Evaluation of the ability of intact platelets to accumulate acridine orange

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Summary. A new approach to the evaluation of the uptake of fluorescent probes by intact cells is described. Acridine orange (AO) was used because it can be selectively accumulated by serotonin-containing granules of platelets. Analysis of the fluorescence signal allows the estimation of the relative volume of the granules and the equilibrium coefficients for AO transport across the cytoplasm and granule membranes. The following results were obtained for human and rabbit platelets: the relative volumes of the granules were 14 ± 1% and 29 ± 2%, the ratios of intragranular-extracellular probe concentration were 2260 ± 382 and 30,000 ± 5550, and the cytoplasm-extracellular medium concentration ratios were 375 ± 60 and 225 ± 60, respectively.
Key words. Platelets; acridine orange; storage granules; fluorescence quenching; accumulation ability.

Accumulation and storage of biologically active substances at high concentrations in the intracellular storage organelles are specific features of platelets [1]. Accumulation inside the granules of intact cells involves the following stages: a) transport across the cell membrane, b) transport across the granule membrane, c) storage inside the granule. In some pathological conditions the mechanism of accumulation is impaired [2, 3]. Generally, the role of disarray remains unclear. This may be attributed to the fact that in clinical and laboratory practice the estimation of the accumulation process has been characterized by the establishment of an 'impaired accumulation ability' [3, 4], and to the fact that there exists no reliable method for the differentiation and evaluation of the three stages of accumulation process.

Two methods have been currently used for the analysis of accumulation, namely, the single isotope radiometric technique and the use of fluorescent probes. The first one has been widely applied to studies of intact cells; however, the most interesting results were obtained in isolated granules [5, 6].

The second method is based on the ability of acridine and its derivatives to be selectively accumulated in platelet-dense bodies [7, 8]. On accumulation in subcellular organelles, reduction of fluorescence results from the complexation of acridines at high concentrations [9, 10]. The fluorescent probes have been currently applied for characterization of the process under investigation [11, 12]. Evaluation of fluorescent probe uptake is based on a model considering granules and the external medium, and suggesting complete quenching of fluorescence inside the granules [13, 14].

In the present study a more adequate model is proposed. We have considered three phases (extracellular medium, cytoplasm and intragranular matrix), and an empirical relationship between probe concentration and fluorescence intensity. This model allows evaluation of acridine orange (AO) accumulation inside intact cells by determining the interrelationship between fluorescence intensity and quantitative characteristics of accumulation; relative volume of storage organelles in platelets and equilibrium coefficients for probe transport across the plasma and granule membranes.

Materials and methods. Platelets from rabbits and from healthy donors were studied. The blood was collected in 0.13 M sodium citrate (pH = 7.4, 9:1, v/v) from rabbits under ether anesthesia. Platelet-rich plasma (PRP) was prepared by centrifugation at 200 × g for 10 min (human blood) and 15 min (rabbit blood). Platelet-poor plasma (PPP) was prepared by centrifugation at 2000 × g for 25 min. All experiments were carried out at room temperature. The relative volume of platelets was determined using a microcentrifuge cuvette [15]. AO and rhodamine 6G were purchased from Polysciences, Inc., USA. Fluorescence of AO was measured with excitation of 480 nm (slit width 5 nm) and emission at 530 nm (slit width 15 nm). For rhodamine 6G excitation and emission, wavelengths were 480 nm and 560 nm respectively, slit widths were 5 nm in all cases. A special spectrofluorometer was designed for the experiments. Fluorescence was detected from the small volume adjacent to the front surface of the cuvette (angle 45°) in order to preclude light scattering in a turbid sample and prevent the internal screening effect. The sample (0.8 ml of the relevant platelet suspension in a round plastic cuvette) was stirred at 13 rps. Calculations were carried out by the steepest descent method [16]. The data are given as a mean ± SEM of the number of experiments indicated.

Results and discussion. As mentioned above, accumulation of AO inside subcellular organelles of intact cells leads to fluorescence decrease. Figure 1 illustrates the changes in fluorescence intensity during AO uptake by intact rabbit platelets. At first, fluorescence rapidly decreases and after about 40 min reaches the plateau which indicates the equilibrium distribution of probe in the suspension. The main cause of fluorescence intensity reduction is concentration quenching of fluorescence at high probe concentration inside granules and possibly in the cytoplasm. At steady state the probe is distributed mainly in the extracellular medium, cytoplasm, and intragranular matrix. Even at low mean concentrations of AO in the sample its concentration in the latter two phases can be high enough for concentration quenching to take place. Quenching can also result from probe binding to membranes [10]; however, it has been shown that only about 5% of AO binds to the membranes in PRP [7]. Therefore, it is essential to know the nature of AO concentration quenching in order to determine the relationship between probe fluorescence intensity and the parameters describing its distribution in platelet suspension.

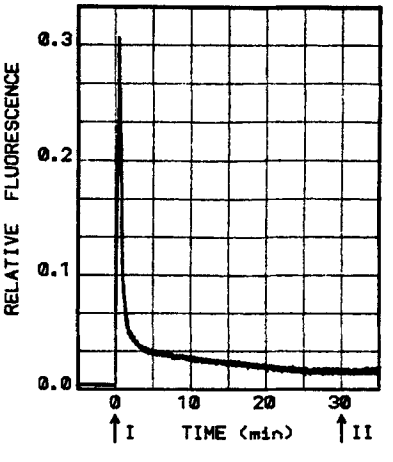

Figure 1. Decrease in fluorescence intensity on AO accumulation by rabbit intact platelets. Arrow I indicates the time of AO (10 μM) injection. Arrow II shows the point where steady state is reached.

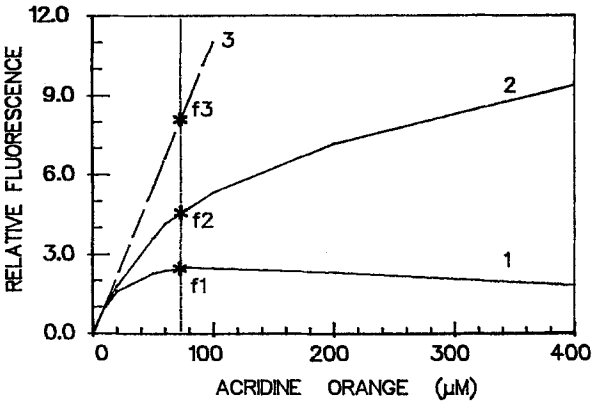

Figure 2. Relationship between fluorescence of probes and AO concentration in slaine. 1, acridine orange; 2, rhodamine 6G; 3, extrapolation of curve 2 initial linear part. Concentration of dyes has been chosen so that the extinctions (E) of corresponding solutions of AO and rhodamine 6G were the same at the exciting wavelength (480 nm). Fluorescence intensity at E = 0.028 mm^{-1} was assumed to be 1 for both probes.

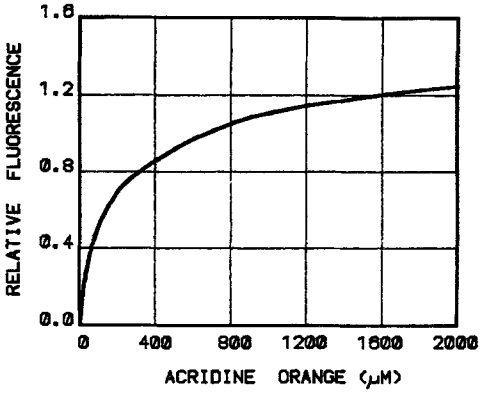

Figure 3. Calculated curve of AO fluorescence in normal saline corrected for internal screening effect. See text for details.

The relationship between AO concentration and fluorescence is shown in figure 2, curve 1. It can be seen that AO fluorescence increases linearly with increasing probe concentration until a concentration of about 20 μM is reached. Beyond that, the growth becomes slower, and at AO concentrations higher then 80 μM the fluorescence decreases. This phenomenon may result from both concentration quenching and from the internal screening effect which occurs at high probe concentrations in solution. At AO accumulation by platelets the second effect is negligible because the light absorption depends on the product of the probe concentration in the sample and the length of the path of light through the sample. Although probe concentration in granules is high the granule size is too small. Therefore the light absorption in the platelet suspension is determined by the average AO concentration in the whole sample and is not changed during AO redistribution. Hence we can suggest that a decrease in AO fluorescence in the cell suspension (fig. 1) is caused only by concentration quenching.

In order to correct for any internal screening effect in the calibration solution, solutions of rhodamine 6G were prepared with extinctions at the exciting wavelength equal to those of AO solutions with known concentrations of the probe. Absorption at emission wavelength was negligible for both rhodamine 6G and AO. Curve 2 in figure 2 shows fluorescence of corresponding solutions of rhodamine 6G. Nonlinear growth of this curve is caused by an internal screening effect. We used this phenomenon to exclude AO fluorescence selfscreening. The fluorescence intensity (f1) of each AO solution has been multiplied by the corresponding correcting coefficient:

$$F(C) = f1 \times f3/f2,$$

where f3 is the calculated fluorescence of rhodamine 6G obtained by extrapolation of the initial linear part of curve 2 to the range of higher concentrations. After this procedure the AO concentration-fluorescence relationship (fig. 3) is obtained from curve 1 in figure 2. Nonlinearity of the relationship is caused only by concentration quenching. The curve was approximated by the polynom $C = \sum_{n=1}^{6} a_n \times F^n$ using the least squares criterion. The dependence of fluorescence on concentration. F (C) used in further calculations is a reverse function of this polynom.

Let us determine the relationship between probe concentration in the three compartments at steady state and AO concentration (C_0) injected in cell suspension (fig. 4). It should be noticed that the model of probe accumulation under consideration does not depend on AO uptake mechanisms on cellular and granular membranes, and describes a steady state when the probe uptake is completed. At this state the mass law for AO distribution can be written as follows:

$$C_0 = C \times (1 - U) + C \times K_1 \times U \times (1 - W) + C \times K_2 \times U \times W \quad (1)$$

or

$$C = C_0 / [1 - U + K_1 \times U \times (1 - W) + K_2 \times U \times W] \quad (1'),$$

where C is probe concentration in extracellular medium; $K_1 \times C$, probe concentration in the cytoplasm; $K_2 \times C = K_1 \times K_1' \times C$, probe concentration in the granules; K_1 and K_1', equilibrium coefficients for AO transport across the plasma and granule membranes; U, relative volume of cells in suspension (determined by centrifugation); W, proportion of the granules volume in the cell; $U \times W$, relative volume of granules in the sample; $U \times (1 - W)$, relative volume of cytoplasm in the sample.

Total fluorescence (F_0) from cell suspension involves three components:

$$F_0 = (1 - U) \times F(C) + U \times (1 - W) \times F(K_1 \times C) + U \times W \times F(K_2 \times C) \quad (2).$$

After the substitution of C from (1') into (2), three parameters remain unknown: K_1, K_2 and W. These unknowns may be calculated from a system of three equations. If parameters K_1 and K_2 are independent of probe concentration (for low concentrations of AO this assumption is correct) the other two equations may be generated by substituting various values for C_0 in equation 1'. Since the function F (C) is non-linear, the three equations will be linearly independent.

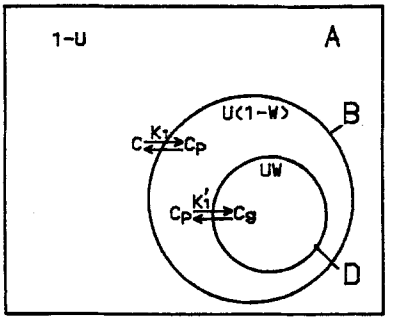

Figure 4. A model for AO accumulation by intact platelets. A, cytoplasm; B, platelet; D, granule. Dye concentration in: extracellular medium (C); cytoplasm (Cp); granule (Cg). Other symbols are indicated in the text.

This approach allows the determination of the relative volume of the serotonin-containing granules and the equilibrium coefficients for AO transport across the plasma and granule membranes. 8 µl of AO were injected into three PRP samples 0.8 ml each (final concentrations were 2, 4 and 8 µM). The samples were incubated for 40 min and AO fluorescence intensities were measured. Fluorescence intensities of corresponding AO concentrations in autologous PPP were also measured to take into account the probe fluorescence changes in plasma compared to its fluorescence in normal saline. The following formula was used for this correction:

$$F_0 = f_0 \times f(\text{saline})/f(\text{ppp}),$$

where f (saline) and f (ppp), AO fluorescence in normal saline and PPP, respectively; f_0, AO fluorescence in PRP. The calculated values F_0 for different concentrations of AO were substituted into three equations type (2), and the set was solved with a computer.

The following results were obtained for healthy donors (n = 5) and rabbits (n = 10):

human platelets $\rightarrow W = 0.14 \pm 0.01$,
$K_1 = 375 \pm 60$, $K_2 = 2260 \pm 382$;

rabbit platelets $\rightarrow W = 0.29 \pm 0.02$,
$K_1 = 225 \pm 60$, $K_2 = 30\,000 \pm 550$.

So far, the granule apparatus has been characterized by the size (average diameter) and the number of granules determined by electron microscopy[17–19]. However, application of these parameters for characterization of the granule apparatus is complicated by the fact that they do not obey the law of normal distribution[20]. It was suggested that the relative volume of granules obeys the normal distribution law and is a more convenient criterion for characterization of the granule apparatus on accumulation and release. The results obtained confirm this suggestion and demonstrate an origin-specifity of the granule apparatus. In human platelets the relative volume of the granules was 14±2%. In rabbit platelets it was 29±2%, which is in good agreement with the results obtained by Pletscher et al. (30%) using differential centrifugation[21].

Rabbit platelets have the highest content of serotonin compared to that in human and guinea pig platelets[1]. Not surprisingly, both W and K_2 (the intragranular/extracellular AO concentration ratio) are greater in rabbit than in human platelets. Our data also indicate that the plasma membrane (parameter K_1) plays an important role in the process of accumulation.

It should be mentioned that the results are valid only for the assumption that F (C) (relationship between fluorescence intensity and probe concentration) is the same in the extracellular, cytoplasmic and intragranular compartments. This assumption was used since it is difficult to develop a model of intragranular space in vitro. Based on good agreement between our results and those reported by others, we have concluded that this assumption does not lead to considerable errors. It is noteworthy that in biomedical investigations not absolute values but the relative changes of the above mentioned parameters may be important.

The proposed method is simple and provides detailed information on accumulation of various substances in a vast array of cells. This approach can be used for the investigation of intestine and adrenal medulla chromaffin cells, synaptosomes, mast cells, and other cells capable of accumulation and storage of biologically active substances.

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The sensitive period for yellow phenocopy induction in *Drosophila melanogaster*

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Summary. Yellow phenocopies of *Drosophila melanogaster* were produced by raising larvae on α-DMT contaminated media. Using a survivorship test, the sensitive period for phenocopy induction was found to occur during the third larval instar of development, with increased survivorship at 1% α-DMT compared with lower concentrations. It was also found that treatment with α-DMT significantly slowed development. These findings are related to the relevant morphological and behavioral developmental pathways and to phenocopy induction.
Key words. Sensitive period; phenocopy; yellow; survivorship; *Drosophila melanogaster*.