UPTAKE OF AMINO DERIVATIVES OF ACRIDINE BY ELECTRON-DENSE PLATELET GRANULES

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The electron-dense granules of platelets contain biogenic amines (serotonin, adrenalin, noradrenalin, and dopamine), nucleotides (mainly ADP and ATP), and calcium and magnesium ions, which they can release under the influence of various activating agents [3]. Because of the presence of a highly efficient system for transporting biogenic amines through plasma and granular membranes, platelets can accumulate these compounds in large quantities. Meanwhile, the low selectivity of the amine uptake mechanism by subcellular organelles makes it possible to study this process by the fluorescent probe method. For example, fluorescent amines such as mepacrine and acridine orange (AO), which are selectively taken up by the electron-dense granules of platelets, can be used for intravital visual observation of the electron-dense granules by fluorescence microscopy [2].

A more detailed study of the accumulation process requires quantitative analysis of changes in the fluorescence spectra of the dyes used in real time. However, when spectral changes take place in the course of counted minutes, limitations of equipment usually do not allow investigations of this kind to be undertaken. The present investigation was carried by means of an OMA-2 multichannel optical analyzer, by means of which it was possible to study the kinetics of uptake of fluorescent amines, although usually these processes take place over a period of several seconds.

EXPERIMENTAL METHOD

The measuring system used in the investigation was based on the OMA-2 instrument (Parc, USA), a tunable laser on M2100 dyes (Parc), and a microcomputer with an 8086 processor. The multichannel optical analyzer enabled the spectral curves to be recorded and transformed into digital information. The microcomputer controlled the course of the experiment and carried out mathematical analysis of the results in real time. Fluorescence was observed from the front of a constantly stirred cuvette. Measurements were made at 37°C. The spectrum was divided into components by computer, using the criterion of least squares. Let us assume that within the volume to be studied there is a mixture containing N fluorescent components, whose spectra $f_i(\lambda)$ are known. In that case, by the term analysis of the spectrum $F(\lambda)$ into its components will be understood the finding of coefficients x_i satisfying the condition of least squares:

$$\int_{\lambda_1}^{\lambda_2}\left[F(\lambda)-\sum_{i=1}^{N}x_i\cdot f_i(\lambda)\right]^2\cdot d\lambda=min.$$

By differentiating the integral with respect to each of the coefficients x_i, as a parameter, we obtain a linear system relative to x_i:

$$A\cdot x=b,\ \text{where}\ a_{ij}=\int_{\lambda_1}^{\lambda_2}f_i(\lambda)\cdot f_j(\lambda)\cdot d\lambda;$$

$$b_i=\int_{\lambda_1}^{\lambda_2}f_i(\lambda)\cdot F(\lambda)\cdot d\lambda.$$

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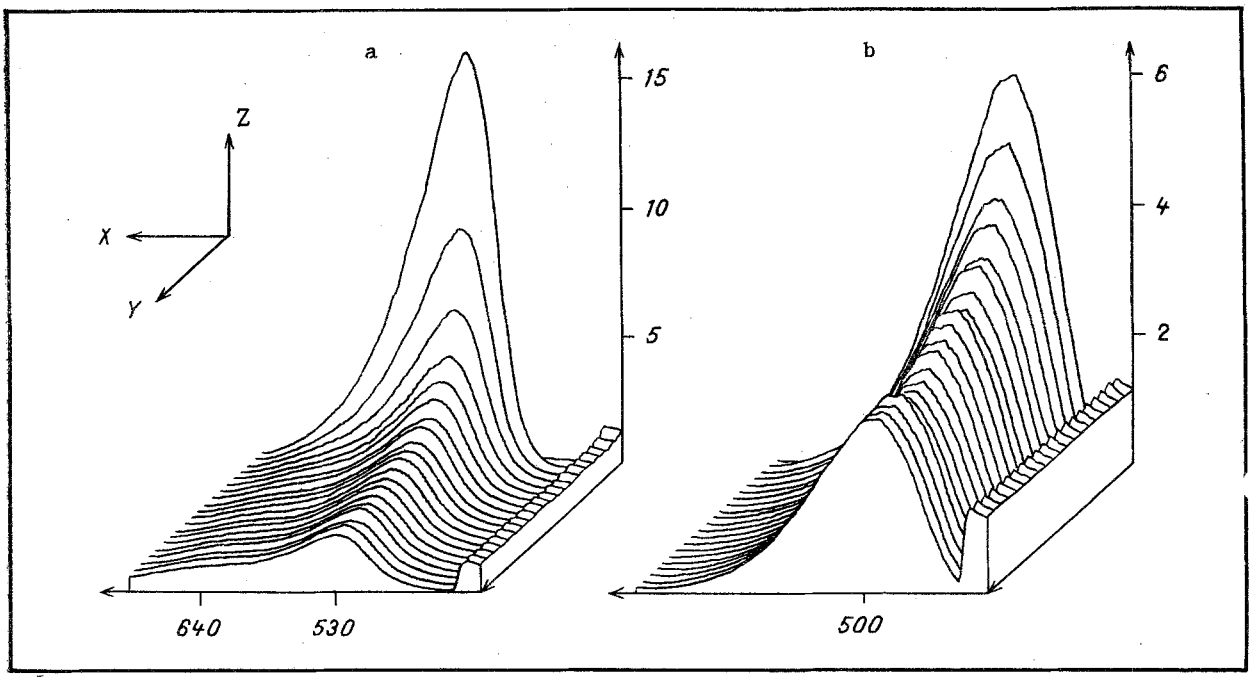

Fig. 1. Changes in fluorescence of AO (a) and mepacrine (b) during their accumulation in platelets. Abscissa, wavelength of fluorescence (in nm); ordinate, time (interval between spectra 5 sec); z axis, intensity of fluorescence (in relative units).

To solve this system during preparation for the experiment, the reciprocal matrix A^{-1} is calculated. This is possible, for the spectrum recorded $F(\lambda)$ appears only in the expression for free terms. The vector of free terms b and the required coefficients x_i of the expansion were calculated during an experiment in real time. The lower limit of integration was chosen so that the wavelengths at which secondary absorption of light (absorption of light emitted by the dye itself) gives a significant contribution, were found to be outside the limits of integration. The upper limit coincided with the longwave limit of the spectrum analyzer.

Blood was obtained from donors by puncture of the cubital vein and stabilized with 0.13 M sodium citrate solution (pH 7.4) in the ratio of 9:1. Platelet-enriched plasma was prepared by centrifugation of blood at 200g for 9 min. By the addition of autologous platelet-deprived plasma (2000g, 15 min) the cell concentration was adjusted to 200,000/ml. The platelet concentration was determined by a phase-contrast method in a Goryaev counting chamber.

During the investigations the following preparations were used: acridine orange (AO), and platelet activation factor (PAF) were from Sigma (USA); mepacrine was from BDH (England), and the remaining reagents were of the chemically pure grade.

EXPERIMENTAL RESULTS

To begin with we studied the character of changes in the fluorescence spectra of AO and mepacrine during their accumulation by platelets. Spectra within the waveband from 437 to 716 nm, recorded every 5 sec, are shown in Fig. 1 in three-dimensional form. As will be clear from Fig. 1a, addition of 15 μM AO to platelet-rich plasma caused the appearance of intense fluorescence in the 530 nm region (excitation 440 nm). In the course of accumulation of AO the intensity of fluorescence in the 530 nm region diminished by 89 ± 3% and fluorescence appeared in the longer wave region with a maximum at 640 nm. Addition of 20 μM mepacrine to platelet-rich plasma (Fig. 1b) induced fluorescence in the 500 nm region (excitation 440 nm). In the course of accumulation of the dye by the platelets the intensity of fluorescence decreased by 56 ± 2%, but the maximum and shape of the spectra remained unchanged.

Similar changes in spectral characteristics were found if the concentration of AO and mepacrine in aqueous solution was increased (physiological saline, pH 7.4). With low AO concentrations (below 50 μM, extinction at wavelength 440 nm below 1 cm^{-1}), the spectrum had a maximum of fluorescence at wavelength 530 nm. With AO concentrations of over 50 μM a second maximum began to appear on the spectrum at a wavelength of 640 nm. A change in the mepacrine concentration did not lead to any changes in shape or displacement of the fluorescence spectrum.

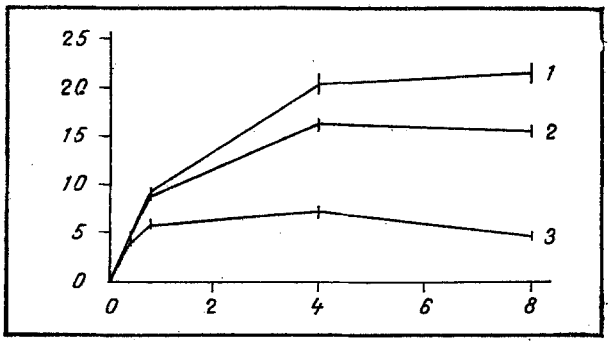

Fig. 2. Dependence of fluorescence of dyes on their concentration in physiological saline (explanation in text). Abscissa, extinction of dye solution at wavelength of 440 nm (in cm^{-1}); ordinate, intensity of fluorescence (in relative units). 1) Rhodamine 6G, 2) mepacrine, 3) AO.

No effect of pH of the medium (between 4.0 and 9.0) on spectral characteristics of the solutions of both dyes likewise could be found.

The diminution of fluorescence of AO and mepacrine during accumulation of the dyes is connected with an increase in their local concentration in the cells. It was found that the fluorescence of AO and mepacrine in physiological saline (pH 7.4) depends on their concentrations. An increase of concentration leads to a nonlinear increase in the intensity of fluorescence (Fig. 2). This may be connected with absorption of the exciting light at high extinction values and with concentration quenching.

The concentrations of the dyes were chosen so that extinction at the wavelength of excitation (440 nm) was the same. The intensities of fluorescence at E = 0.8 cm^{-1} were taken as 1. AO and mepacrine are characterized by a faster decrease in the rise in the intensity of fluorescence with an increase of concentration than rhodamine 6G. This can be explained by the formation of complexes of dye molecules, as a result of which their optical properties are changed [1]. Concentration quenching of fluorescence is more effective for AO than for mepacrine.

The fluorescence spectra of AO were divided into components. The shape of the spectrum of the first component was obtained by recording the fluorescence spectrum of a dilute solution of the dye (concentration 0.5 μM), the second component by subtracting the spectrum of the first component from the fluorescence spectrum of a concentrated solution of AO (500 μM). Later these spectral curves were used for determination of the weighting factors of each component in the combined spectrum by computer.

The kinetics of the change in each component of the spectrum during accumulation of 15 μM AO and after treatment of the platelets with 500 nM PAF is shown in Fig. 3. After the addition of AO to the sample of plasma, the intensity of the first component diminished (Fig. 3, 1). Parallel with this, a long-wave component of the spectrum appeared and subsequently increased in intensity (Fig. 3, 2). After treatment of the cells with PAF (a substance which induces release of the contents of the electron-dense granules), the change in fluorescence of the first component began after a delay (t = 10.0 ± 2.5 sec) relative to the time of addition of PAF. The change in fluorescence of the long-wave component began without any visible delay.

During accumulation of the dye by the cells it was unevenly distributed in the various cellular structures, and the local concentration of the probe could reach high values. This phenomenon should lead to quenching of fluorescence of the dye at sites where it was present, and in the case of AO, to the appearance of fluorescence in the long-wave region of the spectrum, which was indeed observed experimentally. The concentration of dye in the granules may exceed its concentration in the extracellular medium by several thousand times, and its concentration in the cytoplasm by tens of times [4, 5].

The presence of two components in the fluorescence spectrum of AO, whose weighting factors depend on the concentration of the probe enables the mechanism of accumulation of monoamines by the various cell compartments to be studied. This naturally makes certain demands on the apparatus used in the experiment. In the present case the most important demands are:

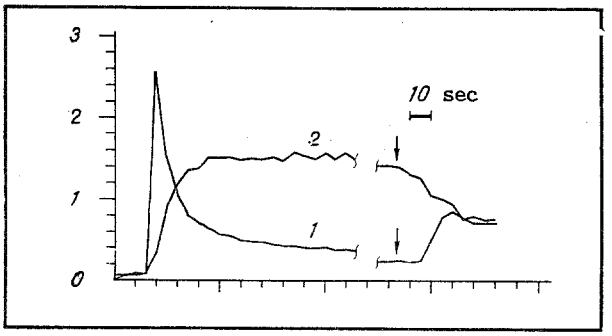

Fig. 3. Dependence of fluorescence of two components of the AO spectrum on incubation time with platelets before and after addition of PAF. Abscissa, time (in sec); ordinate, fluorescence (in relative units). 1) Change in fluorescence of short-wave component of spectrum (wavelength 530 nm), 2) change in fluorescence of long-wave component of spectrum (wavelength 640 nm). Interval of 15 min between AO accumulation and release curves; arrow indicates time of addition of PAG (500 nM).

1) ability to obtain fluorescence spectra during a period of time shorter than that for which the process lasts; 2) ability to analyze the spectra obtained into components.

In the present investigation the use of a computerized method of analysis of the spectrum into components made it possible to record the kinetics of the change in components of the spectra of AO during its accumulation by and release from the platelets in real time (Fig. 3). The decrease in fluorescence in the shorter-wave region and its appearance in the long-wave region took place immediately after the beginning of incubation of the dye with the cells. The observed absence of a lag-period in the change in intensity of fluorescence of the second (long-wave) component during release of AO from the platelets after their treatment with PAF (Fig. 3), must be evidence of a rapid decline in the local AO concentration in the platelets. However, the presence of a lag-period in the change in the first component means that at this time the concentration of the dye outside the cells was not increased. It can be tentatively suggested that before the beginning of release, the dye is redistributed within the platelets. The possibility cannot be ruled out that this process is intimately connected with centralization of the granules in the course of the release reaction.

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