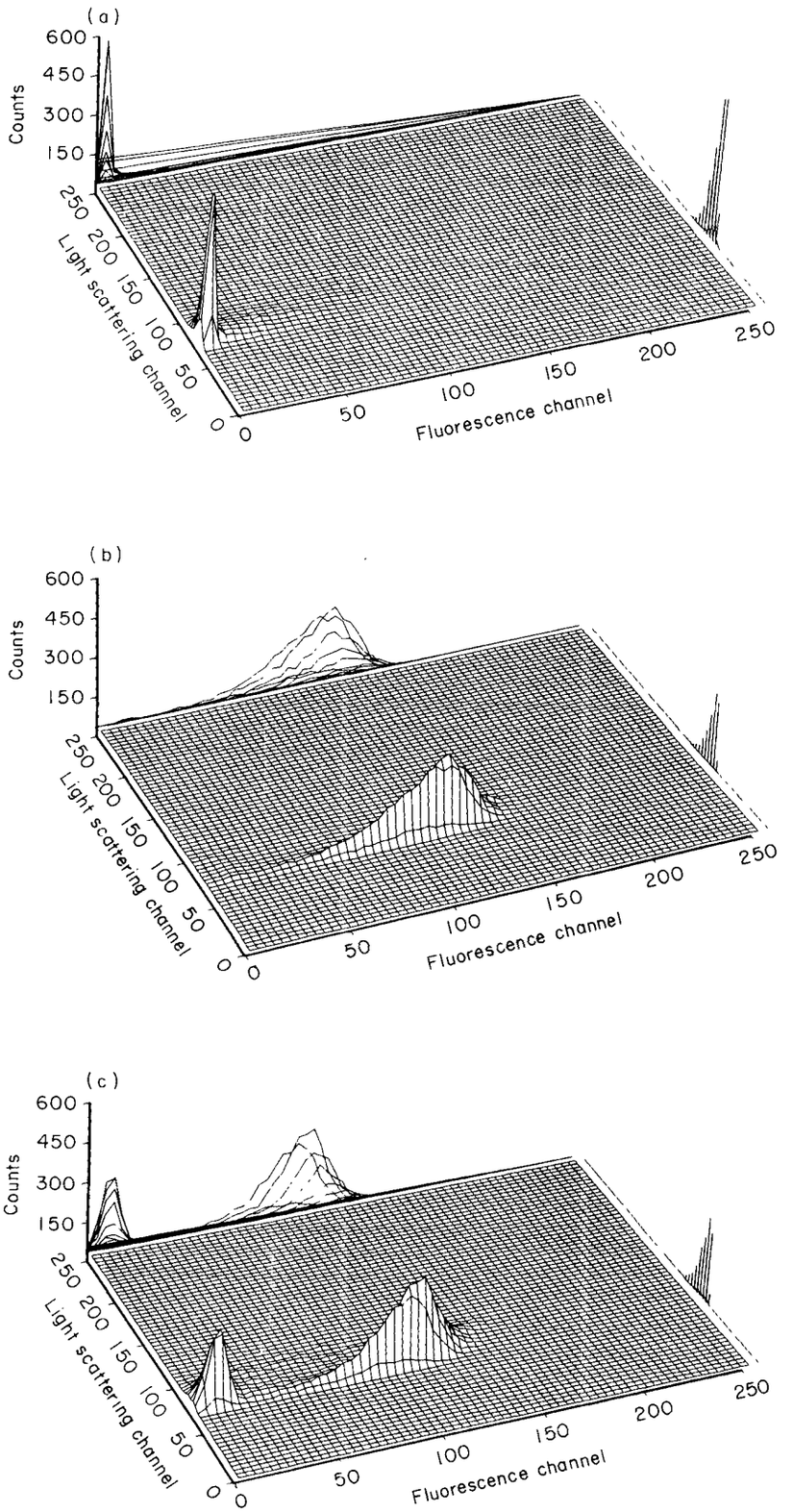

Fig. 6 Flow cytometric assessment of viable and non-viable cells and their mixtures, based on their ability to accumulate rhodamine 123. (a) *Micrococcus luteus* cells were grown to stationary phase in batch culture in lactate minimal medium and starved by storage at 4°C for 1 month. They were then stained with Rh 123 and measured flow cytometrically as described in the text and in the legend to Fig. 4. (b) Cells were grown, taken from the mid-logarithmic phase of the culture, and immediately stained as in (a). (c) Cells from (a) and (b) were mixed in the ratio 1:2 and treated as in (a) and (b)

viable:non-viable ratio of 2 : 1 and then stained. Flow cytometry revealed (Fig. 6c) two peaks of fluorescence whose areas were in the expected ratio. By varying the ratio of the numbers of the two types of cell it was possible to show (Fig. 7) that the flow cytometric behaviour gave a rapid measure of the percentage of 'viable' cells in a population consisting of a mixture of '100% viable' and '5% viable' cells. It is noteworthy, however (Fig. 7), that Rh 123 gave an apparent overestimate of the viability in proportion to the overestimate observable in Fig. 6b.

'Viable' cultures were given a number of treatments that were expected to decrease their viability, including starvation, cycles of freezing and thawing, and the addition of the cytotoxic organic solvent octan-1-ol (see Stoicheva *et al.* 1989). In all cases, the correlation between cell viability as judged by plate counts and the ability to accumulate Rh 123 to give fluorescence in a channel number greater than 19 was excellent and similar to that in Fig. 7: Fig. 8 shows results for both starving cells and those subjected to freeze-thaw cycles. In this case, the line of best fit extrapolates to an apparent viability on the ordinate of some 30%.

As described above, cells can exist in physiological states in which although they will not form colonies on nutrient agar plates they are not 'dead', in that they can be resuscitated by the addition of suitable nutrients. Figure 9 shows an experiment in which cells from a C-limited chemostat culture grown at a very low dilution rate (0·01/h), with an initial 'viability' of some 40%, were resuscitated in lactate minimal medium. Over a period of some 9 h the viability increased to nearly 90%, with no increase in either the total

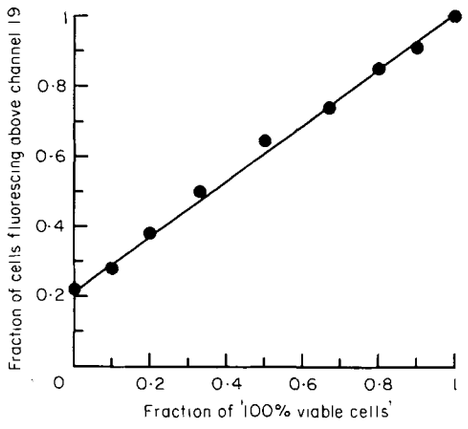

Fig. 7 Effect of cell viability on the flow cytometric properties of cells stained with rhodamine 123. *Micrococcus luteus* cells were prepared and treated exactly as described in the legend to Fig. 6, save that the ratio of 'viable' to 'non-viable' cells was varied as indicated. The ordinate shows the fraction of cells whose fluorescence occurred in channel numbers greater than channel 19 (●)

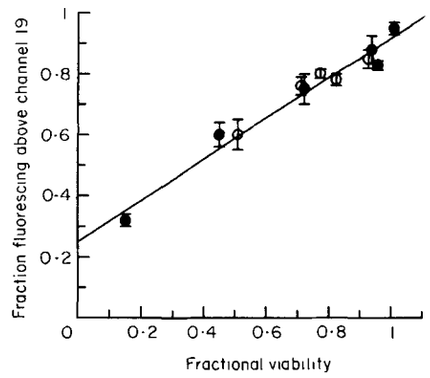

Fig. 8 Effect of starvation and freeze-thaw cycles on the viability and flow cytometric behaviour of a population of *Micrococcus luteus*. Cells were grown in rich medium in batch culture, harvested and resuspended in a buffer consisting of lactate minimal medium lacking both lactate and NH_4Cl and stored. Storage was either at room temperature (for starvation), over a period of 15 days, or cells were subjected to a number of freeze-thaw cycles (up to 15) in which they were normally held in a freezer (at −30°C) but thawed and refrozen every 2 or 3 days. Appropriate samples were taken and their viability assessed both by plating and by flow cytometry, exactly as described in the legend to Fig. 7, except that the medium for flow cytometry also contained 10 mmol/l L-malate. ○, Starved cells; ●, frozen/thawed cells

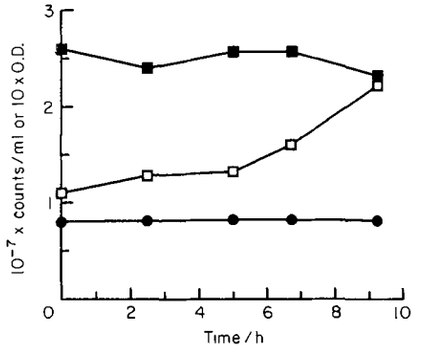

Fig. 9 The resuscitation of *Micrococcus luteus*. Cells were grown in a C-limited chemostat (D = 0·01/h for nine volume changes), harvested and washed once in and resuspended to an optical density of 0·08 in lactate minimal medium and shaken at 30°C at 200 rev/min. At the points indicated, samples were taken for direct and total counts and for optical density measurements. ■, Total counts; □, viable counts; ●, optical density

cell numbers or the optical density of the sample. Samples were also taken, stained with Rh 123 in the usual way, and assessed by flow cytometry; representative results are shown in Fig. 10. It is particularly noteworthy that cells present in the time zero sample (Fig. 10a) which possess a

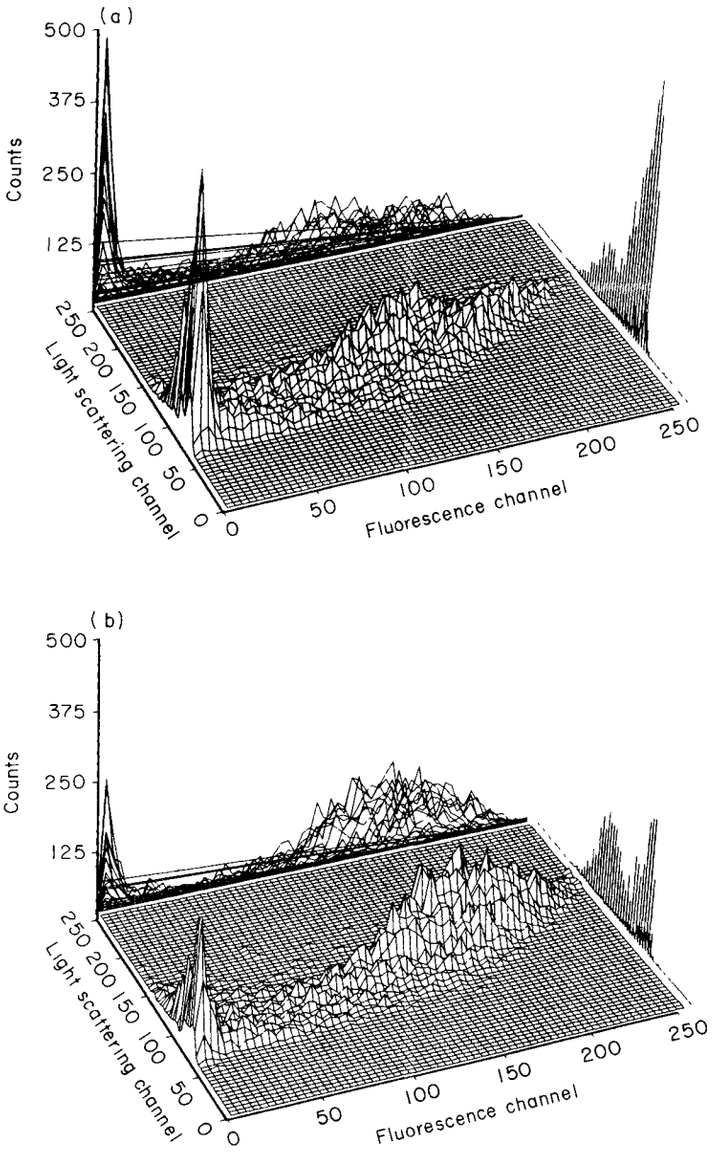

Fig. 10 Flow cytometric behaviour of *Micrococcus luteus* during resuscitation. Cells were taken from the experiment described in Fig. 9 at time zero (a) and at 9·2 h (b), stained with rhodamine 123 and assessed flow cytometrically as described in the legend to Fig. 4, except that the photomultiplier tube voltages for the light-scattering and fluorescence channels were 600 V and 750 V, respectively

fluorescence below a channel number of approximately 90 have greatly increased their ability to accumulate Rh 123 after 9 h of resuscitation (Fig. 10b). From the flow cytometric light-scattering results it may be observed that these cells were amongst the smallest cells in the population. So far as we are aware, this constitutes the first observation of resuscitation in chemostat-grown cells. It is obviously tempting to conclude that the cells with 'intermediate' levels of fluorescence represented cells which were 'viable-but-non-culturable', but which could be resuscitated after incubation with the appropriate nutrients. Figure 11 shows results which support the view that the fluorescence properties of different cohorts of cells rather accurately reflect their behaviour on plates, and indicate a clear reflection of the decrease 'in cell numbers with 'intermediate' fluorescence and their appearance as both viable cells and cells capable of accumulating significantly greater concentrations of Rh 123.

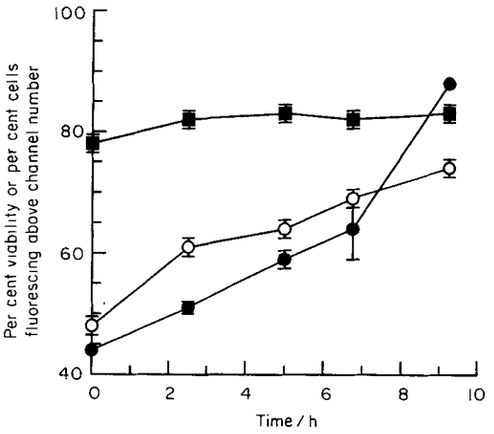

Fig. 11 Relationship between percentage viability and flow cytometric behaviour of *Micrococcus luteus*. The experiment was performed as described in the legend to Figs 9 and 10. The ordinate displays the percentage of viable cells (●) (determined by plating and taking the total counts to be average of those in Fig. 9) and the percentages of cells whose fluorescence lay (■) between channels 14 and 256, and (○) between channels 110 and 256

Although its polarity indicates that Rh 123 should be accumulated in the aqueous cytoplasm of the cells, flow cytometry cannot distinguish whether there are selective sites of accumulation of the dye. Figure 12 therefore shows both phase-contrast and fluorescence micrographs of a sample of cells taken directly from a C-limited chemostat (D = 0·01/h), indicating that the accumulation of the dye in what is a culture grown in a nominally homogeneous chemostat is quite heterogeneous both between the cells (as observed flow cytometrically in Fig. 10a) and within cells. This is in agreement with the observations of Smiley *et al.* (1991), who observed heterogeneity within individual mitochondria of the uptake of a J-aggregate-forming cationic dye.

Finally, to indicate the present method can be applied to Gram-negative as well as to Gram-positive organisms, cultures of *E. coli* were tested. We found (not shown), as did Matsuyama (1984), that batch-grown cultures of *E. coli* were not well stained with Rh 123. However, treatment of the cells with Tris-EDTA, which permeabilizes their outer membrane (Leive 1965; Nikaido & Vaara 1985), permitted good staining with Rh 123. Figure 13 shows a typical flow cytometric analysis of the fluorescence of these cells. Thus, as also pointed out by Shapiro (1990), Gram-positive and Gram-negative bacteria can be distinguished by the ability of Tris-EDTA treatments to allow their staining by fluorescent dye molecules.

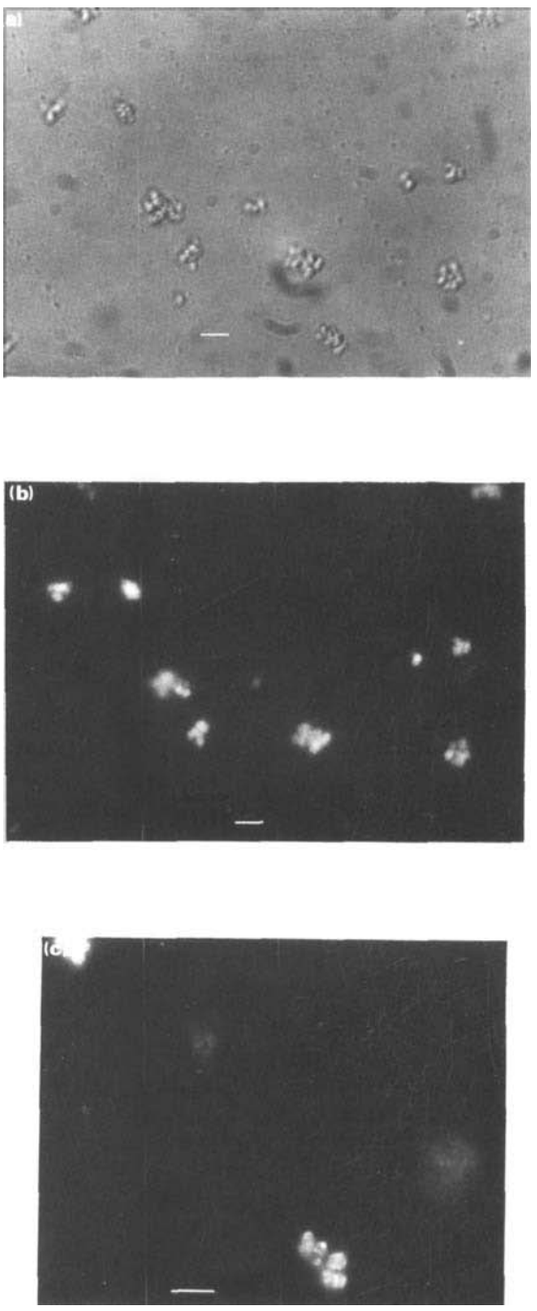

Fig. 12 Microscopic appearance of *Micrococcus luteus* cells stained with Rh 123. Cells were grown in C-limited chemostat culture as described in the text, diluted five-fold in lactate minimal medium and stained with Rh 123 at a final concentration of 0·013 μmol/l. Their appearance was determined with (a) phase-contrast and (b) and (c) epi-fluorescent optics. (a) and (b) represent the same view, to illustrate the heterogeneity in Rh 123 fluorescence observable between cells, whilst (c) represents a close-up view to illustrate the heterogeneity of staining observable within different cells. The bars represent 5 μm

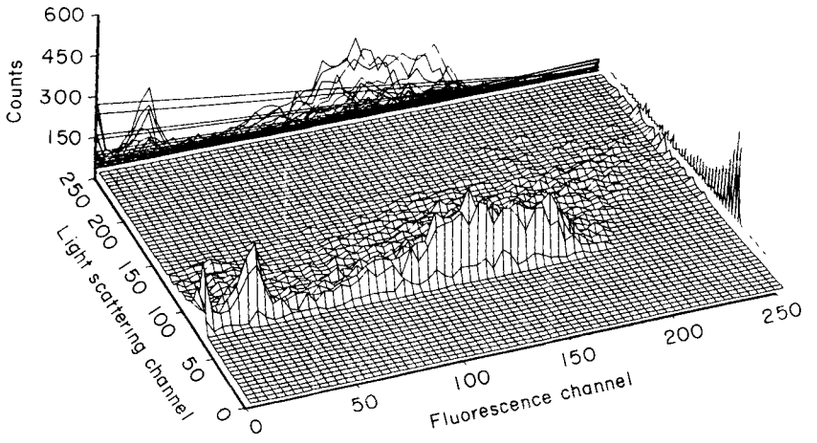

Fig. 13 Flow cytometric behaviour of *Escherichia coli* stained with rhodamine 123. Cells were grown in rich medium, harvested and washed three times in a buffer consisting of 50 mmol/l Tris chloride containing 5 mmol/l disodium EDTA, pH 8·0. The cells were then washed and resuspended in lactate minimal medium containing 10 mmol/l L-malate, and stained in the usual way. Flow cytometry was performed as described in the legend to Fig. 4, except that the photomultiplier tube voltages for the light-scattering and fluorescence channels were respectively 500 V and 700 V

DISCUSSION

It has been shown that rhodamine 123 is concentrated and accumulated by *Mic. luteus* in an uncoupler-sensitive fashion. Provided the added dye concentration was below approximately 1 μmol/l, the uptake as judged by steady-state fluorescence measurements was fully uncoupler-sensitive, and was not accompanied by the significant amount of self-quenching which occurs when high concentrations of the dye are allowed to accumulate intracellularly. This permitted the extent of accumulation of the dye by an individual cell to be assessed quantitatively, using flow cytometry.

As expected, viable and non-viable cells could be rapidly distinguished in a flow cytometer by the extent to which they accumulate the dye, and there was an excellent correlation between the percentage of viable cells in a mixture of '100% viable' and '95% non-viable' cells (Figs 6 and 7). The same was true in populations of cells stressed by starvation or freezing and thawing (Fig. 8), or by exposure to an organic solvent. However, although the correlation between the two methods was good, the flow cytometric method tended to overestimate the viability by some 20%. This suggested the presence in these populations of cells which, although they could effect the accumulation of Rh 123 to a certain degree, still could not form colonies on plates without resuscitation.

It is well known that the viability of a chemostat culture of (copiotrophic) cells tends to decrease with decreasing dilution rate (Sinclair & Topiwala 1970). Indeed, we found that the viability of a very slowly growing chemostat culture of *Mic. luteus* was only some 40–50%, as judged by plate counts. A similar observation was made with *Klebsiella aerogenes* grown at a slightly lower dilution rate (Tempest *et al.* 1967). In the present case, however, we found that most of the apparently 'non-viable' cells could in fact be resuscitated by incubation of the culture in an appropriate nutrient medium before plating them out. Thus care must be taken in seeking to confine the possible states of a cell in a chemostat to a two-valued logic system (alive/dead). We believe this to be the first demonstration that chemostat-grown cells of a low apparent viability can be resuscitated.

Several workers have studied the decline in ATP, adenylate energy charge, and/or the ability to accumulate lipophilic cations in starving cells or in cells grown at low dilution rates (Horan *et al.* 1981; Jones & Rhodes-Roberts 1981; Zychlinski & Matin 1983; Otto *et al.* 1985; Poolman *et al.* 1987), generally finding that none of these bioenergetic parameters could be correlated with the loss of viability (as judged by plate counts). The great problem with these types of measurements, however, is that they represent bulk or ensemble measurements; it is not possible therefore to distinguish whether a decrease in ATP levels or in the uptake of the tetraphenylphosphonium cation, say, is due to the irreversible death of a proportion of the cells or an identical decrease of these parameters, unaccompanied by death, in all cells (or of course any combination of these extreme possibilities (Kell 1988)). In the present work, we have shown (Fig. 10) that cells grown in a chemostat at a very low dilution rate (and even those grown in batch culture at μ_{max} (Fig. 4a)) are extremely heterogeneous with respect to their ability to accumulate the lipophilic dye

Rh 123, and that their viability, and resuscitation, could be quite well correlated with their ability to accumulate the dye. Indeed, it was possible in part to relate the degree to which individual cells accumulated Rh 123 and the distinguishable physiological states ('viable', 'non-viable' and 'non-viable but resuscitable') exhibited by cells in the culture (Fig. 11). Thus flow cytometry of cells stained with Rh 123 allows one rapidly to distinguish not only 'viable' and 'non-viable' cells but the degree of viability of individual cells reflecting the heterogeneity of a culture observable following sublethal starvation, stress or injury.

Gram-negative bacteria do not accumulate rhodamine 123 (nor other dyes such as hexamethylindodicarbocyanine (Shapiro 1990) or 1-*N*-phenylnaphthylamine (Tsuchido *et al.* 1989)) because these dyes do not rapidly cross the outer membrane. However, a simple treatment with Tris and EDTA at alkaline pH largely overcomes this (Leive 1965; Nikaido & Vaara 1985) (Fig. 13). We conclude that flow cytometry of cells stained with rhodamine 123 constitutes a powerful and convenient method for the rapid assessment of bacterial viability and vitality.

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