QUANTITATIVE ESTIMATION OF FLUORESCENT DYE UPTAKE BY INTACT PLATELETS

A. G. Mezhlumyan, I. Yu. Gavrilov, and E. G. Popov

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One of the principal functional characteristics of platelets is their ability to accumulate and store biologically active substances in high concentrations in specialized serotonin-containing granules (5-HT organelles) [9]. In some pathological states this mechanism is disturbed [1, 6]. However, the role of the changes discovered still awaits explanation in many cases.

The principal methods which are widely used to study the accumulation process can presently be distinguished: the radioactive labeling method and the fluorescent dye method. The first is widely used for research with intact cells, but the most interesting investigations have been on isolated granules; however, the procedure of obtaining them is highly complex and laborious [5, 11]. The second method is based on the fact that many monoamines (acridine orange and its derivatives, in particular) are fluorescent dyes and are taken up selectively in electron-dense granules of the platelets, like biogenic amines [8, 15]. During accumulation of the dye by subcellular organelles, quenching of the fluorescence is observed, due to the formation of complexes of dye molecules [2]. The fluorescent dye method is nowadays used mainly to obtain qualitative results [3, 15]. Quantitative estimates of accumulation are based on an approximate model, which examines a biphasic system of granules and surrounding medium, and assumes complete quenching of fluorescence inside the granules [2].

In this paper the authors suggest a more adequate model, which examines the distribution of the dye in three phases: external, cytoplasmic, and granular, and takes account of the true dependence of fluorescence of the dye on concentration. With this model it is possible to determine accumulation of the dye by intact cells numerically, and to establish correlation between the intensity of fluorescence of the dye and the quantitative parameters of the accumulation process, such as the coefficient of transfer of the dye from the cytoplasmic and granular membranes in the equilibrium state, and the relative volume of granules in the cell. Estimation of the first two values has hitherto been linked with the technique of obtaining isolated granules [5, 10], but the state of the granular system as a rule has been characterized by the number of granules in the cell and (or) the size of the granules (their mean diameter) [7, 13].

EXPERIMENTAL METHOD

Platelets from healthy blood donors and rabbits were used as the test object. Blood was stabilized with 0.13 M sodium citrate solution (pH 7.4) in the ratio of 9:1. Platelet-enriched plasma was obtained by centrifugation of the blood at 200g. The duration of centrifugation was 10 min for human blood and 15 min for rabbit blood. The supernatant was kept at room temperature and the relative volume of platelets was estimated by means of a microcentrifuge cuvette [12]. The fluorescent dye used in the experiments was acridine orange (from Polysciences Inc., USA). The amount of dye added to the specimen of platelets was not more than 1% of the total volume of the samples. Fluorescence was excited at a wavelength of 480 nm (the half-width of the salt was 5 nm), and recorded at a wavelength of 530 nm (half-width of slit 15 nm). The measurements were carried out on a spectrofluorometer which was specially designed for the purpose of this investigation. The cuvette

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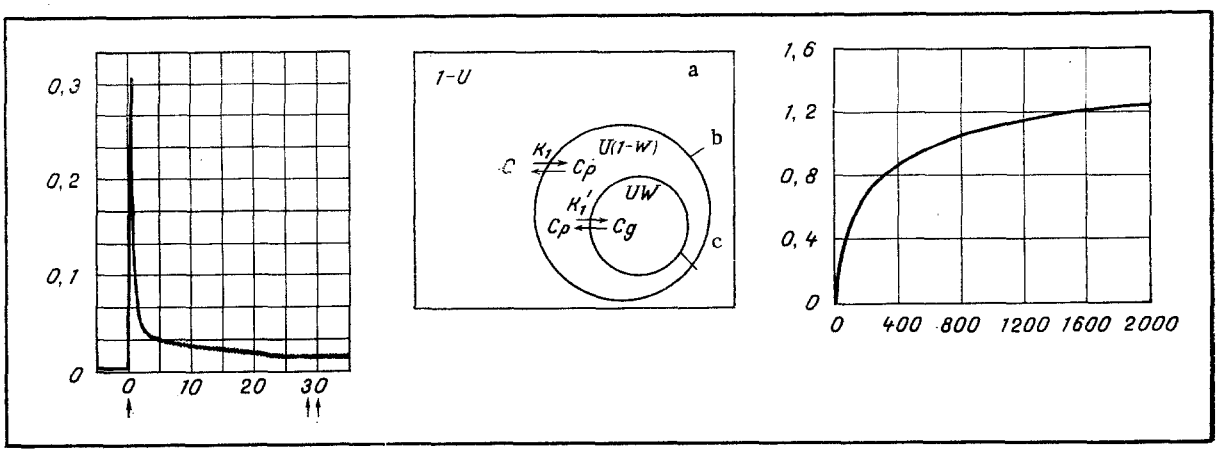

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Decrease in the intensity of fluorescence during acridine orange accumulation by intact rabbit platelets. Abscissa, time (in min); ordinate, intensity of fluorescence (in relative units). Arrow indicates time of addition of 10 μM acridine orange, two arrows indicate time when equilibrium distribution of the dye is established.

Fig. 2. Model of acridine orange accumulation by intact platelets: a) plasma, b) platelet, c) granule. C, Cp, Cg) Concentrations of dye in the extracellular medium, cytoplasm, and inside the granules respectively. Explanation in text.

Fig. 3. Calculated curve of fluorescence of acridine orange monomer in physiological saline. Abscissa, acridine orange concentration (in μM); ordinate, intensity of fluorescence (in relative units).

portion of the apparatus differed from the standard model in that, to avoid scattering of light in a turbid specimen and to avoid an internal screening effect, the fluorescence signal was measured from a small volume, adjacent to the front surface of the cylindrical cuvette. A combination of branch and bound search and steepest ascent [4] method was used for computer processing of the results.

EXPERIMENTAL RESULTS

As was pointed out above, during acridine orange accumulation by subcellular organelles of intact cells quenching of fluorescence is observed, because molecules of the dye in high concentrations form complexes with different spectral characteristics. The maximum of fluorescence of the complexes is shifted into a longer wavelength region (640 nm) than the maximum of fluorescence of the monomer (530 nm). The characteristic appearance of the curve of the change in the intensity of fluorescence of the monomer during acridine orange accumulation by platelets is shown in Fig. 1.

A suspension of intact cells contains three phases in which the dye may be distributed [8]: the external medium, cytoplasm, and intragranular matrix (Fig. 2). Let us determine the numerical relationship between the concentration of dye in these three phases in the equilibrium state and the initial concentration of dye C_0. Let the concentration of dye in the equilibrium state in the extracellular medium be C. In that case, its concentration in the cytoplasm will be $K1 \cdot C$, and in the granules $K1 \cdot K1' \cdot C = K2 \cdot C$, where K1 and K1' are coefficients of transfer through the cytoplasmic and granular membranes. Let the fraction of the volume of suspension accounted for by cells be U. This value is estimated in the course of the experiment by means of a microcentrifuge cuvette. The part of the cell volume occupied by granules is denoted by W. In that case the fraction of volume of the specimen accounted for by granules will be $U \cdot W$, and by cytoplasm $U(1 - W)$. If the dye is added to the suspension in concentrations C_0, the equation of conservation of mass will be as follows:

$$C_0 = C \cdot (1 - U) + C \cdot K1 \cdot U \cdot (1 - W) + C \cdot K2 \cdot U \cdot W \quad (1)$$

$$\text{or} \quad C = C_0 / [1 - U + K1 \cdot U \cdot (1 - W + K2 \cdot U \cdot W]. \quad (2)$$

An increase in the quantity of added dye C_0 by several times leads to an increase in its concentration externally, in the cytoplasm, and inside the granules by the same number of times. However, dependence of fluorescence of the acridine orange on its concentration F(C) is nonlinear. This dependence, which was obtained in a preliminary experiment, is illustrated in Fig. 3.

The intensity of fluorescence of the test specimen is made up of three components: external — F(C), cytoplasmic — F(K1•C), and granular — F(K2•C);

$$F=(1-U)\cdot F(C)+U\cdot(1-W)\cdot F(K1\cdot C)+U\cdot W\cdot F(K1\cdot K2\cdot C). \quad (3)$$

After substitution of the expression for C from equation (2) into equation (3) three unknowns remain: K1, K2, and W, and to find them a system of three independent equations is necessary. Two other equations can be obtained simply by taking two other values of C_0. Since the function F(C) is nonlinear, all three equations will be linearly independent, so that it is possible to determine the parameters K1, K2, and W.

This approach was used to estimate the relative volume of 5-HT-containing granules and the coefficients of transfer of the fluorescent dye acridine orange through the cytoplasmic and granular membranes of the platelets. To three equal specimens of platelet-enriched plasma (each 0.8 ml in volume) 8 µl of acridine orange solution was added. The final concentrations of the dye were 2, 4, and 8 µA. The samples were incubated for 40 min at room temperature. Next, the intensity of fluorescence of each sample was measured. In that way a system of three equations of type (3) could be created and solved by computer. The following results were obtained in a series of experiments for human (n = 5) and rabbit (n = 10) platelets.

Human: W = 0.14 ± 0.1, K1 = 375 ± 60, K2 = 2260 ± 382;
Rabbit: W = 0.29 ± 0.02, K1 = 225 ± 60, K2 = 30,000 ± 5550.

These results clearly illustrate species differences between the granular apparatus of human and rabbit platelets. The granular apparatus is more fully developed in the rabbit, in which it occupies 29 ± 2% of the cell volume compared with 14 ± 1% in man. The value for the relative volume of the granules of rabbit platelets agrees well with the result obtained by Pletscher et al. (30%), who used a technique of differential centrifugation [14].

The results show that the cytoplasmic membrane is also important in the accumulation process (parameter K1), but its role has hitherto been neglected. The method we have developed provides fresh prospects for research in this direction.

According to data in the literature, among the vertebrates the rabbit's platelets contain the largest quantity of serotonin, whereas in man and the guinea pig the smallest quantity was found [9]. Values for the parameter K2 (the ratio of the concentration of dye inside the granules to the extracellular concentration) demonstrate this difference in uptake ability well. The method we have developed enabled this parameter to be estimated for the first time in intact cells, using fluorometric measurements.

Clearly the method described above is simple in use and at the same time it leads to a marked increase in informativeness of the fluorescent dye method. It can be used widely for the study of objects such as the chromaffin cells of the stomach and adrenal, synaptosomes, mast cells, etc., which can accumulate and store biologically active substances in specialized organelles.

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