# Kinetics of the Accumulation of Aluminum(III)-Sulfophthalocyanine by Human Leukocytes Measured with a Scanning Flow Cytometer

I.G. Scribunov*, P.A. Tarasov, K.A. Semianov, V.P. Maltsev, A.V. Chernyshev, Institute of Chemical Kinetics and Combustion, Institutskaya 3, Novosibirsk 630090, Russia.
E.R. Chernych, M.V. Tichonova, Institute of Clinical Immunology, Yadrintsevskaya 14, Novosibirsk 630099, Russia.
C.D. Nikonov, Siberian Center of Laser Medicine, Vavilova 14, Novosibirsk 630122, Russia.

## ABSTRACT

The kinetics of aluminum(III)-sulfophthalocyanine uptake by human leukocytes was measured with a scanning flow cytometer (SFC) during the initial period of accumulation, 40 min. The individual cells were distinguished by SFC from theirs light scattering traces. The dye fluorescence in the cells was excited by N2 pulse laser, and the kinetics of the cell distribution on the amount of the accumulated dye was obtained. A mathematical model of endocytosis was applied in order to describe the dynamics of cell distribution in the system during the cellular uptake. The main kinetic parameters of the dye accumulation were evaluated.

**Keywords:** sulfonated aluminum phthalocyanine, photodynamic therapy, cellular uptake, scanning flow cytometry.

## 1. INTRODUCTION

Phthalocyanines are second-generation photosensitizers, which absorb strongly at 675 nm within the "therapeutic window". The photosensitizing properties of phthalocyanines *in vitro* have been described for a variety of tumors, and aluminum phthalocyanine, AlPc, was found to be more efficient than hematoporphyrin derivatives.[1-3, 8-10] Most phthalocyanines are not soluble in water. This problem can be solved by synthesizing and applying sulfonated derivatives. Sulfonated aluminum phthalocyanine, AlPcS, is rather effective photosensitizer for PTD.[4-8, 11-15] With AlPcS, changing the state of sulfonation alters the pharmacokinetics and localization properties *in vivo*.[9-12, 16-19] Effective clinical trials for solid tumor destruction of PDT with AlPcS of mixed sulfonation, *Photosens*, have been reported.[13, 20]

In many cases photosensitizers are delivered to tumors via a lipoprotein receptor-mediated pathway.[7, 14] But that is not the only possible route: pinocytosis and passive diffusion and could also contribute to the drug uptake.[1, 10] Nevertheless, it is known[3, 15] that the cellular uptake of such photosensitizers as hematoporphirin derivatives is provided whether by receptor-mediated or direct endocytosis.

Since AlPcS has a wide potential application for PDT, the kinetics of AlPcS uptake by different cells is under intensive investigation *in vitro* and *in vivo*.[4-20] Generally, in such experiments, the long-time (a few hours) uptake dynamics is studied, but the dynamics of the cell population during the initial (first 40 minutes) uptake remains unclear.

Flow cytometry is widely used for the kinetic study of endocytosis[11-14, 16-18] and photosensitizer accumulation.[1, 10] A flow cytometer provides the measurement of the cell distribution on the amount of accumulated dye. Recently, a scanning flow cytometer[19-22] was developed, and it expanded the performance of an ordinary flow cytometer to distinguish cells from theirs light scattering traces.

This paper presents the application of the scanning flow cytometry for the study of the cellular uptake in a complex cell system. We investigated the kinetics of the uptake of sulfonated aluminum phthalocyanine of mixed sulfonation (Photosense) by human leukocytes during the initial 40 minutes of the process. For this purpose we have combined scanning flow cytometry with fluorescence assay based on pulsed $N_2$ laser.[22] The population of leukocytes was considered as a complex cell system, in which different cells (lymphocytes, monocytes, neutrophils, eosinophils, basophils) have different rate of uptake. The kinetics of cell distribution on the amount of accumulated dye was measured, and a statistical mathematical approach was applied in order to describe the kinetics of photosensitizer uptake in such complex system.

* E-mail: Ilya@cyto.kinetics.nsc.ru


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## 2. MATERIALS AND METHODS

### 2.1 Cells

Leukocytes were obtained from heparinized peripheral blood of healthy donors in the Institute of Clinical Immunology, Novosibirsk, Russia. Cells were washed three times and resuspended in the buffered saline (0.01 M phosphate buffer, pH 7.2 with 0.15 M NaCl). All kinetics experiments were carried out at room temperature 22°C.

### 2.2 Chemicals

AlPcS, Photosens, was obtained as a powder (NIOPIK, Moscow, Russia). The wavelength 337 nm of the pulse $N_2$ laser fits the short-wavelength band of the absorbance spectrum of the dye. A stock 0.05% solution of AlPcS in water isotonic buffered saline was prepared and kept in the dark. For experiments the stock solution was diluted to the concentrations 5 μg/ml.

### 2.3 Scanning Flow Cytometer

The optical system of the SFC was discussed in details elsewhere [19-21] and presented in Fig.1. The SFC was equipped with the pulse $N_2$ laser 1 (wavelength 337 nm, average power 10 mW, pulse duration 10 ns) to excite the fluorescence of the dye in a cell. A 650 nm emission (longpass) filter was used to detect AlPcS red fluorescence. The He-Ne laser 2 (632nm, 10 mW) provided the trigger pulse to control the location of a cell within the optical cuvette. The same He-Ne laser 3 was used to measure the light scattering pattern. The optical set-up allowed the measurement of the light scattering pattern at polar angles ranging from 5° to 100° with integration over the azimuthal angles from 0° to 360°. The angular resolution of the indicatrix measurement was about 0.5°. The data acquisition system provides accurate measurement of the indicatrix with a rate of 300 particles/s. The SFC allowed the measurement of the light scattering patterns with the rate of 300 particles per second.

### 2.4 Measurement of dye uptake by Scanning Flow Cytometer

Samples contained $3x10^5$ cells/ml were incubated 60 or 30 min at room temperature (22°C) with fresh dye-containing medium at specified concentrations. During the incubation the cells were continuously taken from the sample and measured by Scanning Flow Cytometer. Fluorescence background signal from an environmental medium of a cell was negligible because of the mask with pin-hole in front of PMT2 and exciting the pulse $N_2$ laser exactly on a cell. As a result, the kinetics of cell distribution on the amount of accumulated dye was obtained from the experiments.

## 3. RESULTS

Light scattering traces and fluorescence from single cells were measured by SFC during the process of incubation of leukocytes with the dye-containing medium. On the record of the experimental signal (Fig. 2) the light scattering trace of a cell is followed by the trigger pulse and the fluorescence signal. The trigger signal was appeared when a cell crossed the orthogonal laser beam, and corresponds to the certain location of the cell in the measuring zone of the SFC. Fig 3. shows examples of light scattering traces of different human blood cells measured by the SFC. These examples demonstrate the potential ability of the SFC to distinguish different types of blood cells measuring light scattering traces.

It is shown in Fig. 4 that the leukocytes were distinguished into two main populations by means of the integrated intensity of a light scattering trace. The integrated intensity was calculated by integrating the light scattering trace over time started from the reference point (trigger signal). In our experiments, the integration of the light scattering trace over time corresponds to the integration of the light scattering pattern over angle from 5 to 60 degree. The populations were called as "small" and "large" leukocytes, respectively.

The kinetics of cell distribution on the amount of accumulated dye was obtained for different concentration of the dye ($8x10^{-6}$ M and $1.6x10^{-5}$ M) and for "small" and "large" leukocytes separately. Fig. 5 shows the example of the kinetics of cell distribution for "large" leukocytes.

## 4. DISCUSSIONS

The present study demonstrates that scanning flow cytometer is a useful and reliable technique in the kinetic analysis of cellular uptake. The distinction of cells is done on the basis of their light scattering traces (Fig. 2 and Fig. 3). The light scattering trace of a cell corresponds to the light scattering pattern transformed by an apparatus function of the SFC. The inverse transformation of the trace to the scattering pattern can be done easily, taking into account the reference time point provided by the trigger signal. The light scattering trace is a native characteristic of a cell and it can be used for cell separation.

According to the size of cells, all leukocytes in the sample can be distinguished into two main populations: "small" leukocytes (lymphocytes, 6 - 9 μm) and "large" leukocytes (monocytes and all granulocytes, 10 – 13 μm). Taking into account the relative amount of different types of leukocytes in the sample, it is possible to consider two main populations - lymphocytes (16-48%) and neutrophils (50-70%). The amount of all other leukocytes is less then 13 %, and all of them are large cells (monocytes, eosinophils, and basophilsand). Since all small leucocytes (6-9 micron) are lymphocytes, and the main amount of large leukocytes (10-13 micron) are neutrophils, "small" and "large" leukocytes may be considered for some approximation as lymphocytes and neutrophils, respectively.

The intensity of light scattering pattern depends strongly on cell size, and Fig. 4 shows that it is possible to distinguish leukocytes on "small" and "large" cells using the integral of the light scattering trace over time. The numeric estimation gives the following value of the relative amount of "small" leukocytes in our experiments:

$$\frac{\textit{Amount of "small" leukocytes}}{\textit{Total amount of leukocytes}} \approx 0.39$$

This value is in a good agreement with the typical relative amount of lymphocytes in human blood.

For the kinetic evaluation, it is assumed that AlPcS is taken up by endocytosis, according to the formula[23] for a single cell:

$$\frac{dQ}{dt} = \nu\left(1+\frac{\theta R}{K+C}\right)C - kQ \qquad (1)$$

where $Q$ is the amount of the dye taken up per unite volume of the cell, in 1/cm$^3$; $t$ is the time, in min; $\nu$ is the pinocytotic rate, in min$^{-1}$; $\theta$ is the ratio of the average surface to the average volume of pinocytic vesicles, in cm$^{-1}$; $R$ is the concentration of binding sites on the membrane, in 1/cm$^2$; $C$ is the AlPcS concentration, in 1/cm$^3$; $K$ is the AlPcS concentration giving half-saturation of the binding sites; $k$ is the rate constant of a first-order process (exocytosis) of degradation of stored AlPcS, in cm$^3$/min. If all other variables remain constant, the kinetics of cellular uptake is represented by the following equation

$$Q = Q_0\left(1-e^{-kt}\right) \qquad (2)$$

where

$$Q_0 = \frac{\nu}{k}\left(1+\frac{\theta R}{K+C}\right)C \qquad (3)$$

It follows from our experimental data that we can neglect the autofluorescence of cells, since we used high concentration of the dye in the medium. Generally, the Eq. (2) is used to describe the kinetics of the mean value of accumulated dye in a homogeneous cell population.

If the concentrations of the dye in the medium is high, i.e.

$$\frac{\theta R}{K+C} << 1 \qquad (4)$$

then the amount of the accumulated dye is the linear function of the concentration:

$$Q_0 = VC \qquad (5)$$

where $V$ is the effective uptake volume:

$$V = \frac{\nu}{k} \qquad (6)$$

We applied Eq. (2) to fit the experimental data on the kinetics of mean fluorescence for "small" (Fig. 5) and "large" (Fig. 6) leukocytes, separately, in order to find the accumulation time $\tau$:

$$\tau = \frac{1}{k} \qquad (7)$$

As a result, shown in Table 1, it was found that the accumulation time $\tau$ is shorter for "large" leukocytes than for "small" ones. Moreover, the accumulation time is rather different for different cells within a population, since we observed wide distribution of accumulated dye for both populations of "large" and "small" leukocytes. In order to obtain the information about the differentiation of cells on the uptake parameters (accumulation time, amount of accumulated dye), a statistical approach should be applied for the treatment of the experimental data.

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**Table 1**. Parameters obtained by fitting from the kinetics of cellular uptake.

| | Lymphocyte | Nutrophils |
|---|---|---|
| τ, min | 11.6 ± 2.5 | 8.3 ± 2.2 |

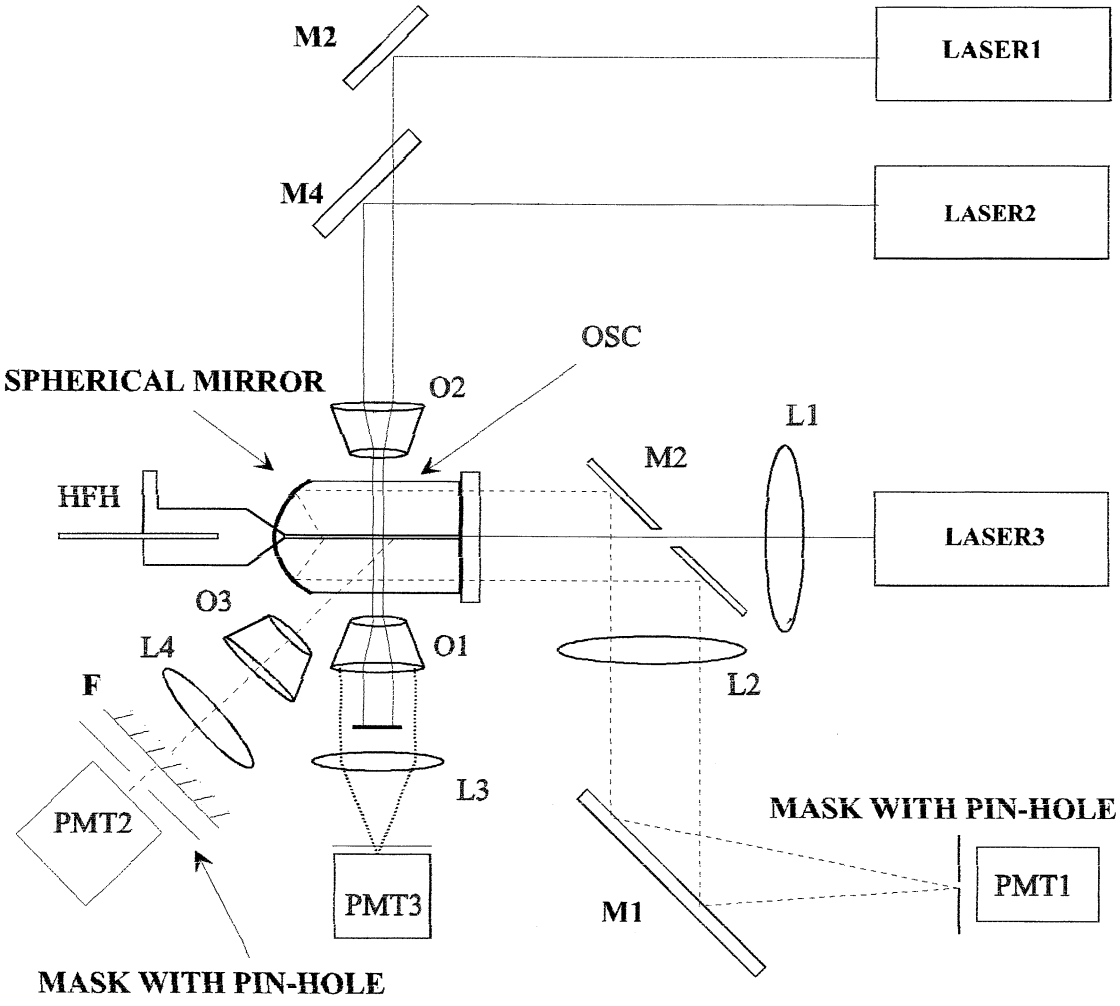


**Fig. 1.** Optical system of the scanning flow cytometer. The following elements are shown: laser1-3, lenses (L1, L2, L3, L4) , hydrofocusing head (HFH), photomultiply tubes (PMT1, PMT2, PMT3), objectives (O1, O2, O3), optical scanning cuvette (OSC), longpass filter on 650 nm. (F), plain mirrors (M1, M2, M3) and dichroic mirrors (M4).

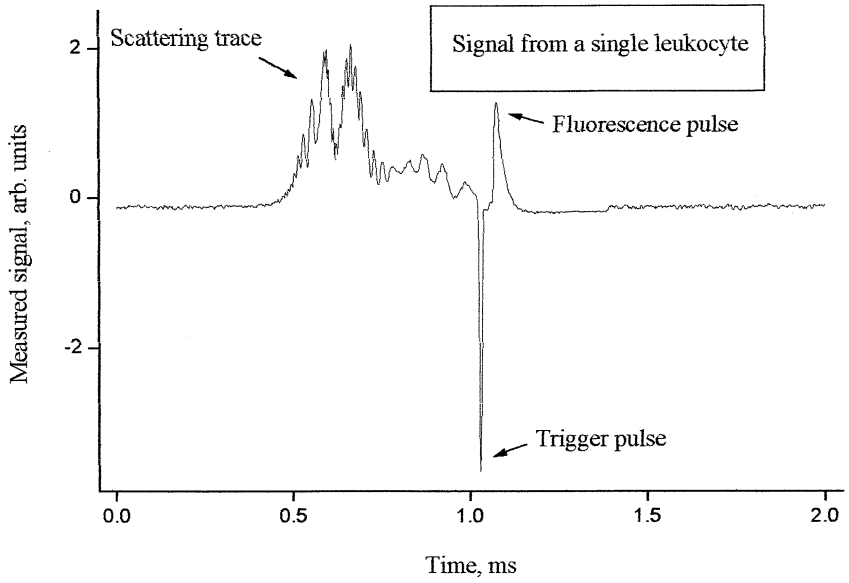


**Fig. 2.** Experimental signal from a single cell.

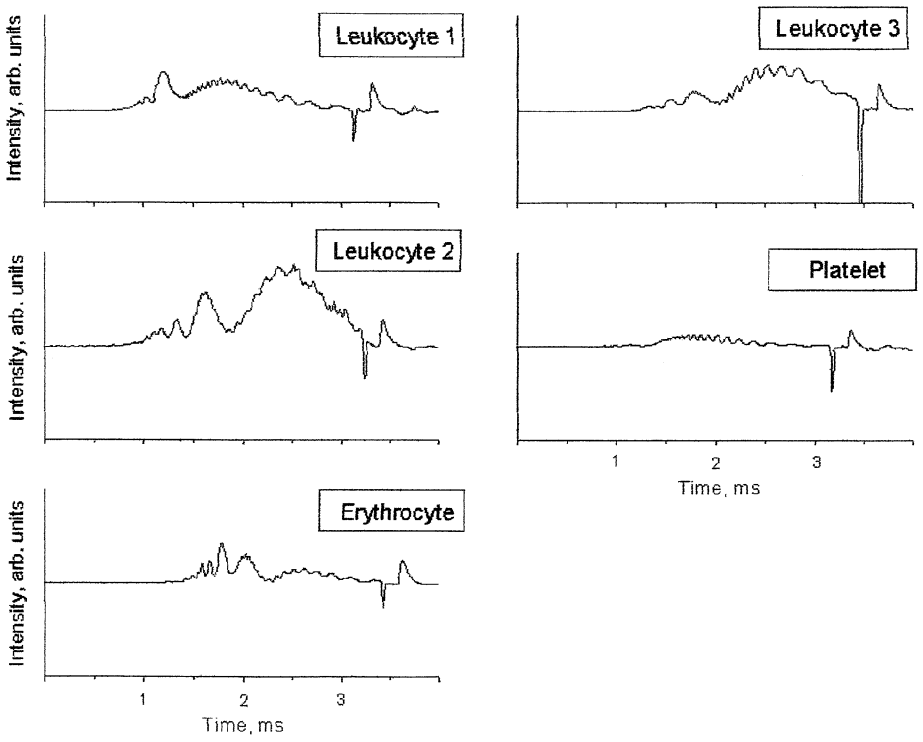


**Fig. 3.** Examples of light scattering traces of different human blood cells measured by the Scanning Flow Cytometer.

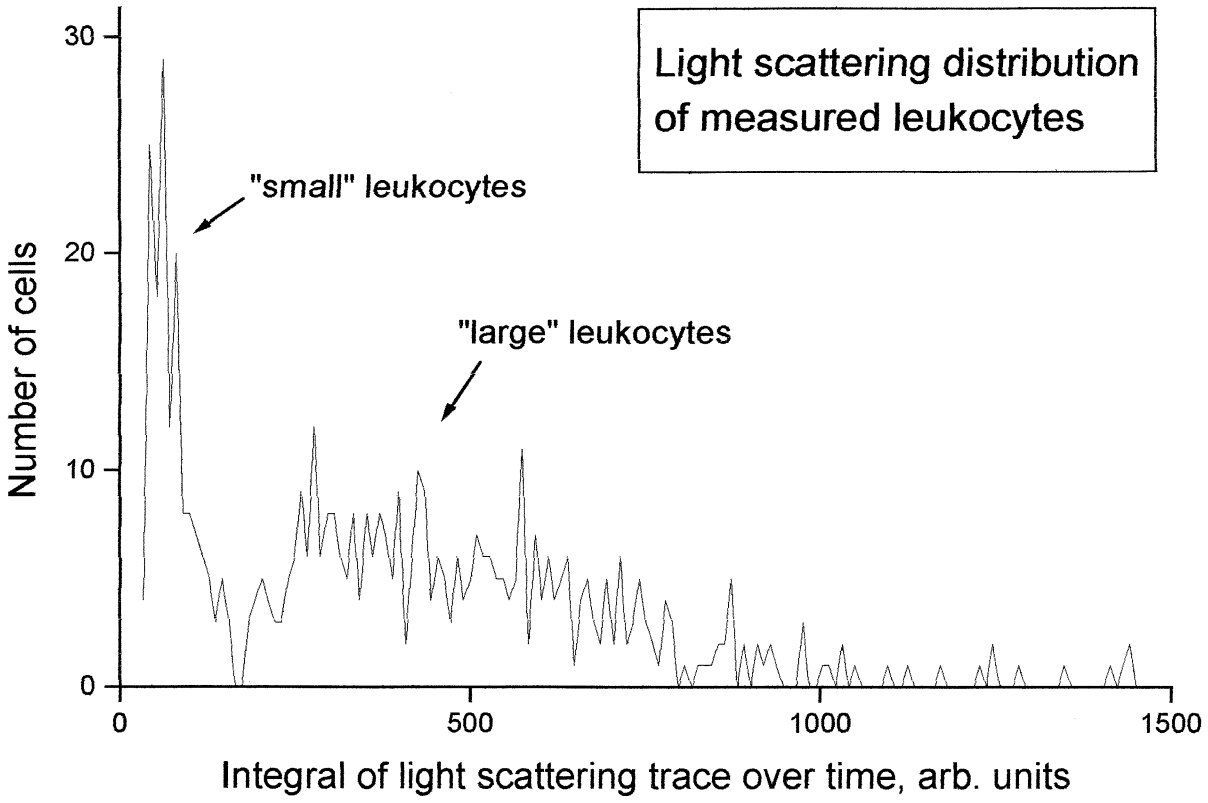


**Fig. 4.** Distribution of leukocytes on the intensity of light scattering traces.

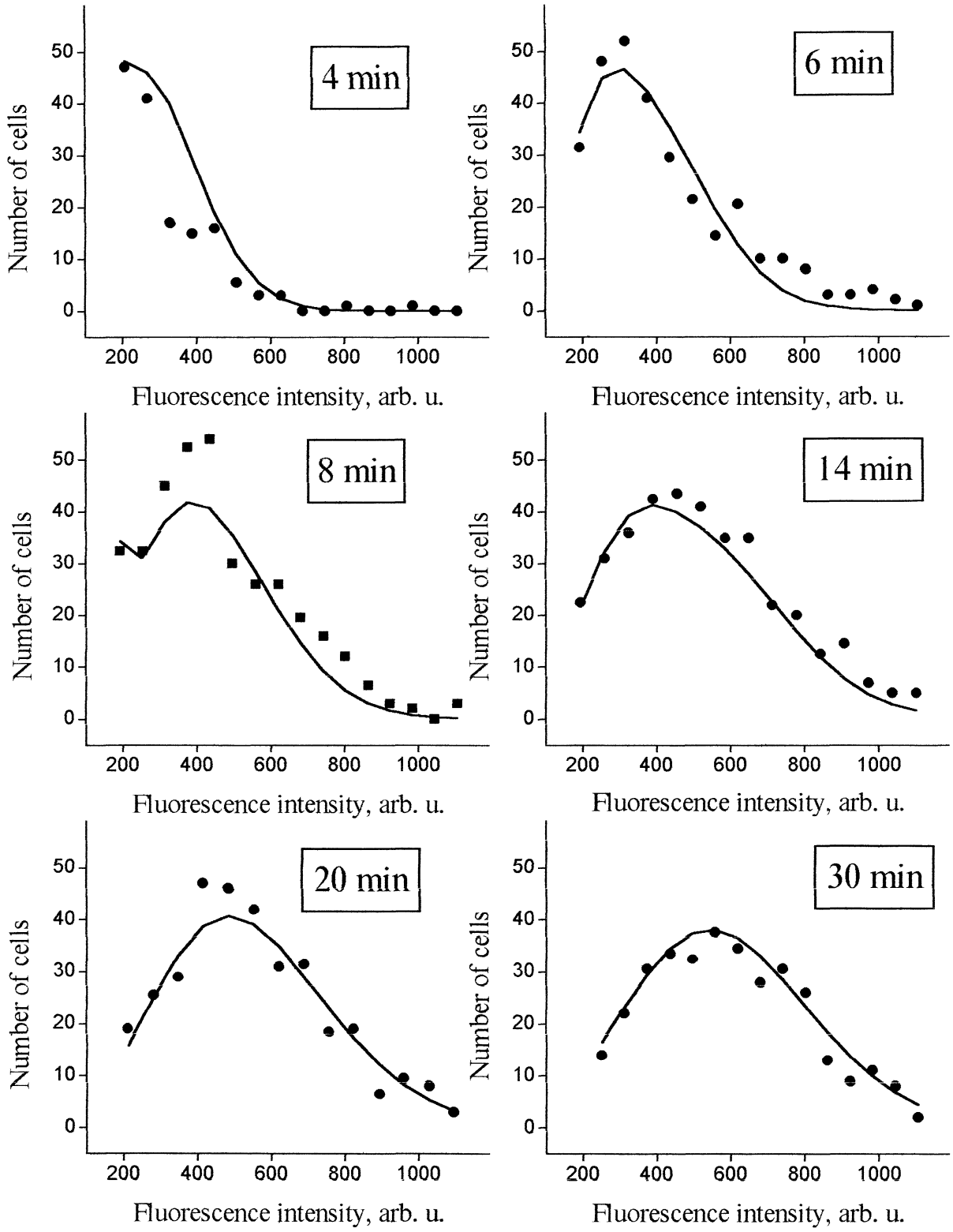


**Fig. 5.** Example of the kinetics of fluorescence intensity histogram for "large" leukocytes (neutrophils); photosensitizer concentration - 5μg/ml (points – experiment, solid line – theory).

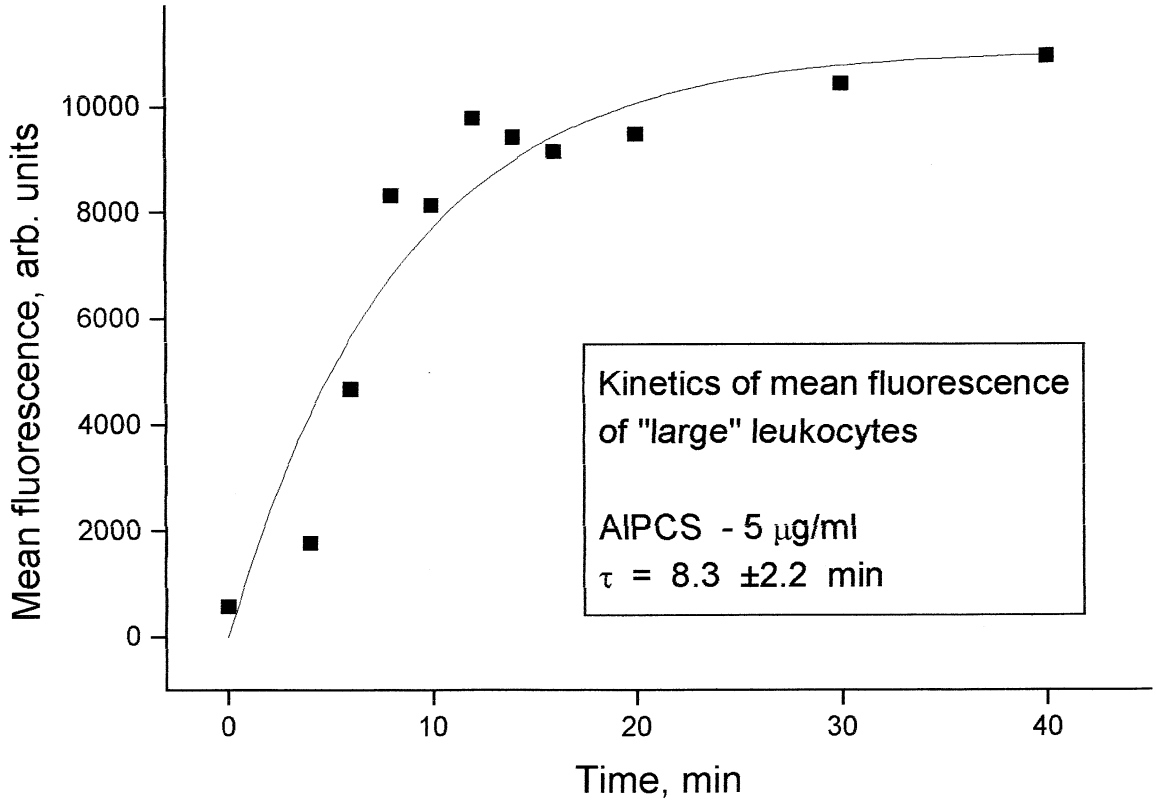


**Fig. 6.** Kinetics of the mean fluorescence intensity for "large" leukocytes (points – experiment, solid line – theory).

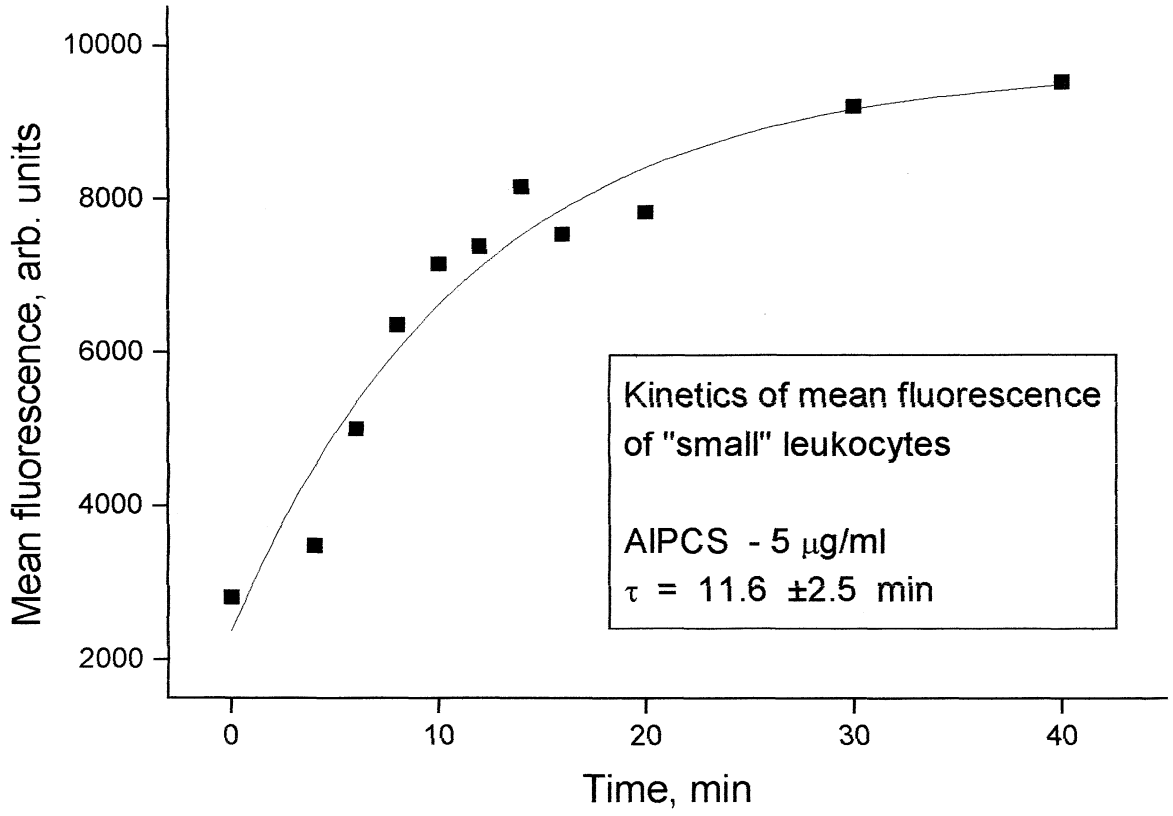


**Fig. 7.** Kinetics of the mean fluorescence intensity for "small" leukocytes (points – experiment, solid line – theory).