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Flow Cytometric Determination of Esterase and Phosphatase Activities and Kinetics in Hematopoietic Cells with Fluorogenic Substrates[1]

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Viable and formaldehyde fixed rat bone marrow or spleen cells and rat or human blood leukocytes were incubated with fluorescein (fluorescein-diacetate, -dibutyrate and -laurate) or umbelliferone (4-methylumbelliferyl acetate and -phosphate) fluorogenic substrates for subsequent flow cytometric determination of cell fluorescence and cell volume. Formaldehyde-fixed cells preserved between 14 and 20% of the enzyme activity of the unfixed cells and the number of cell clusters for fixed and unfixed cells was the same. The esterase substrates revealed one cell cluster for spleen cells, two for bone marrow cells and four for peripheral blood leukocytes. Phosphatase activity was only associated with a cell cluster of large cells. The time course of substrate cleavage was linear during the first 10 min of incubation. Later on a plateau was reached. Specific enzyme activities were calculated on a single cell level from the simultaneous cell volume and cell fluorescence measurement. Two enzyme activities could be measured simultaneously by using a substrate mixture of umbelliferone acetate and fluorescein acetate.

Key terms: Flow cytometry, enzyme kinetics, esterase, phosphatase, hematopoietic cells

Enzymatic activities of cells or tissues can be determined by biochemical or by histochemical methods. For biochemical determination the cells or tissue are usually homogenized, the enzymatic activities can then be precisely quantified. Such measurements are, however, bulk measurements, therefore the results are difficult to interpret on a cellular level if the tissue or the cell suspension contain different cell populations. Furthermore the homogenization destroys the cells as functional units which may influence the enzymatic activities. The histochemical approach avoids these problems because enzymatic activities become morphologically apparent on a single cell level. The quantitation of histochemical stains is, however, difficult and time consuming. Since the first cytochemical demonstration of esterase activity in blood cells by Gomori 1953 (5), many enzyme staining techniques mostly based on light absorption have been developed (12). The methods are applicable in investigative as well as in diagnostic pathology and play an important role in the identification of hematopoietic cells (15), the investigation and diagnosis of enzyme abnormalities (7), the recognition of cell maturation processes (11) and the classification of leukemias (2, 4).

Flow cytometric measurements combine the advantages of biochemical and histologic enzyme determination. They are single cell measurements at high speed (1000–2000 cells/second) and the enzyme activity can be precisely quantified. Flow cytometers have furthermore the advantage to be capable of evaluating simultaneously other cell parameters as cell volume, DNA content or cell membrane receptors. Due to these possibilities flow cytometers can be used for automated differential white cell counting (1). With absorptive histochemical esterase stains one can distinguish flow cytometrically between monocytes and granulocytes (11). Attempts have also

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been made to use enzyme stains for the characterisation of preneoplastic (21) and neoplastic cells (22).

Flow cytometry in connection with cell sorting allows the preparative isolation of subpopulations of cells. The aim of sorting is often to isolate viable cells. Usually, however, the cells are devitalized during fixation and preparation for absorptive histochemical stains. The advantage of fluorogenic substrates (6) in view of cell sorting is that they indicate the enzyme activity in viable cells (13). The property of viable cells to accumulate the fluorescent reaction product distinguishes them from damaged cells where the cell membrane is leaky, a fact which is used for cell viability tests with fluorescein-diacetate (FDA) (13). Fluorogenic substates indicate also the enzyme activity in fixed cells (13), where it is in general significantly lower than in viable cells. Therefore, the activity loss on cell fixation (8) can be evaluated with fluorogenic substrates. This is usually not possible with absorptive stains.

The aim of the present study was (*a*) to characterize the enzyme kinetics of unfixed and fixed cell populations of the hematopoietic system, (*b*) to compare the total and specific intracellular enzyme activities of these populations and (*c*) to attempt to measure two enzyme activities in addition to cell volume simultaneously.

Materials and Methods

Fluorogenic Substrates

Fluorescein-diacetate (FDA), -dibutyrate (FDB) and -laurate (FL) (Sigma Chemical Co., St. Louis, MO.) and 4-Methyl-umbelliferyl acetate (UMA) and -phosphate (UMP) (Polysciences Inc., Warrington, PA) were dissolved in N-N1-dimethyl-formamide (Baker Chemicals, Deventer, Holland) at a concentration of 2 mg/ml.

Cell Preparation

Healthy young adult female BDIX rats (Berlin-Druckey, Freiburg, F.R.G.) of 160 g weight were bled under ether anesthesia through the abdominal aorta until respiratory arrest. Both the femurs and the spleen of the rat were immediately removed. Clotting of the rat blood was prevented by the addition of 5% v/v Vetren (Promonta, Hamburg, F.R.G., 100 I.E. heparin/ml). Human blood was obtained from a healthy donor and was also anticoagulated with heparin. Leukocyte suspensions were prepared from this blood by lyzing the erythrocytes with ammonium chloride according to the method of Schwartz (16) with the following modifications: one part blood was incubated with 4 parts of 0.155 *M* ammonium chloride solution at 4°C for 20 min in order to hemolyze the red cells. After centrifugation at 200 × *g* at 4°C for 10 min, the leukocytes were washed twice (200 g) and resuspended in 0.15 *M* NaCl solution buffered with 5 m*M* Tris/HCl to pH 7.4 (TBS). The blood erythrocytes were lyzed, because they are approximately by a factor of 1000 in excess of the blood leukocytes in normal blood. Although one can discriminate erythrocytes and leukocytes in the unlyzed blood with the flow cytometer by appropriate threshold setting, it is difficult to collect a sufficient number of leukocytes for kinetic measurements within 30 sec at a flow rate of 1000–2000 cells/sec if dilutions of whole blood are used. A single cell suspension of the spleen was prepared by mincing the organ with a tissue chopper (Mickle Lab. Engineering Co., Gomshall Surrey, England) and subsequently passing the tissue pieces (approximately 1 mm^3) several times in TBS through needles with inner diameters of 1.0, 0.8 and 0.6 mm. Cells were then sieved once through a nylon filter (100-μm pore diameter), and washed twice in TBS. The bone marrow was similarly processed after having been flushed out of the femurs with TBS. All cell suspensions were adjusted to a final concentration of 1×10^6 cells per ml TBS. Cell viability was determined by the dye exclusion test using 0.02% w/v trypan blue. It was never less than 93% in the unfixed samples. An aliquot of each cell suspension was fixed in freshly prepared TBS solution containing 1.7% v/v formaldehyde (Merck, Darmstadt, F.R.G.) adjusted to pH 7.4 with 1.0 *N* NaOH. The cells were stored in the fixative for at least 3 hr and approximately 1×10^7 cells were washed once in 10 ml TBS, centrifuged and resuspended to 10 ml with TBS before incubation with substrate.

Cell Staining

Two hundred fifty μl of unfixed or fixed cells (10^6 cells/ml) were incubated with 5 μl substrate solution at 25°C and measured during 30 sec after incubation times from 1–60 min. The time indicated in the graphs refers to the start of the measurement. The substrate concentration in the cell suspensions was between 0.05 and 0.10 m*M* depending on the substrate. For kinetic studies, a 10 ml sample of cells was incubated with substrate at 25°C. One-ml aliquots of this cell suspension were used for measurements at different times after the start of the incubation period. Spontaneous hydrolysis of the fluorogenic substrate in TBS was negligible during a 3-hr incubation period at 25°C.

Centrifugal Elutriation

Blood leukocytes were separated according to cell volume with an elutriator centrifuge (Beckman, Fullerton, CA) at 3000 rpm by stepwise increase of the buffer flow (TBS). The volume distribution curves of the cells in the eluted fraction were determined in a METRICELL flow cytometer (9). An aliquot of each fraction was centrifuged on a microscope slide with a cytocentrifuge (Shandon Elliott, Frankfurt, F.R.G.). Cell morphology was evaluated microscopically after May Grünwald-Giemsa staining of the slides.

Flow Cytometry

The fluorescence and the volume of the stained cells were measured simultaneously in a FLUVO-METRICELL flow cytometer (10) which combines electrical cell sizing in a hydrodynamically focussed orifice with an epi-illumination fluorescence measurement of cellular fluorescence. An orifice of 100-μm diameter and 100 μm length was used for the electrical volume measurements with an orifice current of 1.10 mA and a particle flow rate between 800 and 1000 particles per second. The sheath flow was TBS. Fluorescence was excited by an HBO 100 mercury lamp using a combined 450 nm high pass and a 490 nm low pass interference filter for fluorescein excitation, a 500 nm dichroic mirror to separate exciting and fluorescent light and an additional 500 nm highpass filter in front of the photomultiplier. Fluorescence excitation during the simultaneous fluorescein and umbelliferone measurements was between 300 and 400 nm (BG12 filter). The fluorescent light was collected between 400 and 500 nm for umbelliferone and between 500 and 580 nm for fluorescein using a 400 nm and a 500 nm dichroic mirror and a 580 nm low pass filter to separate the blue and yellow fluorescences. The dichroic mirrors were from Zeiss (Oberkochen, F.R.G.) and the filters from Schott (Mainz, F.R.G.).

All measurements had a fluorescent background which corresponded to the fluorescent noise generated by the extracellular fluorescence in the sample beam. Cellular fluorescent signals could therefore not be resolved as long as their amplitude did not exceed the amplitude of the noise signals. For this reason the background noise was not subtracted from the enzyme activities (Tables 2 and 3), because otherwise the relationship between the intracellular amount of fluorescein and the measured fluorescence signal would have been altered.

The stability of the electrical volume sizing was monitored by electronic calibration (9). The stability of the fluorescence measurements was controlled by reference particles and by checking the

position of the sample beam carefully before and after each measurement. The reference particles were either erythrocytes which were covalently labeled with fluorescein isothiocyanate (FITC) and then formaldehyde fixed, or fresh erythrocytes taken from always the same donor and stained with FITC-poly-ornithine which binds to the cell surface charges (18). Fluorescent latex particles were also used in some instances; it was, however, not easy to have them in the desired range of photomultiplier sensitivity with the linear pulse amplifiers of the flow cytometer. The electrical volume determination was stable within 2% on a day to day basis. The fluorescence calibration on a given day was stable within 5% and was typically maintained over several hours provided the position of the sample beam was controlled before and after each measurement and readjusted if necessary.

Data Processing

Two Parameter Measurements: The maximum amplitude of both fluorescence and volume signals of each cell were measured, digitized and stored in a 64 × 64 channel array of a multichannel analyzer (AEG Telefunken, Ulm, F.R.G.). The sampling of the volume and fluorescence pulses were performed in such a manner that fluorescence pulses were only accepted, if the particle volume was greater than the erythrocyte volume. Thus only nucleated cells were incorporated into the two parameter histograms. After the end of the measurement the data were transferred to magnetic tape for further analysis by Fortran IV computer programs (200 kbyte core memory in a Siemens 4004/150 computer (Siemens Co., München, F.R.G.). The programs allow plotting of the experimental data as three dimensional histograms (Fig. 1) and printing of the projection of these histograms on the volume/fluorescence plane as isoamplitude maps (Fig. 2), as described elsewhere (14, 18–20). Furthermore the fluorescence per unit cell volume and the absolute and relative number of cells within one or more limited areas of the histograms may be calculated.

Three Parameter Measurements: The pulse height of the analogue signals of the cell volume, fluorescence 1 and 2 from each individual cell were digitized with a resolution of 128 steps and stored on-line with a maximal rate of 6000 cells/sec via an Interdata 74 computer (Interdata Corp., Oceanport, NJ; 64 kbyte core memory) on magnetic tape (3). Only cells with a fluorescence 1 (FDA) signal above background were accepted for on-line storage. The sequential list mode data on the tape were then corrected on a cell by cell basis for fluorescence overlap (5% UMA overlap into the fluorescein channel and 26% FDA overlap into the umbelliferone channel[2]) and classified as a 22 × 22 × 22 channel three parameter histogram by a Fortran IV computer program (3) in the same Interdata 74 computer. No correction was made for the quantum efficiency of the fluorescein and umbelliferone fluorescences. The three parameter histogram was evaluated by a newly developed Fortran IV computer program (17) (260 kbyte core memory in a Siemens 4004/150 computer (Siemens Co., München, F.R.G.) or in a VAX 11/780 computer (Digital Equipment Co., Maynard, MA)).

UV-Microscopy

All cell samples were examined by a Leitz fluorescence microscope with epi-illumination (Leitz GmbH, Wetzlar, F.R.G.), equipped with a 150 watt HBO Xenon lamp. Dichroic mirrors and filters were matched so as to obtain conditions similar to those used in the flow cytometer.

Results

The intracellular esterase activity decreased after fixation of the cells with formaldehyde. The fixation affected the enzyme activity of different hematopoietic cells to varying degrees; between 14 and 20% of the enzyme activity of the viable cells were preserved after fixation (Table 1). The number of cell clusters in the histograms was the same for unfixed and fixed cells, but the speed of substrate cleavage was lower with fixed cells. The reproducibility of the assay and measurement was tested by incubating several aliquots of the same rat bone marrow cell suspension for 10 min at 25°C with FDA. The coefficient of variation for five sequential assays was 6.9%.

Assay with One Enzyme Substrate: Two cell clusters (Fig. 1*a*) were detected with FDA, FDB, FL and UMA in suspensions of unfixed rat bone marrow cells (small and large nucleated cells). Rat spleen cells formed only one cell cluster after incubation with these substrates (Fig. 1*b*). Four cell populations were found in the case of rat blood leukocytes (Fig. 1*c*). The upper cell cluster (S2) of the small cells was observed with all substrates. It did not appear in the bone marrow and also not in most of the spleen samples (Fig. 1*a* and *b*). The population of large nucleated cells which has only little enzyme activity (L1) was found with FDA incubation (Fig. 1*c*), but not with FDB or FL incubation of rat blood leukocytes. Human blood leukocytes revealed three populations of nucleated cells (Fig. 1*d*), a fourth cell cluster of large cells with low activity analogeous to the L1 cluster in rat leukocytes was sometimes detectable with FDA. The small nucleated cells of human and rat blood leukocytes were lymphocytes and the large nucleated cells were granulocytes and monocytes. The cells were identified microscopically after the small and large cells had been preparatively separated by centrifugal elutriation. Phosphatase activity after incubation with UMP was generally low and was only associated with the large cells of the rat bone marrow and the rat and human blood leukocytes. The specific enzyme activities for the various cell populations differed depending on the substrate. Rat cells (Table 2) and human cells (Table 3) cleaved the FDA substrate faster and to a greater extent than the longer chain esters FDB and FL. The UMP- and FL-cleavage was low and the fluorescence signals were close to background noise. The intracellular accumulation of the fluorescent product could be followed as a gradual migration of the cell clusters to higher fluorescence intensities (Fig. 2). The kinetics of the enzyme activities in large rat bone marrow cells for FDA, FDB and FL were linear during the first 10 min of incubation after which a plateau was reached (Fig. 3). The mean volume and the volume distribution curve of the unfixed cells did not significantly change during the intracellular accumulation of fluorescein and the later plateau phase suggesting that the introduction and the cleavage of the fluorogenic substrate in the cells did not measurably interfere with cell viability for a period of 30 min. Later on the percentage of nonfluorescent (dead) cells increased. Large cells cleaved more substrate than small cells. However, the specific enzyme activities for large and small rat bone marrow cells were similar (Fig. 3). The UV-microscopic examination of the cell samples was in agreement with the flow cytometric measurements. Incubation with FDA and FDB resulted in a bright and homogeneous fluorescence of the cytoplasm. The less intense fluorescence of FL and UMA stained cells showed a granular pattern of the cytoplasm and the shadow of the unstained nucleus was

[2] Valet G: Manuscript in preparation.

Table 1
Specific Esterase Activity (Fluorescence/Unit Cell Volume) of Unfixed and Formaldehyde Fixed Rat Cells after 10 Min of Incubation with FDA at 25°C[a]

	Rat Bone Marrow		Rat Spleen	Rat Blood Leukocytes		
	Small Cells (S)	Large Cells (L)	(S)	Small Cells (S1)	Small Cells (S2)	Large Cells (L1 + L2)
Unfixed	0.92 ± 0.22	1.00 ± 0.22	0.93 ± 0.30	0.80 ± 0.21	0.87 ± 0.23	0.69 ± 0.19
Formaldehyde fixed	0.12 ± 0.03	0.14 ± 0.03	0.16 ± 0.04	0.16 ± 0.03	0.14 ± 0.02	0.10 ± 0.02
% activity of unfixed	13.0	14.0	17.2	20.0	16.1	14.5

[a] One histogram per enzyme assay was recorded (between 12243 and 37585 cells), and the mean specific esterase activity for each cell cluster in this histogram was determined (16). The SD in this and the following tables indicates the biologic scatter of the enzyme activities in the cells of each cell cluster. The standard error of the mean values (SE = SD/√total cells) is, however, much lower, since a high number of cells was measured per histogram. This means that 3-4% difference of the mean specific enzyme activity of various cell clusters within one histogram are statistically significant. Since the coefficient of variation of the mean values for repetitive assays of the same cell suspension is 6.9%, the differences of the mean values have to be 10 to 15% to be statistically significant if cell clusters of various histograms are compared. Explanation of S, S1, S2, L, L1, L2 see Figure 1*a-d*.

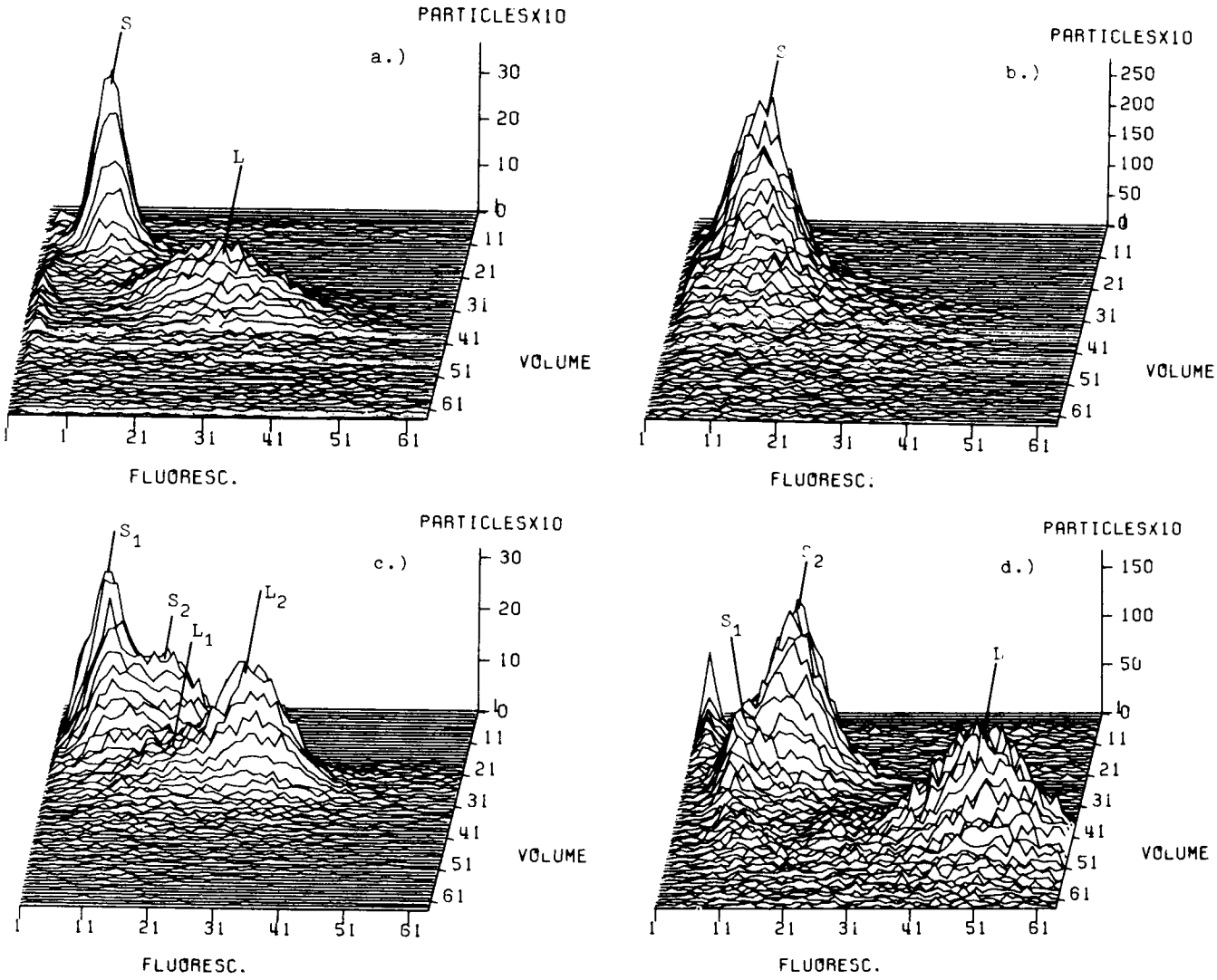

FIG. 1. Intracellular esterase activity versus cell volume of unfixed cells of the rat bone marrow (*a*) (total cell count (TC) = 33774, contribution S = 36.3%, L = 63.7%), rat spleen (*b*) (TC = 37585), rat blood leukocytes (*c*) (TC = 64929, S1 = 20.9%, S2 = 31.2%, L1 = 15.6%, L2 = 32.3%) and human blood leukocytes (*d*) (TC = 57053, S1 = 19.1%, S2 = 25.8%, L = 55.1%) after 13 min of incubation with FDA at 25°C. The TC and contribution percentage were calculated by fitting two dimensional Gaussian distributions to the experimental curve (18).

Table 2
Specific Esterase Activity (Fluorescence/Unit Cell Volume) of Unfixed Rat Cell Populations after 10 Min of Incubation with Substrate at 25°C (between 3019 and 12724 Cells were Measured to Determine each Mean Value)

Substrate	Rat Bone Marrow		Rat Spleen	Rat Blood Leukocytes		
	Small Cells (S)	Large Cells (L)	(S)	Small Cells (S1)	Small Cells (S2)	Large Cells (L1 + L2)
FDA	0.96 ± 0.23	1.02 ± 0.17	1.17 ± 0.46	1.75 ± 0.42	0.93 ± 0.24	1.01 ± 0.36
FDB	0.73 ± 0.24	0.80 ± 0.17	0.89 ± 0.28	1.28 ± 0.31	0.77 ± 0.18	0.94 ± 0.21
FL	0.45 ± 0.15	0.22 ± 0.06	0.28 ± 0.11	0.40 ± 0.12	0.40 ± 0.12	0.18 ± 0.06
Background (See Methods)	0.41 ± 0.14	0.17 ± 0.05	0.28 ± 0.08	0.40 ± 0.13	0.40 ± 0.13	0.16 ± 0.04

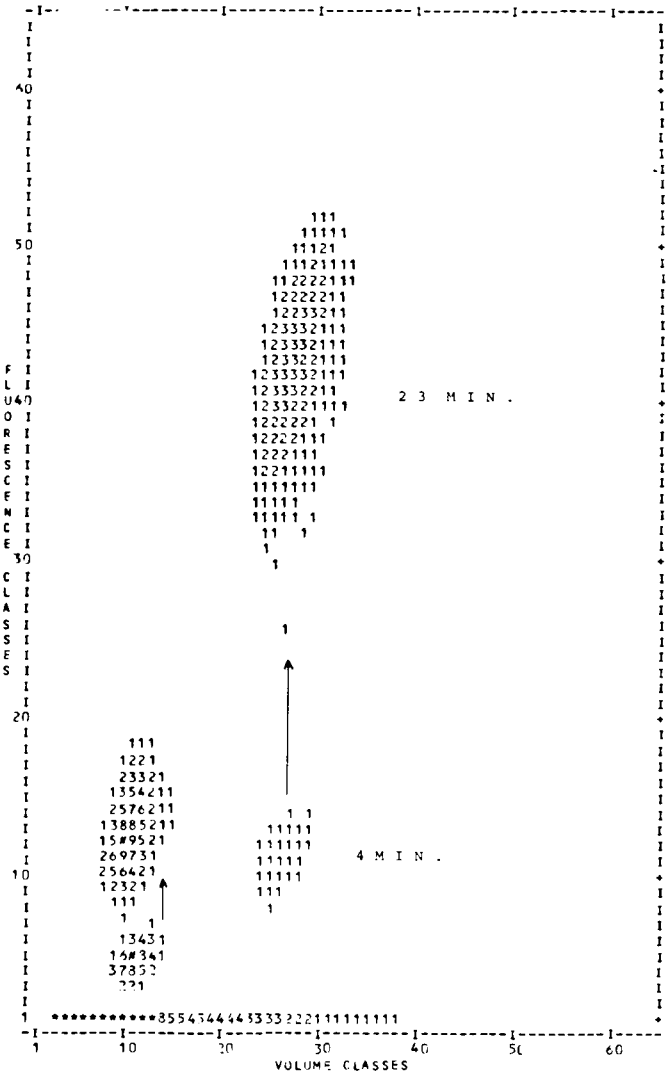

FIG. 2. Intracellular esterase activity versus cell volume of unfixed rat bone marrow cells measured after 4 and 23 min of incubation with FDA at 25°C. The isoamplitude print is normalized to the channel content designated by (§). The amplitude range between 0 and 100% is divided equally into 10 steps (number 1–9). Channel contents higher than the channel content used for standardization (§) are indicated by (*). One volume channel corresponds to 9.3 μm^3.

visible. The intensity of background fluorescence increased in the samples containing unfixed cells after 15–20 min FDA incubation period, but not in the formaldehyde fixed cell suspensions. Unfixed and fixed cells were incubated with 0.10 and 0.01 m*M* fluorescein solution in TBS over a 20-min period to exclude nonspecific adsorption of free fluorescein from the medium to unstained cells. No detectable adsorption to the cell membrane or incorporation of fluorescein into the cells were observed.

Table 3
Specific Esterase and Phosphatase Activity (Fluorescence/Unit Cell Volume) of Unfixed Human Blood Leukocytes after 10 Min of Incubation with Substrate at 25°C (between 3343 and 9670 Cells Were Measured to Determine each Mean Value)

Substrate	Human Blood Leukocytes	
	Small Cells (S1 + S2)	Large Cells (L)
FDA	0.85 ± 0.25	1.24 ± 0.20
FDB	0.29 ± 0.07	0.36 ± 0.07
FL	0.27 ± 0.07	0.20 ± 0.12
UMA	0.31 ± 0.10	0.11 ± 0.03
UMP	0.22 ± 0.06	0.11 ± 0.03
Background (See Methods)	0.22 ± 0.03	0.08 ± 0.03

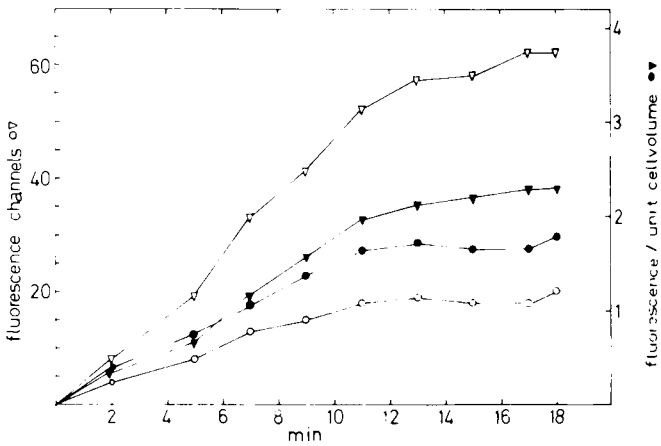

FIG. 3. Kinetics of total esterase activity (fluorescence channels (○,▽)) and specific esterase activity (fluorescence/unit cell volume (●,▼)) of unfixed small (○,●) and large (▽,▼) rat bone marrow cells after incubation with FDA at 25°C.

Assay with Two Enzyme Substrates: As an extension of the preceding experiments, unfixed rat bone marrow cells were incubated at the same time with FDA and UMA. Both fluorescences and the cell volume were measured. The graphical display of these measurements showed two distinct populations of nucleated cells (Fig. 4). The correlation between the esterase activities for FDA and UMA was linear, indicating that both substrates were metabolized with similar time course by the esterases of the bone marrow cells.

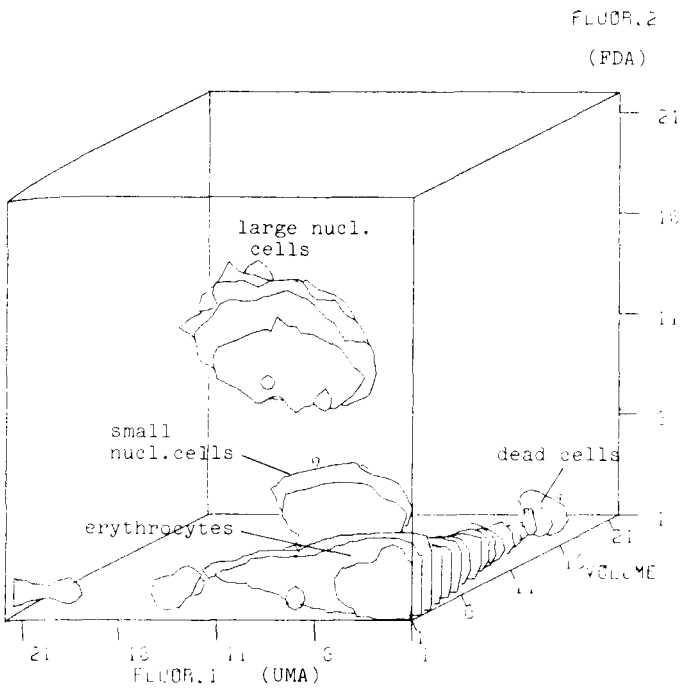

FIG. 4. Graphical display of a three parameter measurement of FDA and UMA esterase activity and cell volume of unfixed rat bone marrow cells after 10 min incubation at 25°C.

Discussion

Flow cytometric measurements with fluorogenic substrates allow the investigation of enzyme activity and enzyme kinetics of unfixed cells. It is important to note that the number of subpopulations observed for enzyme stained rat bone marrow, rat spleen and rat and human blood cells is the same in the native and in the fixed state. The esterase activity is, however, reduced by formaldehyde fixation to 14–20% of the values in unfixed cells. The simultaneous measurement of the cell volume enables one to calculate the specific enzyme activities (fluorescence/unit cell volume or fluorescence/μm^3 cell content). This facilitates the comparison of different cell populations in a standardized manner (Tables 2 and 3), and detects slight variations of enzyme activity per unit cell volume for the various cell clusters.

Unfixed cells accumulate the fluorescent product and release it in small amounts that are proportional to the intracellular concentration, whereas cells damaged by freezing and thawing or by aging lose the fluorescent product very rapidly (13). This rapid loss of fluorescent product by cells with leaky membrane is the basis for the recognition of damaged cells in cell viability tests with fluorogenic substrates. The degree of accumulation of fluorescent product in the viable cells depends mainly on the intracellular enzyme activity and the speed of product excretion (13). The conditions to measure the intracellular enzyme activity are best during the first minutes after addition of substrate to the cell suspension, as long as the intracellular concentration of the fluorescent reaction product is low. After 15–20 min of incubation a plateau of the concentration of the reaction product in the cell is reached (Fig. 3), which means that substrate cleavage and product excretion are in equilibrium. During this plateau phase it is difficult to decide whether different levels of the intracellular product concentration in different cell populations are due to the enzyme activity or to the rate of excretion of the fluorescent product. Therefore, in the present study the enzyme activity measurements were always performed between 1 and 10 min after addition of substrate. An advantage of the simultaneous assessment of an enzyme activity together with cell volume is to identify more subpopulations in heterogenous cell samples than by one parameter measurement. This is demonstrated by the detection of four distinct cell clusters of rat blood leukocytes (Fig. 1) instead of only two populations by either a cell volume distribution analysis (small and large cells) or by a fluorescence analysis (low and high activity cells) alone. Not only qualitative differences, such as the presence or absence of enzyme activity, but also relatively small quantitative differences of cell enzyme activity in the different cell clusters can be evaluated by fitting one or several two-dimensional Gaussian distributions to the experimental curve and calculating the mean value, the standard deviation and the mean fluorescence per unit cell volume (16) of the various overlapping cell clusters (Fig. 1).

The simultaneous determination of a third parameter which can be either an additional enzyme activity or the nuclear DNA or a cell membrane marker provides additional information about cell subpopulations in comparison to the two parameter histogram. Even four parameters can be simultaneously measured and displayed (17), since the data can be related to the cell volume and can be reduced for graphical representation to three parameters by the calculation of specific activities, concentration values or surface densities. In this way it is possible to characterize and to identify small subpopulations of cells which may show only slight quantitative differences to other cells within a heterogeneous population. In analogy to protein chemistry one is able to produce cellular fingerprints by flow cytometry. The location of each cell cluster in the three or four parameter histograms under standardized condition should be highly characteristic for a given subpopulation of cells and be of importance for the recognition of a small proportion of abnormal cells in an excess of normal cells.

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