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A new method for studying platelets, based upon the low-angle light scattering technique. 1. Theoretical and experimental foundations of the method

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Abstract. A new method is presented for simultaneous monitoring of changes in shape and aggregation of platelets. The signal of light scattering alterations at angles below six degrees was shown to be determined by platelet aggregation dynamics (aggregation, disaggregation, coagulation). Over a range of larger angles (6–15 degrees), cell shape changes also contributed to the signal: (i) spherization, and (ii) pseudopodia formation. The first stage was shown to be fast ($t_{1/2}$ of few seconds) and correlated with [Ca^{2+}] increase. It was characterised by a narrow signal fluctuation and by a rapid increase (30–40%) in signal intensity. During the second stage, which was much slower, the signal decreased describing the aggregation process. The EC_{50} value for ADP-induced spherization was 40 nmol l^{-1}. Aggregation kinetics in saline solution under turbulent flow showed second order kinetics in relation to initial cell concentration. The rate constant depended on stirring conditions and on calcium concentration in the medium. Standardisation of the testing conditions made it possible to characterize the initial functional state of platelets by their sensitivity to agonists, with estimation of EC_{50} and maximum velocity of aggregation (U_{max}) values. The method has potential applications in pharmacology and toxicology research and in clinical practice, as a simple and highly sensitive functionality test for platelets.

Keywords: Platelets, aggregation, light scattering, hemostasis, spherization

1. Introduction

Platelets are an excitable population of blood cells, critical for the processes of coagulation, reparation of vascular wall, deposition and transport of biologically active substances carrying out the immune reactions [1]. This polyfunctionality of platelets is due to the existence of many different receptor complexes on their plasma membrane. It has been shown that there are about 30 sites for interaction of

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agonists of different chemical nature (purines, catecholamines, tryptophane derivatives, prostaglandins, peptides, etc.) [2–6].

Platelets, being highly-labile structures, can be activated or inhibited under small influences. Despite the prevailing understanding of the role of platelets in the general system of hemostasis, and a variety of methods for investigating their functionality, there remains a need for simple quantitative estimation of their sensitivity to the basic physiological agonists: adenosine diphosphate (ADP), thrombin, platelet activating factor (PAF), etc. For a long time, the basic method of studying platelets has been the method of Born – registering the aggregation of cells by changes in light transmission [7]. The advantages of the method are its simplicity and low requirements for equipment. However, the method is only semiquantitative: treatment of the data is reduced to relative evaluation of finite signal amplitude, which in practice does not characterize a change in the sensitivity of platelets to the agonists. There are also difficulties associated with Born's method when comparing results obtained from different series of experiments.

In 1994, a new method for assessment of platelet aggregation with the help of light scattering technique was described [8]. The method enabled recording of backward scattering from separate cells or their aggregates consisting of up to 100 cells (or even more); aggregates were classified into classes. The method was suitable for measurements on platelet rich plasma and has subsequently been applied to a range of aggregation studies, e.g. see [9–13]. Limitations of the method are the inability to register the shape change of platelets and the very restricted application in registering kinetic processes (especially the fast ones).

In the present work we report on a low-angle light scattering method for qualitative and quantitative assessment of the functional status of platelets. Both theoretical and experimental foundations of the method are discussed.

2. Materials and methods

2.1. Preparation of platelet rich plasma (PRP)

Blood from male Wistar rats, which were preliminary narcotized with nembutal at the dose of 40 mg kg^{-1} or with urethane at the dose of 1 g kg^{-1}, was taken from the carotid artery; from the boundary vein of "chinchilla" rabbits and, in man, from the cubital vein. Sodium citrate (3.2%; pH 6.0) was used as anticoagulant at a 9 : 1 (blood : citrate) ratio. The PRP was obtained by centrifugation of the blood for 10 min at 200g.

2.2. The light scattering measuring device

The intensity of the scattered light was measured with a Laser Particle Analyzer, "LaSca" (Lumex Ltd, St-Petersburg, Russia). A schematic of the analyzer is shown as Fig. 1. The light source (1) illuminated a suspension of cells in a transparent cuvette (2), which was mixed and thermostatically controlled (3). The intensity of light scattered at various angles was detected with a multiple photodiodes array (4); the photodiodes were located at different angles from 1 to 14 degrees with a step of 0.5 degree. A multichannel 24-bit sigma-delta ADC (5) converted the analog signal from the photodiodes, amplified the signal and transmitted to a computer. A special algorithm with original software, LaSca_32, was used to register the data and to calculate experimental parameters in Excel. The magnetic stirring device used in the cuvette had a special cylindrical construction, without blades or paddles. It ensured a homogeneous

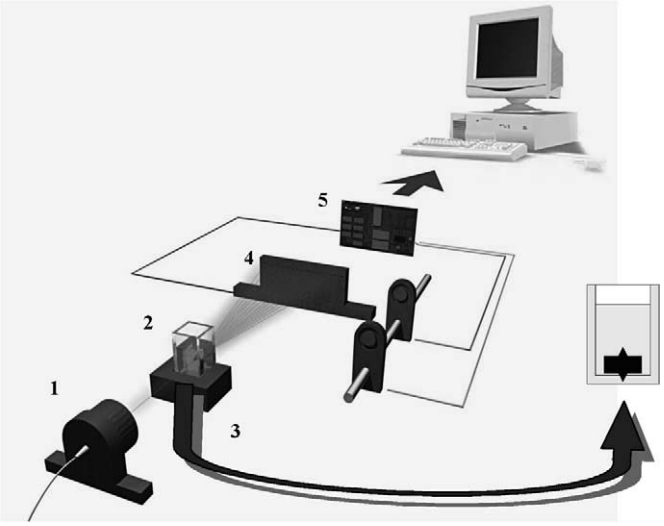

Fig. 1. Schematic of low-angle light scattering device: (1) light source – laser diode at 670 nm; (2) cuvette with stirrer bar; (3) magnetic stirrer and thermostat; (4) photodiode array and multiple channel amplifiers; (5) analogue-to-digital converter (ADC).

isotropic turbulent mixing of the suspension without formation of bubbles; the level of signal oscillations was no more than 1–2%.

3. Results and discussion

3.1. Use of the low-angle light scattering technique for investigating platelets

Born's method is based on measurement of multiple-scatter of light upon aggregation of platelets; detailed studies on multiple-scattering have been reported [14]. Our analysis by Born's method used cell concentrations of 2.5×10^8 and 1.0×10^9 ml^{-1}. The ratio of the intensities of light at angles of 0 and 2 degrees was enhances with the formation of small aggregates (2 to 10 cells per aggregate), and then dropped with further increase in aggregate size, i.e. results obtained by Born's method depended strongly on the aperture of the recorded angle. Furthermore, the method did not clearly distinguished aggregation from coagulation (the activation of fibrinogen and subsequent formation of agglomerate). The dynamics of signals in these processes is of the same direction and, therefore, the treatment of the results obtained by Born's method is reduced to the relative assessment of the finite signal amplitude. Kinetic parameters are essentially not recorded; at best the two-phase nature of the process of aggregation is noted.

To avoid the uncertainties connected with multiple-scattering of light, experiments were conducted under single-scatter conditions. The retention of a linear dependence between the intensity of light scattering and the particle concentration is the criterion of single-scattering [15]. Figure 2 shows that this dependence was obtained for cell concentrations of $\leqslant 10^7$ cells ml^{-1}. It is also noted that the lower the angle of light registration, the lower the cell concentration at multiple-scattering is observed (Fig. 2). Thus, the criterion for single light-scattering in the method is ensured by dilution of PRP 30 to 100-fold in physiological isotonic salt medium of the following composition: NaCl, 140 $mmol\,l^{-1}$; Tris-HCl buffer (or HEPES-buffer, pH 7.4), 10 $mmol\,l^{-1}$; $CaCl_2$, 1 $mmol\,l^{-1}$.

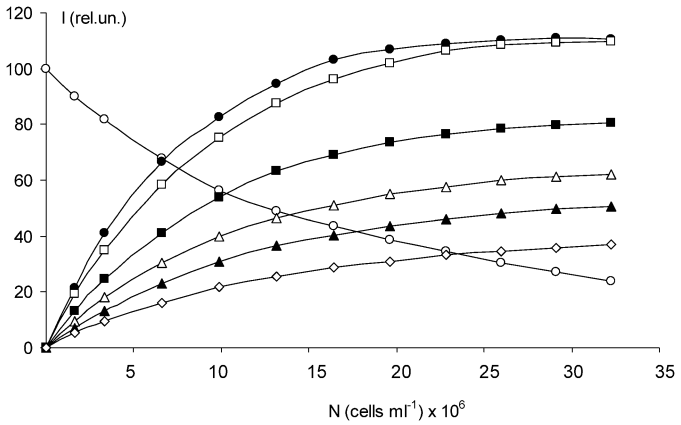

Fig. 2. Dependence of light intensity at various angles [$I(0)$, $I(2)$–$I(10)$] upon concentration of human platelets in NaCl-medium. Angles (degrees): 0 (○), 2 (●), 3 (□), 4 (■), 5 (△), 7 (▲), 10 (◇).

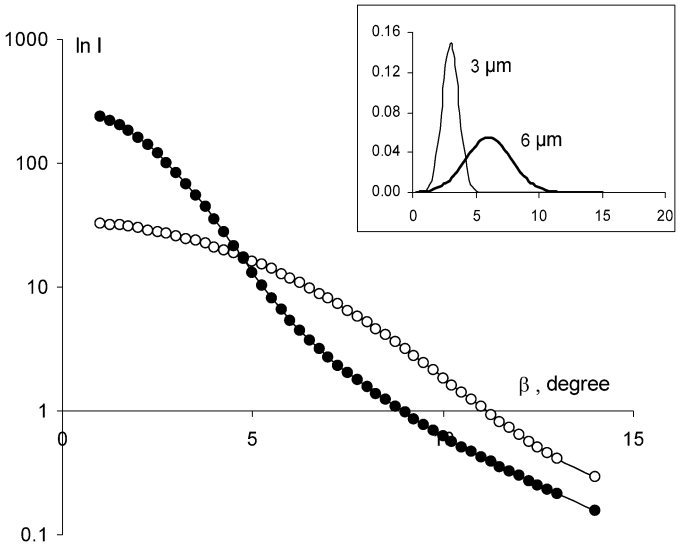

Fig. 3. Calculated angular diagram for particles of 3 (○) and 6 μm (●) diameter with the assigned characteristics of particle distributions according to size (insert). The calculated intensity for the 6 μm particles is reduced by a factor of two (model of dimmer formation from monomers). Light scattering angle, β.

The theory of single light scattering (Mie theory) describes dependence of the scattered light intensity upon relation of the particle size to the wavelength of the source of light (the so-called diffraction parameter, $\rho = \pi d/\lambda$, where d is diameter of particle). This dependence can be represented in the form of a special angular diagram. For several particular cases, there are approximated equations (approximation of Rayleigh–Debye–Gans for small particles $\rho \sim 10$; approximation of Fraunhofer for $\rho \gg 10$). Thus the scattering of 670 nm wavelength laser light is satisfactorily described for particles with sizes up to 10 μm by the approximation of Rayleigh–Debye–Gans; represented in the monographs of Shifrin [16], van de Hulst [15] and Bohren [17].

Figure 3 shows the dependences of logarithm of intensity ($\ln I$) upon the scattering angle (β), calculated according to the equation of Rayleigh–Hans, for particles of 3 μm diameter (approximate model

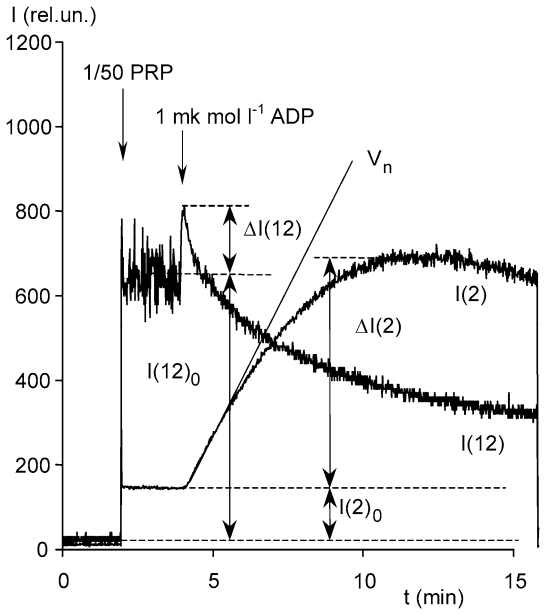

Fig. 4. Dynamics of change in intensity of light scattering by platelets under the action of ADP, at angels of 2 and 12 degrees. Intensity of scattered light at angels of 2 and 12 degrees, $I(2)$ and $I(12)$ respectively; increase in the intensity of light diffusion, ΔI; initial velocity of aggregation, V_n (evaluated throughout a change of intensity of scattered light at an angle of 2 degrees).

for thrombocytes), and particles of 6 μm diameter (approximation for the dimer). The absolute intensities of light in the latter case are two-fold lower, which is caused by decrease of particle (monomer) concentration with formation of dimers. These calculated dependences are represented for the particles with the assigned particle-size distribution functions (Fig. 3, insert), which leads to smoothing of diffraction pattern. The calculated characteristics make it possible to predict the dynamics of the aggregative response: at very low angles (to 5 degrees) the intensity will increase upon aggregation, whereas at larger angles (about 10 degrees) the intensity will be reduced. The process of disaggregation will give a reverse picture. Use of wide-aperture photodiodes [18] for registering is not adequate, since the dynamics of the signals obtained does not allow unambiguously treatment of aggregation and coagulation.

The dynamics in the intensities of the light scattering under the action of ADP is shown in Fig. 4. Introducing PRP into the salt medium lead to increase in the initial signals of $I(2)$ and $I(12)$, in proportion to cell concentration in the cuvette. A rapid increase of the intensity of light scattering at an angle of 12 degrees upon action of the inductor of aggregation is connected with spherization of platelets, caused by raising the level of intracellular calcium; the subsequent decrease of the value reflects formation of pseudopodia and development of aggregation. The differently directed dynamics of signals in the different angles upon the aggregation reflects the forecast behavior of signals, made in the model calculations (Fig. 3). Figure 4 also illustrates the calculated parameters – $I(12)$, $I(2)$, ΔI, V_n – used for quantitative estimations.

3.2. Study of shape change by the low-angle light scattering method

3.2.1. Identification of light scattering signals

The dependence of light scattering intensity upon concentration of platelets is linear over the range from 10^6 to 10^7 cells ml^{-1} in the saline medium, using the 20 mm cuvette and angles of 1–15 degrees

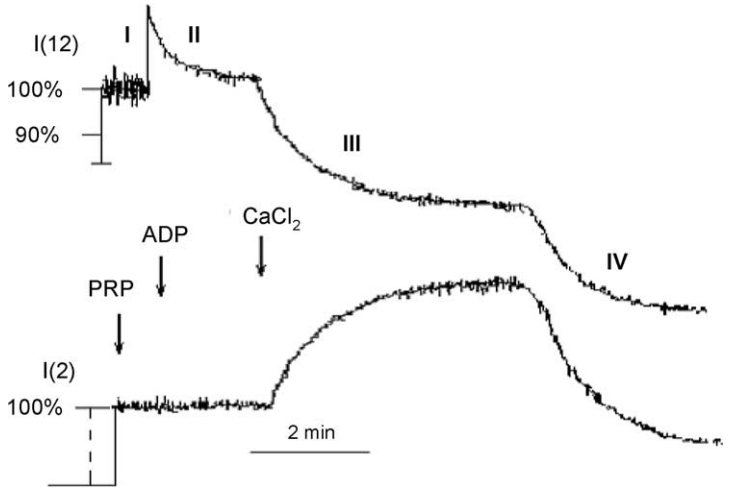

Fig. 5. Change in light scattering intensity during platelet transformation under the action of ADP in [Ca^{2+}]-free medium initially (in contrast to that presented in Fig. 4). Lower and upper traces are at 2 and 12 degree angles respectively. The different phases (I to IV) are discussed in the text.

to monitor the signal. At higher cell concentrations the dependence is not linear because of multiple-scattering effects.

Using the conventional light transmission technique (Born's method), the concentration of calcium ions should be very low (ca. 30 nmol l^{-1}). In our conditions, the aggregation can occur only at external calcium concentrations close to 1 mmol l^{-1} (such concentration of free calcium in PRP is impossible because of coagulation and fibrin sedimentation), which are similar to *in vivo* conditions.

The dynamics of signal intensity under the action of ADP at 2 and 12 degrees, initially in calcium-free medium, is presented in Fig. 5. When there is no aggregation (before introduction of calcium into the medium) the signal dynamics at the 12 degrees angle has a biphasic pattern. It apparently conforms to two phases: cell spherization (i) and pseudopodia formation (ii). Aggregation (iii) (after introduction of calcium) and the spontaneous clotting reaction (iv) are monitored at both angles; in addition, the signal is inverted at 12 degrees [19].

It is known that the platelet spherization occurs with increase in intracellular free calcium concentration ([$Ca^{2+}]_i$) [1]. We propose therefore a dependence of the signal monitored at the angle of 12 degrees on factors increasing internal calcium concentration: agonist action (ADP), calcium ionophore action and alteration in external ionic composition. The ionophore ionomycine (0.2 μmol l^{-1}) increases the signal, which is caused by shape change (and/or by the alteration of internal refraction coefficient) rather than increase in cell volume (Fig. 6). Supporting this are the following facts: (1) the signal dynamics is fast (seconds), but significant enhancement of the cell volume by entry of ions (Na^+ and Cl^-) into the cells is unlikely over such short time; (2) the signal dynamics induced by ADP (0.6 μmol l^{-1}) is identical for sodium chloride medium and isotonic choline-chloride medium.

3.2.2. Action of ADP on platelet spherization

Low ADP concentrations (around 10 nmol l^{-1}) cause an initial signal increasing only, i.e. platelet spherization only. Therefore the study of dependence of the spherization process upon the agonist (ADP) concentrations was carried out by analysis of the signal response on ADP addition (Fig. 7). For rats, the half-maximum effect (EC_{50}) of ADP was found to be 18.8 ± 4.5 nmol l^{-1} ($n = 8$), and for rabbits it was found to be 40.2 ± 8.3 nmol l^{-1} ($n = 12$). These values, obtained in the medium with calcium

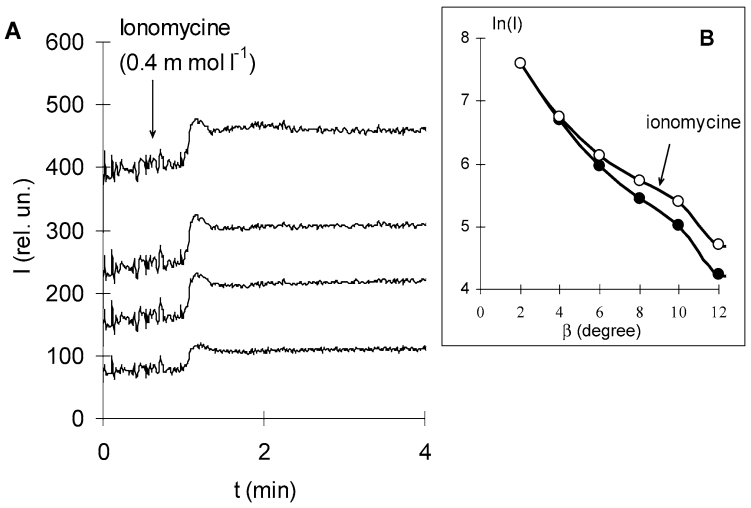

Fig. 6. Activation of rabbit platelets in NaCl-medium ($[Ca^{2+}]_0 < 30$ mmoll^{-1}). (A) Dynamics of signals at four different degrees (6, 8, 10, 12 degrees, from top to bottom). (B) Scattering indicatrixes for platelets before and after addition of ionomycine.

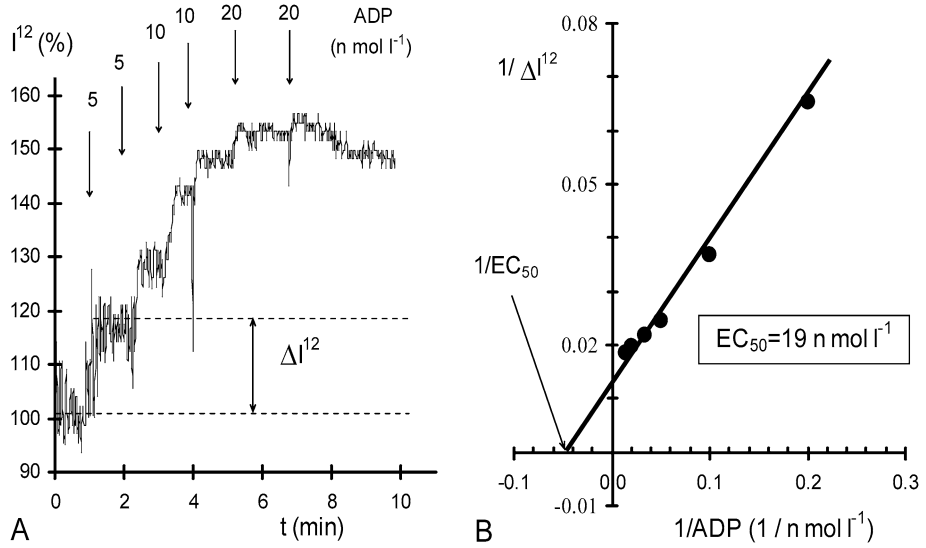

Fig. 7. Platelet spherization induced by low ADP concentrations (nmoll^{-1}) and monitored by light scattering intensity at an angle of 12 degrees (A). Additions of ADP to the saline medium are indicated by arrows. The EC_{50} value is determined with double reciprocal dose–effect coordinates (B).

ions (1 mmoll^{-1}), reflect P_2X_1-purinoceptor status. In the calcium-free medium (1 mmoll^{-1} EDTA) the EC_{50} values were as follows: for rats 93.4 ± 6.6 ($n = 5$) and for rabbits 104.7 ± 11.2 ($n = 4$). These date reflect the status of P_2Y_1-purinoceptor. Furthermore, studies were carried out on ATP and β,γ-methyl-ATP action [20].

3.3. Study of the platelet aggregation

In the saline medium (140 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl buffer pH 7.8, 1 mmol l^{-1} $[Ca^{2+}]_0$), at a platelet concentration 10^6 cells ml^{-1}, the aggregation monitored by light scattering technique at the angle of 2 degrees showed a monophasic pattern over the designated ADP concentration range. This enables evaluation of aggregation in terms of two values: the initial concentration of cells (N_0) and the initial velocity of aggregation (V_0), the former being proportional and the latter being squared proportional to the initial $I(2)$ value. The velocity of the reaction (aggregation) was determined by probability and efficiency of the impingements between cells followed by formation of the paired platelets (dimers), which has second order reaction kinetics in respect to cell concentration. This hypothesis is corroborated experimentally (Fig. 8). The order of the aggregation reaction with respect to platelet concentration (N_0) is represented graphically at different mixer rotation speeds (the aggregation is induced with 0.6 μmol l^{-1} ADP, which is a saturating concentration for the activator). The shape change is not the rate-limiting step (it runs for a few seconds, while aggregation proceeds for minutes), therefore the aggregation (dimerisation) velocity is specified by the second order constant k_{ex}. The k_{ex} value is proportional to the V_n/N_0^2 ratio and depends on the cell encounter probability and efficiency. Therefore the influence of the mixer rotation speed (W) (the encounter probability factor) and the external calcium ion concentration $[Ca^{2+}]_0$ (the efficiency factor) on the aggregation rate are evaluated in respect to the V_n/N_0^2 value.

The dependence of the V_n/N_0^2 value on the external calcium ion concentration $[Ca^{2+}]_0$ at different mixer rotation speeds is shown on double reciprocal coordinates in Fig. 9. The half-saturation constant for calcium was evaluated graphically to be 0.25 mmol l^{-1} (0.24 $\pm$ 0.7 mmol l^{-1}, based on five replicates). The dependence of the V_n/N_0^2 value on the mixer rotation speed can be linearized over a wide range.

The equation representing the maximal initial aggregation rate in the saline medium under conditions for maximal platelet shape change and developed of turbulent flow takes the following form:

$$V_n = k_{ex} \cdot N_0^2, \tag{1}$$

$$V_n = const \cdot \frac{[Ca^{2+}]_0}{K_{0.5}^{Ca} + [Ca^{2+}]_0} \cdot W^m \cdot N_0^2. \tag{2}$$

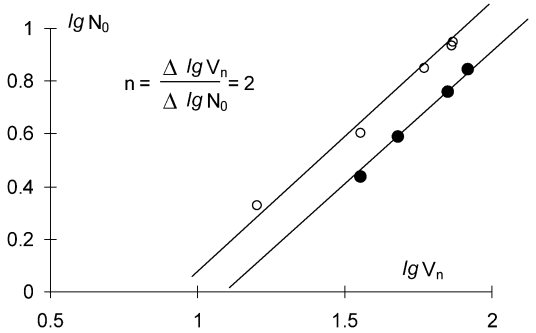

Fig. 8. Graphical representation of the order (n) of the aggregation process in relation to platelet concentration at two different mixer rotation speeds. (○) 750 and (●) 450 rev min^{-1}. The aggregation was induced by ADP (0.6 μmol l^{-1}). Initial velocity of aggregation, V_n; initial platelet concentration, N_0.

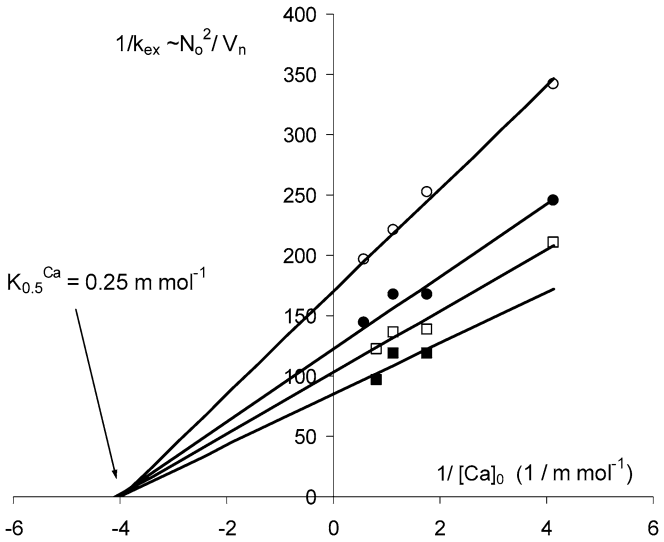

Fig. 9. Dependence of the constant (2nd order) of the aggregation velocity (close to V_n/N_0^2) upon external calcium ions concentration at different rotation speed of the mixer, presented on double reciprocal coordinates. [ADP] $= 0.6$ μmol l^{-1}.

3.4. Low-angle light scattering as a method for evaluating the functional state of platelets

The method of low-angle light scattering makes it possible to standardize study conditions by the salt composition (tonicity, pH, calcium ions). Under these conditions in all the experiments the dose dependence of the initial velocity of aggregation on the concentration of ADP has a saturating nature of the Langmuir type. Due to the fact that the initial velocity depends on the concentration of cells (formula (1)), using proportionality of this concentration from value $I(2)_0$, we introduced the standardized initial velocity of aggregation, U_n, computed from formula (3). During this standardization, a good correlation of the dependence of U_n upon ADP concentration was observed experimentally, given as formula (4). This dependence is quantitatively characterized by two values: EC_{50} (substance concentration necessary for achievement of half-maximum effect) and $U_{\max}$ (maximal normalized velocity of reaction at the saturating dose of a substance). It is convenient that these parameters can be determined by graphical assessments in the double reciprocal dose–effect coordinates and the statistical method of linear regression. These parameters (EC_{50} and $U_{\max}$) characterize the functional status of platelets: EC_{50} reflects the sensitivity of receptors to agonist, while $U_{\max}$ reflects the maximally possible quantity of binding sites on the surface of cells under the action of a saturating dose of an agonist.

The systematic sequence for experimental determination of EC_{50} and $U_{\max}$ is as follows:

(1) Registration of the initial velocity of aggregation caused by different doses of ADP (Fig. 10; usually 4–6 doses of ADP in the range of concentrations from 100 to 1000 nmol l^{-1}; at different disturbances of the platelets' status the range of concentrations can differ).
(2) Computation of the initial velocities of aggregation and normalized according to the equation:

$$U_n = \kappa \cdot \frac{V_n}{I(2)_0^2}. \tag{3}$$

(3) A table of data is compiled with the dependences of the standardized velocities U_n upon the concentration of ADP.

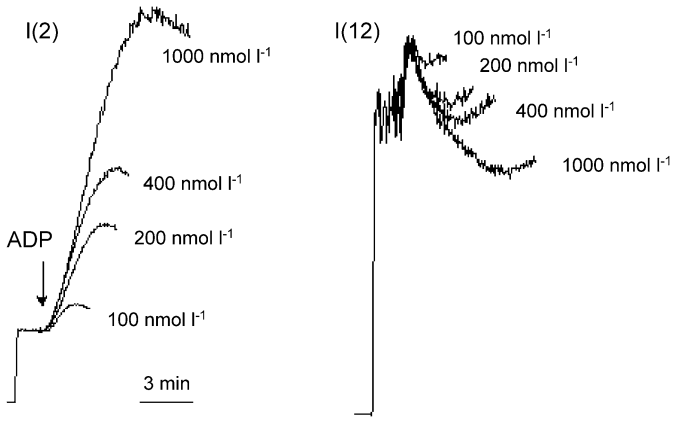

Fig. 10. The dose-dependent nature of the action of ADP on the initial velocity of aggregation, recorded at angles of 2 and 12 degrees.

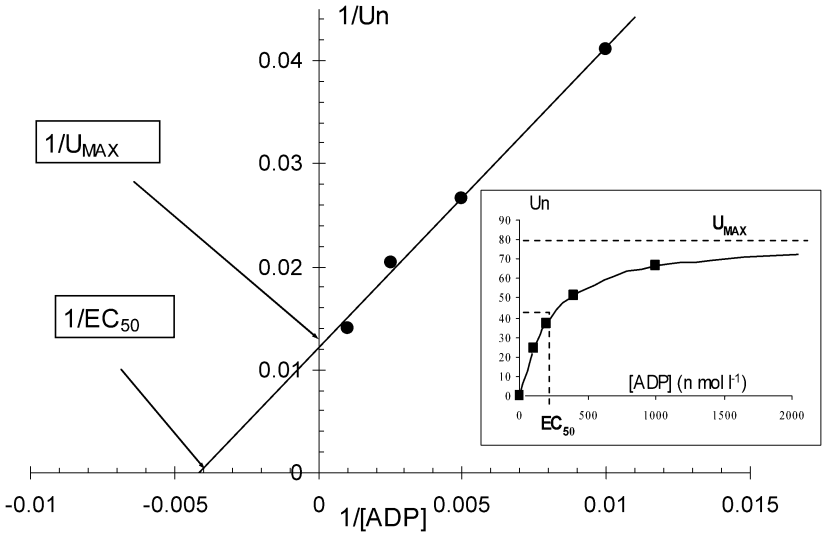

Fig. 11. Standard protocol for quantitative estimation of the status of platelets. Microsoft Excel was used to plot data and to determine values for the parameters EC_{50} and U_{max}.

(4) Reciprocal values are calculated ($1/U_n$ and $1/[\mathrm{ADP}]$).
(5) By statistical methods of linear regression, estimation of the EC_{50} and U_{max} is accomplished on the basis of the equation:

$$U_n = U_{\max}\frac{[\mathrm{ADP}]}{EC_{50} + [\mathrm{ADP}]}. \tag{4}$$

The use of computer technologies makes it possible to simplify the procedure of this estimation (Fig. 11).

The new method based of low-angle light scattering opens the prospects of using a simple and highly sensitive functional test in fundamental and applied research in the fields of pharmacology and toxicology, as well as in clinical practice. Using the new method, we had previously revealed

Table 1
Advantages of the low-angle light scattering method and comparison with Born's method

Properties of measuring system	Low-angle light scattering (new method)	Method of Born
Salt medium	140 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl (or HEPES) buffer pH 7.4 1 mmol l^{-1} $CaCl_2$	Platelet rich plasma (PRP) $[Ca^{2+}]$ – ? pH – ?
Possibilities for registering	Shape change Aggregation Disaggregation Coagulation	Aggregation Disaggregation
Platelet concentration	10^6–10^7 cells ml^{-1}	$>10^8$ cells ml^{-1}
Experimental conditions	Physiological pH and concentration of calcium ions	Unphysiological concentration of calcium ions
ADP concentration used for testing	5–1000 nmol l^{-1} (physiological range)	1–10 μmol l^{-1} (unphysiological range)

a sequence of ADP concentrations acting on purinergic receptors of platelets [20]: for P_2X_1 receptors $EC_{50} \sim$ 20–40 nmol l^{-1}, for P_2Y_1 receptors $EC_{50} \sim$ 90–110 nmol l^{-1}, and for P_2Y_{12} receptors $EC_{50} \sim$ 120–240 nmol l^{-1}. Subsequently, other researchers [21] showed a similar pharmacological profile for these receptors using a molecular cloning approach. The possibility to activate platelets with nmol l^{-1} concentrations exceeds the scope of possibilities of the traditional method of Born, according to which the concentration zone for testing these cells is within the range 1 to 10 μmol l^{-1}.

3.5. Summarized advantages of the low angle light scattering method in comparison with the traditional method of Born

The new method makes it possible to qualitatively and quantitatively characterize the functional status of platelets, which is especially important for investigating the complex pathologies of those connected with disturbances of the cellular component of hemostasis. Table 1 gives the comparative characteristics of the traditional (Born) method and the new method of low-angle light scattering. Probably, the most important advantage of the new method is the possibility to test platelets at the physiological concentrations of calcium ions that substantially influencing velocities of a number of enzyme- and receptor-bound processes. It should be noted that the possibilities of the method can be substantially increased in case of other (different from ADP) agonists, such as thrombin, PAF, epinephrine.

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