Flow cytometric determinations of cellular substances in algae, bacteria, moulds and yeasts

K.-J. HUTTER AND H. E. EIPEL

Fraunhofer-Gesellschaft, Institut für Aerobiologie, Grafschaft, D-5948 Schmallenberg, W. Germany and Biophysics Systems GmbH, D-6140 Bensheim 3, W. Germany

HUTTER, K. -J. and EIPEL, H. E. 1978. Flow cytometric determinations of cellular substances in algae, bacteria, moulds and yeasts. Antonie van Leeuwenhoek **44**: 269–282.

The practical use of flow cytometry is shown in several microbial assays. Recent technical improvements in the optics and electronics of flow cytometric systems as well as in staining techniques permit the measurements of minute cellular components such as the cellular DNA and the protein content of bacteria, algae, moulds and yeasts. Single cell ingredients can be measured by this assay according to their specific stainability. The cell DNA was stained by propidium iodide while the cell protein was fluorochromed by fluorescein-iso-thiocyanate. The DNA synthesis of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* runs discontinuously while the protein content increases continuously during the vegetative growth. The different stages of DNA synthesis of yeast cells can be divided into two 'gap' phases, a synthesis and a mitosis period, corresponding to Howard and Pelc's model of DNA synthesis. Living and dead cells can be counted differentially after staining with Erythrosine B. The red fluorescence of the chlorophyll in algae can readily be used to determine the chlorophyll content of these cells.

INTRODUCTION

The flow cytometric method was extensively used to determine intracellular substances (DNA, RNA, proteins and enzymes) of mammalian cells (Göhde and Dittrich, 1970; Berkhan, 1972; Darzynkiewicz et al., 1975; Kaplow et al., 1976).

Within the scope of microbiology the first measurements of the RNA and protein content of yeasts were performed by using an Impulscytophotometer (Hutter et al., 1975). Through recent technical improvements in the optical and electronic sections of flow cytometer apparatus, determinations of such minute amounts of substances as DNA in bacteria and yeast cells became possible (Paau et al., 1977*a*, 1977*b*; Slater et al., 1977; Ortho Instruments, 1977; Hutter and Eipel

1978*a*). Paau et al. (1977*a*, 1977*b*) measured the nucleic acid content in populations of free-living and symbiotic *Rhizobium meliloti* by flow microfluorometry. Their results show that a population of symbiotic *Rhizobium meliloti* contains a significant number of bacteroids with a higher nucleic acid content than free-living rhizobia. Cell cycle studies at different stages of the vegetative growth of *Saccharomyces cerevisiae*, based on DNA determinations, were carried out by Slater et al. (1977) and Hutter and Eipel (1978*a*). Employing immunological fluorescent staining with fluorescein-iso-thiocyanate diverse yeast strains can be distinguished by flow cytometry (Hutter et al., 1978*a*; Ortho Instruments, 1978).

The rapidity of flow cytometric measurements guarantees exact determinations in large populations. several parameters, such as cell size, number of cells and cellular ingredients, may be studied according to their specific stainability. The aim of this investigation is to test the usefulness of flow cytometry in microbiological assays.

MATERIALS AND METHODS

Microorganisms. The alga *Chlorella kessleri* 8k was obtained from Prof. Dr. Kessler (Botanisches Institut, Universität Erlangen). Subcultures of *Escherichia coli*, *Saccharomyces cerevisiae* 211, *Saccharomyces pastorianus* and *Schizosaccharomyces pombe* were donated by Prof. Dr. Emeis (Institut für angewandte Biologie, RWTH Aachen). Baker's yeast (*Saccharomyces cerevisiae*) was commercially available (Monheimer Presshefe- und Spritwerke GmbH, 4019 Monheim). The mould, *Nectria coccinea* Pers. ex Fr. was obtained from the collection of Dr. Lang (Forstbotanisches Institut, 8000 München).

Cultivations. The alga, *Chlorella kessleri* 8k, was grown in 100 ml culture medium of Kessler and Czygan (1970) for 7 days at 25 C and 6000 lux in 300 ml Erlenmeyer flasks.

The bacterium, *Escherichia coli*, was cultivated in 100 ml Standard I Nutrient Broth (Merck, Art. 7882) for 24 h at 37 C in 300 ml Erlenmeyer flasks in a shaking water bath.

The yeast cells were grown in 100 ml YPG-solution [1% yeast extract (Difco, 0127–01), 2% bacto peptone (Difco, 0118–01) and 2% glucose in distilled water] for three days at 28 C in 300 ml Erlenmeyer flasks.

For determinations of different stages of DNA synthesis during the cell cycle a small quantity of dry baker's yeast stored at 4 C was dispersed in 500 ml YPG-solution and shaken in a water bath at 28 C. The cell density amounted to $8.8 \cdot 10^5$ cells/ml at the start of the growth. Every 15 min a sample of 1 ml of the cell suspension was transferred into a test tube. The cells were washed in 0.18 M tris buffer, pH 7.5 and finally fixed in 70% ethanol.

The mould, *Nectria coccinea*, was incubated at 25 C for five days in a medium containing 3% malt extract (Merck, Art. 5391) and 0.2% yeast extract (Difco,

0127–01) in distilled water. The growth of these microorganisms was filamentous and the cell suspension was therefore filtered through a polyamide sieve (Vereinigte Seidenwebereien AG, Speefeld 7, 4152 Krefeld) with a mesh size of 65 μm (Hutter et al., 1978*b*).

Staining procedures. For the determination of the DNA content the cells were fixed in 10 ml 70% ethanol for 1 h. The cells were washed in 0.2 M tris buffer, pH 7.5. Then, the cells were incubated in 10 ml 0.1% RNase (Serva, 34390) in 0.2 M tris buffer, pH 7.5, for one h at 37 C. The RNase solution was removed by centrifugation at 3000 × *g*. The yeast cells were resuspended in 2 ml pepsin solution (5.5 ml 1 N HCl + 0.5 g pepsin + 94.5 ml distilled water) for 5 min at room temperature. Without centrifugation, the cells were resuspended in 5 ml propidium iodide solution (5 mg propidium iodide in 100 ml 0.18 M tris buffer, pH 7.5, and stained for 1 h.

For determination of the protein content, the yeast cells were fixed (see above) and then fluorochromed overnight in a fluorescein-iso-thiocyanate solution containing 0.03 mg fluorescein-iso-thiocyanate (Merck, Art. 24546)/ml in 0.18 M tris buffer of pH 7.5. Following centrifugation at 3000 × *g*, the staining solution was discarded and the cells were washed three times in 0.18 M tris buffer, pH 7.5 (Hutter and Eipel, 1978*a*, 1978*b*).

The chlorophyll content of *Chlorella kessleri* 8 k was measured by flow cytometry after washing the cells twice in 0.18 M tris buffer, pH 7.5 for 10 min.

Two parameter analysis. For two parameter analysis, *Saccharomyces cerevisiae* 211 was cultivated for 24 h at 28 C in YPG-solution. After a fixation in 70% ethanol for one h, the yeast cells were treated in a 0.1% RNase solution (see staining procedures). Subsequently the protein content was stained with 0,03 μg fluorescein-iso-thiocyanate/ml 0.18 M tris buffer of pH 7.5 for 30 min at room temperature. After centrifugation (3000 × *g*) and rinsing in tris buffer the DNA content was fluorochromed for 20 min in propidium iodide solution (see staining procedures) in an ice bath. The dye solution was discarded after centrifugation and the cells were resuspended in 2 ml 0.18 M tris buffer of pH 7.5. The cells were then measured by flow cytometry.

Viability test. After 24 hours' growth of *Saccharomyces cerevisiae* 211, the cells were washed twice in 0.18 M tris buffer, pH 7.5 and resuspended in 1 ml phosphate-buffered saline (PBS) (1.02% Na_2HPO_4 and 0.437% $NaH_2PO_4.2\,H_2O$ in 0.9% NaCl solution adjusted to pH 7.6). The first sample of the yeast cells was fixed in 70% ethanol for one h, finally washed twice in distilled water and resuspended in 1 ml PBS. The second sample consisted of one part of living cells and one part of fixed cells. The viability of the yeast populations was tested after staining in an Erythrosine B solution (Gurr, 1971). The stock solution was prepared by dissolving 400 mg of Erythrosine B in 100 ml PBS. Two min before measurement, 50 μl of stock solution of Erythrosine B was added to the three samples (Munch et al., 1971).

Flow cytometry. The single cellular components were measured with the CYTO-

FLUOROGRAF FC–200–50 (Ortho Instruments, Westwood, Mass., USA). The apparatus was equipped with a 50 mW argon laser. The excitation wavelength was selected at 514.5 nm for propidium iodide (DNA-staining) and at 488 nm for fluorescein-iso-thiocyanate (protein-staining).

A dichroic mirror with a dividing edge at 570 nm was inserted into the fluorescence analysis path to separate green and red fluorescence. For propidium iodide measurements, a 590 nm barrier filter was used in front of the red channel photomultiplier. A special interference filter with a band pass from 520 nm to 540 nm was used for fluorescein-iso-thiocyanate (Hutter and Eipel, 1978*a*, 1978*b*). The chlorophyll content of algae was measured with an Impulscytophotometer (Phywe AG, 3400 Göttingen). For this measurement a barrier filter OG 630 was used.

Photography. The photographs of the cytograms and histograms were made with a polaroid camera and a black and white polaroid film (Type 107 Land Film) was used.

RESULTS AND DISCUSSION

Fluorescence microscopy has become an important tool for microbiological analysis, especially by Strugger's pioneer work (1949). The strength of the statements based on fluorochromed microorganisms was, however, poor, due to the lack of instrumentation for quantitative determinations.

Quantitative cytophotometry introduced a change in this field. To avoid time-consuming single-cell measurements on slides, rapid flow through systems have been developed which allow the analysis of more than 1000 cells per second (Kamentsky et al., 1965; Dittrich and Göhde, 1969). The time needed to measure the fluorescence of single-cell ingredients according to their specific stainability is in the order of 5.10^{-6} s only.

By introduction of flow cytometry, quantities of cellular compounds of mammalian cells in the order of 10^{-12} g could be accurately measured (Kamentsky et al., 1965; van Dilla et al., 1969; Dittrich and Göhde, 1969; Göhde and Dittrich, 1970; Sprenger et al., 1971; van Dilla et al., 1976).

The measurements of DNA content were performed with *Escherichia coli* (Fig. 1a), *Chlorella kessleri* 8k (Fig. 1b), *Nectria coccinea* Pers. ex Fr. (Fig. 1c), *Saccharomyces cerevisiae* 211 (Fig. 1d), baker's yeast (Fig. 1e) and *Saccharomyces pastorianus* (Fig. 1f). Each DNA measurement in Fig. 1 consists of a cytogram (with exception of Fig. 1b) and a corresponding histogram superimposed on each other.

The cytograms (consisting of clouds of dots; each cell being represented by one dot) show the cell size on the vertical axis and the amount of fluorescence on the horizontal axis. The superimposed histograms (punctuated continuous line) show the number of cells versus the amount of fluorescence. The cytograms show the

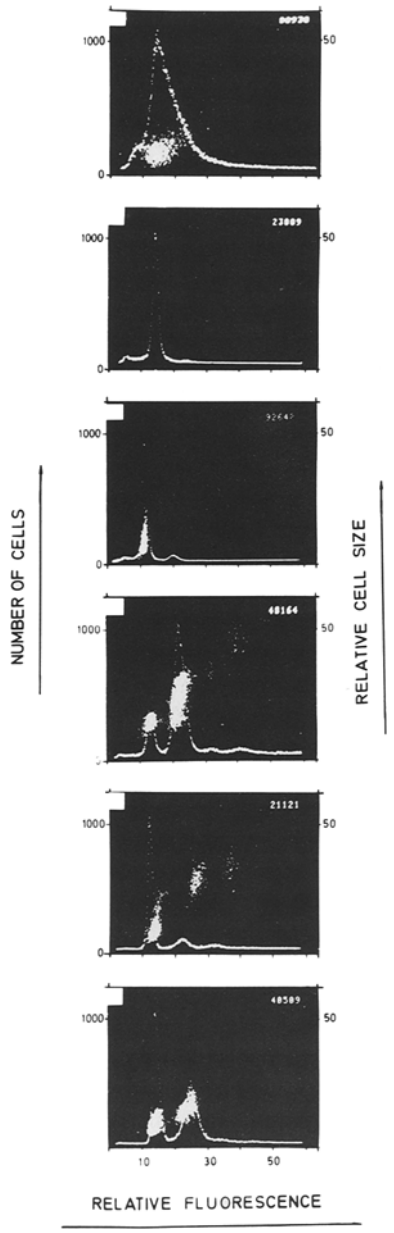

Fig. 1. Flow cytometric measurements of the DNA content of *Escherichia coli* (a), *Chlorella kessleri* 8k (b), *Nectria coccinea* Pers. ex Fr. (c), *Saccharomyces cerevisiae* 211 (d), Baker's yeast (e) and *Saccharomyces pastorianus* (f).

results from about 5000 measured cells. The number of measured cells of the histograms amounts to about 80000 (bacterium), 23000 (alga), 92000 (mould), 48000 (*Saccharomyces cerevisiae* 211), 20000 (baker's yeast) and 40000 (*Saccharomyces pastorianus*) (Fig. 1a–1f).

While the DNA histograms of the bacterium, the alga and the mould show a single-peak-distribution, the histograms of the yeasts consist of two or more peaks, which represent the two-fold, three-fold and four-fold DNA content as compared with the first peak. This DNA distribution corresponds to the statement of Howard and Pelc (1953) which separate the cell cycle into four periods, i.e. G1, S, G2 and M phases. The DNA replication occurs in the S phase, mitosis in the

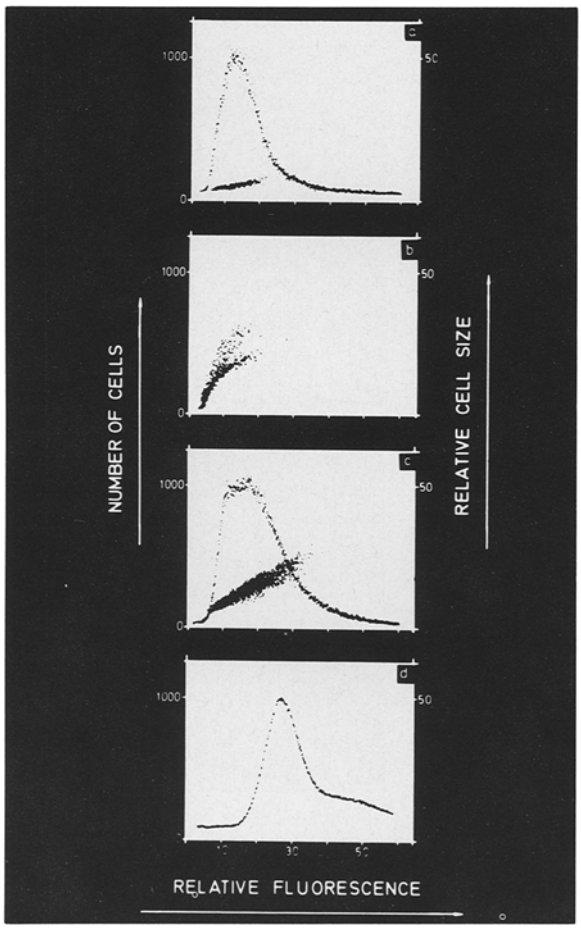

Fig. 2. Flow cytometric measurements of the protein content of *Escherichia coli* (a), *Chlorella kessleri* 8k (b), *Saccharomyces pastorianus* (c) and baker's yeast (d).

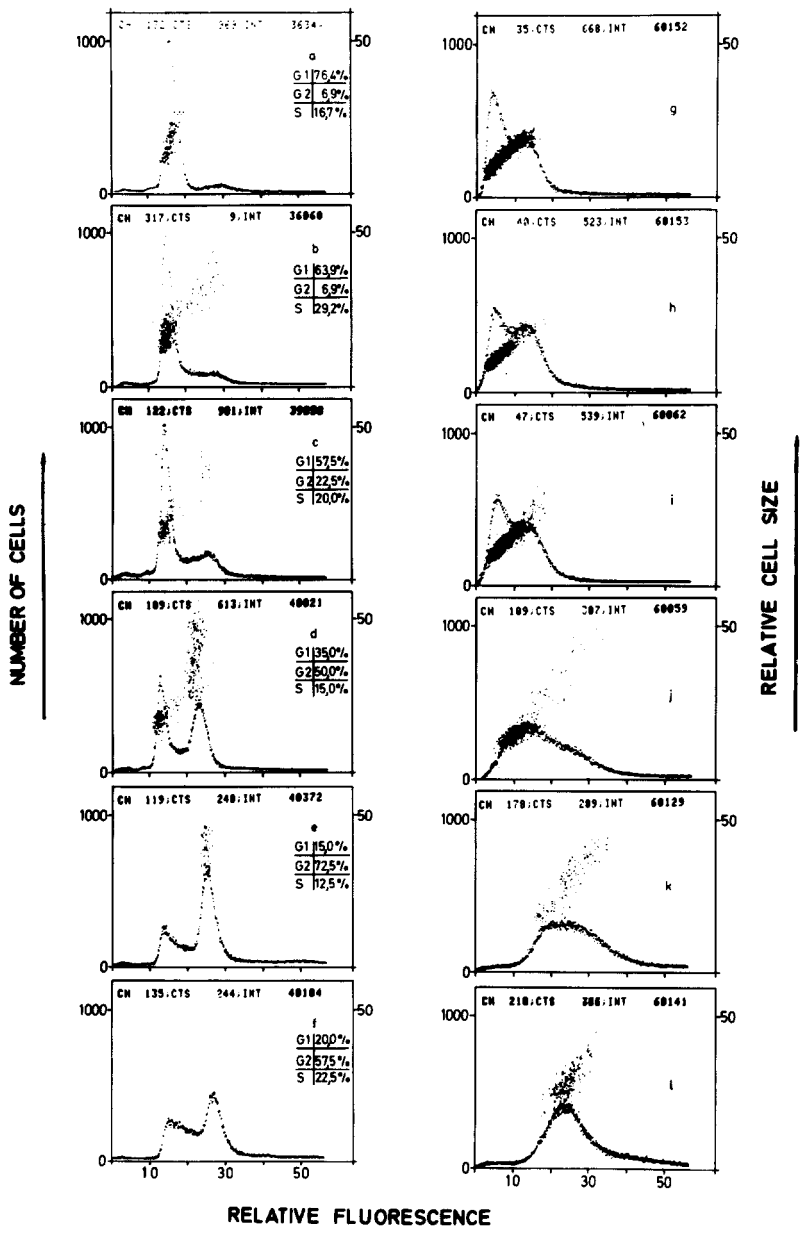

Fig. 3. DNA (a–f) and protein (g–l) distributions of baker's yeast at different stages of cell cycle. Samples were taken after 15, 30, 45, 60, 120 and 150 min of growth.

M phase, while the G1 and G2 phases are 'gap' periods. The discontinuous DNA synthesis cannot be confirmed by means of the histograms of the measured bacterium, alga and mould cells.

The distributions in Fig. 2 show cytograms and histograms (with exception of Fig. 2d) of the protein content of *Escherichia coli* (Fig. 2a), *Chlorella kessleri* 8k (Fig. 2b), *Saccharomyces pastorianus* (Fig. 2c) and Baker's yeast (Fig. 2d). The cytograms of Fig. 2 show the results from 5000 measured cells. The corresponding histograms give the results from about 70000 to 80000 cells. The protein content of these microorganisms increases continuously during the cell cycle.

The different stages in DNA and protein content during the growth of baker's yeast are demonstrated by the cytograms and histograms of Fig. 3. The cytograms again show the results from 5000 measured cells while the histograms of DNA and protein content give the results from about 36000 to 40000 cells and about 60000 cells respectively. The six distributions of DNA content and the corresponding protein content were obtained from samples taken after 15, 30, 45, 60, 120 and 150 min of growth in nutrient broth.

After 15 min of growth in YPG-solution, the DNA content of the yeast cells consists of two distinct peaks (Fig. 3a). Although appearing uniform under the microscope, some cells start earlier than others with the cell cycle. Under the first peak 76,4% appear with an approximately homogenous DNA content (G1 phase). About 6.94% are located under the second peak representing the G2 period with twice the DNA content as compared with the location of the G1 peak. The rest of the population consists of S-phase cells (16.7%). At this phase of growth, only a few yeast cells synthesize DNA. After 30, 45 and 60 min of growth, the DNA replication starts vigorously. That means that more and more cells are found in the S phase and under the second peak (Fig. 3b–3d). This statement becomes most apparent in the histogram shown in Fig. 3e, obtained after 120 min of growth. About 72.5% of the yeast cells have replicated their DNA and arrived in the G2 phase. In the G1 phase 15% and in the S phase 12.5% of the cells are found. At this stage of the cell cycle most of the baker's yeast cells possess buds. The end of the G2 phase is marked by cell membrane separation. This event completes the cycle with the production of two unbudded cells (Mitchison, 1971). Cell division again produces more cells with an uniform low DNA content. The return to the G1 phase of the cells is shown in fig. 3f after 150 min of growth.

In Fig. 3g – 3l the protein distributions do not show separate peaks in comparison with the differentiated peaks in the corresponding DNA distribution.

Biochemical studies indicate that cellular components may be synthesized either discontinuously or continuously. While the DNA content of *Saccharomyces* cells is synthesized periodically (Halvorson et al., 1971; Duffus, 1971; Hartwell, 1974; Smith and Berry, 1974), the protein content is synthesized continuously during the cell cycle (Williamson and Scopes, 1961; Williamson, 1965).

The chlorophyll content of *Chlorella kessleri* 8k was measured without any cell fixation and staining. The algal cells were only washed twice in 0.18 M tris buffer of

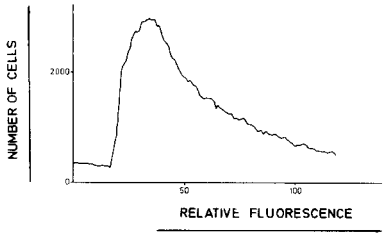

Fig. 4. The histogram of the chlorophyll content of *Chlorella kessleri* 8k was obtained by measurement with an Impulscytophotometer.

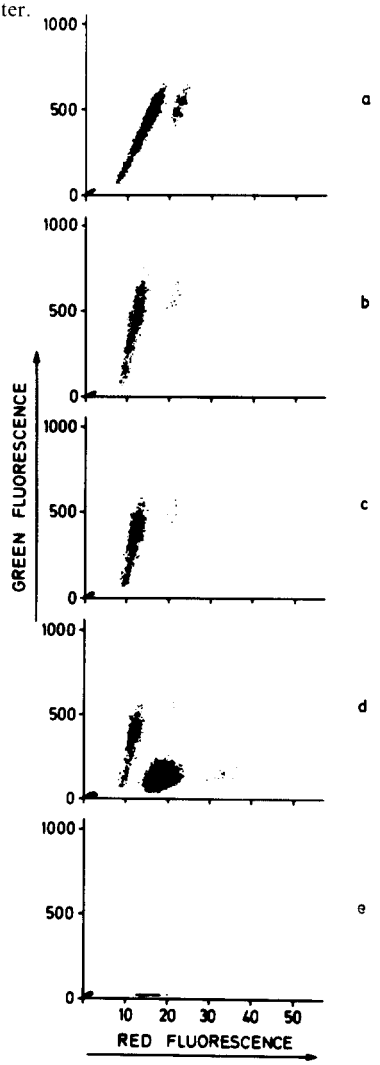

Fig. 5. Simultaneous measurements of DNA and protein content of *Saccharomyces cerevisiae*. Red (DNA) fluorescence is shown in the horizontal direction; green (protein) fluorescence is recorded in the vertical direction. (a) 3 μg FITC/ml tris buffer, (b) 0.15 μg FITC/ml tris buffer, (c) 0.03 μg/FITC ml tris buffer, (d) the two clusters at the bottom were obtained with 514.5 nm excitation and all other conditions like (c) remaining constant, (e) control without fluorescein-iso-thiocyanat.

pH 7.5 before introducing them into the flow cytometric system (Fig. 4). The results of these measurements of algae prove that this method permits the determination of chlorophyll on the living object.

The cytograms Fig. 5a–5e show *Saccharomyces cerevisiae* cells after double-staining with fluorescein-iso-thiocyanate for proteins and with propidium iodide for DNA. The amount of green fluorescence, corresponding to the protein content, is given on the vertical axis; the amount of red fluorescence, corresponding to the DNA content, is shown on the horizontal axis of the cytograms.

Fig. 5a shows the result obtained with a fluorescein-iso-thiocyanate concentration of 3 μg/ml 0.18 M tris buffer, pH 7.5, which is 1/10 of the concentration used for mammalian cells with a much higher protein/DNA ratio than the yeast cells. Ideally, the two clouds which represent the G1 and G2 + M populations, should stand exactly vertical. The inclination is caused by spurious emission of the fluorescein-iso-thiocyanate in the red region of the spectrum. This emission is added to the red DNA fluorescence, resulting in a shift to the right of those cells

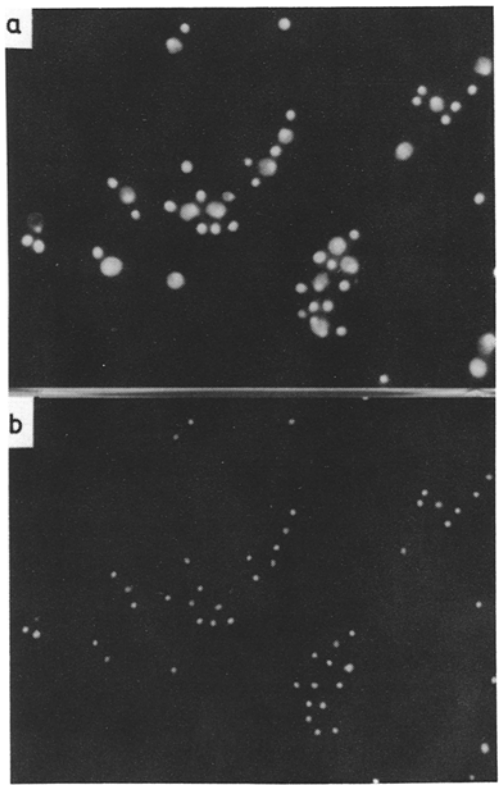

Fig. 6. Simultaneous staining of DNA and protein in yeast cells. (a) shows the green fluorescence (protein), (b) shows the red fluorescence (DNA).

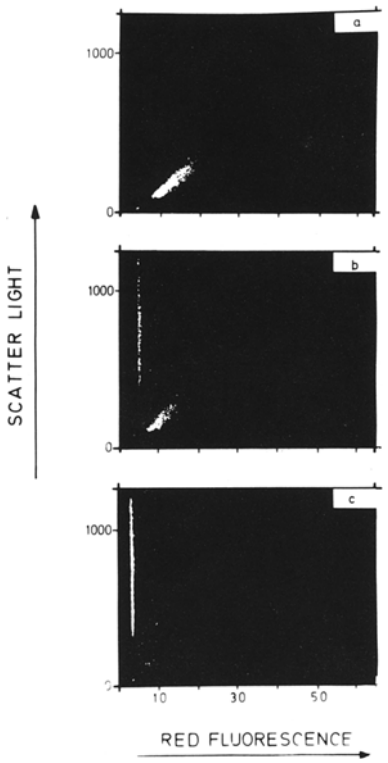

Fig. 7. The cytograms show a flow cytometric measurement of the viability of baker's yeast. Only damaged cells are shown in Fig. a, a mixture of living and dead cells is shown in Fig. b. Fig. c shows fresh cells with very few dead cells.

with a high protein content. Therefore, the fluorescein-iso-thiocyanate concentration was reduced further to 0.15 μg/ml tris buffer (Fig. 5b) and 0.03 μg/ml tris buffer (Fig. 5c). The measurement with 514.5 nm excitation is superimposed on a cytogram obtained with the normal 488 nm excitation, and with all other parameters remaining unchanged (Fig. 5d). The big and small cluster at the bottom were obtained with 514.5 nm excitation, the other two big and small elongated clusters have been obtained with the usual 488 nm excitation. The DNA fluorescence has increased due to the optimal excitation wavelength for propidium iodide, whereas the protein fluorescence has decreased due to poor excitation of fluorescein-iso-thiocyanate. Fig. 5e shows a control with cells stained with propidium iodide only. No green fluorescence could be detected in this sample.

Fig. 6a and 6b present the *Saccharomyces cerevisiae* cells under the microscope. Fig. 6a shows the green and Fig. 6b the red fluorescence, corresponding to the protein and DNA content of the cells.

The cytograms of the viability test (Fig. 7) represent the light scatter signal on

the vertical axis. The red fluorescence of the stained dead cells is recorded on the horizontal axis. In Fig. 7a all the cells exhibit fluorescence, since this sample consisted of fixed cells. A total of 5000 cells was measured for the three cytograms. Fig. 7b shows a sample consisting of living and fixed cells mixed in a ratio of 1:1. The cytogram in Fig. 7c is recorded using fresh cells. Here, only a small fraction of the cells are stained dead cells. Most of the cells appear on the vertical axis.

Fluorochrome Erythrosine B does not penetrate intact cells, while damaged cells take up Erythrosine B and become stained with a strong red hue. The stained cells absorb the laser light at 488 nm very strongly. The light scatter signal from the living cells is stronger than the light scatter signal from the dead cells due to strong light absorption in the stained cells. Therefore, the stained dead cells appear in a lower position in the cytogram than the intact cells, in addition to their displacement to the right which is due to the red fluorescence of Erythrosine B. Stained and unstained cells can be counted separately in two counters to obtain the percentage of injured cells.

These flow cytometric measurements can be correctly interpreted only on the condition that the microbial populations consist of single cells. Microorganisms which grow in chains, pairs, clusters, tetrads, or in filamentous forms and clumps, or those which produce a mycelium or pseudomycelium cannot be measured without pretreatment. Large cell aggregates, with a diameter of more than 150 μm, may block the flow channel. The mould cells were, therefore, filtered through a polyamide sieve with a mesh size of 65 μm. The filtered suspension consists of spores, macro- and microconidia.

These investigations have shown that flow cytometry can be a very valuable assay to determine cellular ingredients of different microorganisms. The rapidity of the measurement (5.10^{-6} s to measure the fluorescence intensity of a single cell), the sensitivity of the method (indication of 10^{-14} g/cell) and the possibility for evaluation of large populations are the main advantages of flow cytometry for use in microbiological problems.

We are greatly indebted to Miss U. Punessen and Mr. Th. Götz for their skilful technical assistance.

Received 9 *March* 1978

REFERENCES

BERKHAN, E. 1972. DNS-Messung von Zellen aus Vaginalabstrichen. — Ärztl. Lab. **18**: 77–79.

DARZYNKIEWICZ, Z., TRAGANOS, F., SHARPLESS, T. and MELAMED, M. R. 1975. Conformation of RNA *in situ* as studied by acridine orange staining and automated cytofluorometry. — Exptl. Cell Res. **95**: 143–153.

VAN DILLA, M. A., TRUJILLO, T. T., MULLANEY, P. F. and COULTER, J. R. 1969. Cell microfluorometry: A method for rapid fluorescence measurement. — Science **163**: 1213–1214.

VAN DILLA, M. A., GRAY, J. W., CARRANO, A. V., MINKLER, J. L. and STEINMETZ, L. L. 1976. New directions for flow cytometry: Chromosome analysis. pp. 57–62. *In* W. Göhde, J. Schumann, Th. Bücher (eds), 2 nd International Symposium Pulse-Cytophotometry. — European Press, Ghent, Belgium.

DITTRICH, W. und GÖHDE, W. 1969. Impulscytophotometrie bei Einzelzellen in Suspension. — Z. Naturforschg. **24b**: 360–361.

DUFFUS, J. H. 1971. The cell cycle in yeast. — J. Inst. Brew. **77**: 500–508.

GÖHDE, W. und DITTRICH, W. 1970. Simultane Impulscytophotometrie des DNS- und Proteingehaltes von Tumorzellen. — Z. Anal. Chem. **252**: 328–330.

GURR, E. 1971. (ed.). Anionic dyes, wholly acid. p. 197–198. *In* Synthetic dyes in biology, medicine and chemistry. — Academic Press, London & New York.

HALVORSON, H. O., CARTER, B. L. A. and TAURO, P. 1971. Synthesis of enzymes during the cell cycle. — Adv. Microbiol. Physiol. **6**: 47–106.

HARTWELL, L. H. 1974. *Saccharomyces cerevisiae* cell cycle. — Bacteriol. Rev. **38**: 164–198.

HOWARD, A. and PELC, S. R. 1953. Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. — Heredity **6**: (Suppl.); 261–273.

HUTTER, K. -J., GÖHDE, W. und EMEIS, C. -C. 1975. Untersuchungen über die DNS-, RNS- und Proteinsynthese ausgewählter Mikroorganismenpopulationen mit Hilfe der Zytophotometrie und der Impulscytophotometrie. I. Mitteilung: Methodische Untersuchungen über geeignete Färbeverfahren. — Chem. Mikrobiol. Technol. Lebensm. **4**: 29–32.

HUTTER, K. -J. and EIPEL, H. E. 1978*a*. DNA determination of yeast by flow cytometry. — FEMS Microbiol. Lett. **3**: 35–38.

HUTTER, K.-J. and EIPEL, H. E. 1978*b*. Protein distribution in population of baker's yeast. — Europ. J. Appl. Microbiol. Biotechnol. **5**: 203–206.

HUTTER, K. -J., EIPEL, H. E. and HETTWER, H. 1978*a*. Rapid determination of the purity of yeast cultures by flow cytometry. — Europ. J. Appl. Microbiol. Biotechnol. **5**: 109–112.

HUTTER, K.-J., GÖRTZ, Th., OLDIGES, H. und EIPEL, H. E. 1978*b*. Durchflußzytophotometrische Bestimmung des DNS-Gehaltes von *Nectria coccinea* Pers. ex Fr. bei Fungizideinwirkung. — Chemosphere **1**: 51–58.

KAMENTSKY, L. A., MELAMED, M. R. and DERMAN, H. 1965. Spectrophotometer: New instrument for ultrarapid cell analysis. — Science **150**: 630–631.

KAPLOW, L. S., DAUBER, H. and LERNER, E. 1976. Assessment of monocyte esterase activity by flow cytophotometry. — J. Histochem. Cytochem. **24**: 363.

KESSLER, E. und CZYGAN, F. -C. 1970. Physiologische und biochemische Beiträge zur Taxonomie der gattung *Chlorella*. IV. Verwertung organischer Stickstoffverbindungen. — Arch. Mikrobiol. **70**: 211–216.

MITCHISON, J. W. 1971. (ed.). *In* Biology of the cell cycle. — Cambridge University Press.

MUNCH, R., BECK, G. und MANOJLOVIC, N. 1971. Untersuchung über die Reaktion *in vitro* gezüchteter Zellen nach Inkubation in SiO_2-Staub. — Beitr. Silikoseforschg. **23**: 4.

ORTHO INSTRUMENTS. 1977. Measurement of DNA in yeast cells. *In* Ortho Instr. (eds), Protocols No. 24. — Ortho Instr., Westwood, Mass., USA.

ORTHO INSTRUMENTS. 1978. Determination of purity of yeast cultures. *In* Ortho Instr., (eds), Protocols No. 26. — Ortho Instr., Westwood, Mass., USA.

PAAU, A. S., LEE, D. and COWLES, J. R. 1977*a*. Comparison of nucleic acid content in populations of free-living and symbiotic *Rhizobium meliloti* by flow-microfluorometry. — J. Bacteriol. **129**: 1156–1158.

PAAU, A. S., COWLES, J. R. and ORO, J. 1977*b*. Flow-microfluorometric analysis of *escherichia coli*, *Rhizobium meliloti*, and *Rhizobium japonicum* at different stages of the growth cycle. — Canad. J. Microbiol. **23**: 1165–1169.

SLATER, M. L., SHARROW, S. O. and GART, J. J. 1977. Cell cycle of *Saccharomyces cerevisiae* in populations growing at different stages. — Proc. Natl. Acad. Sci. USA. **74**: 3850–3854.

SMITH, J. E. and BERRY, D. R. 1974. The vegetative state. p. 106–151. *In* J. E. Smith and D. R. Berry

(eds), An introduction to biochemistry of fungal development. — Academic Press, London & New York.

SPRENGER, E., SANDRITTER, W., BÖHM, N., SCHADEN, M., HILGARTH, M. und WAGNER, D. 1971. Durchflußfluoreszenzzytophotometrie: Ein Prescreening-Verfahren für die gynäkologische Zytodiagnostik. — Beitr. Path. **143**: 323–344.

STRUGGER, S. 1949. (ed.). p. 89–92 *In*: Fluoreszenzmikroskopie und Mikrobiologie. — Verlag M. & H. Schaper, Hannover.

WILLIAMSON, D. H. and SCOPES, A. W. 1961. Protein synthesis and nitrogen uptake in synchronously dividing cultures of *Saccharomyces cerevisiae*. — J. Inst. Brew. **67**: 39–42.

WILLIAMSON, D. H. 1965. The timing of de oxyribonucleic acid synthesis in the cell cycle of *Saccharomyces cerevisiae*. — J. Cell. Biol. **25**: 517–528.