Antiaggregant Effects of Biogenic Chloramines

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Alanine and taurine sharply potentiate antiaggregant effects of hypochlorite on platelets in platelet-rich plasma. This effect is determined by more pronounced action of chloramine derivatives, products of interaction of added amino acids with hypochlorite. Platelets are more sensitive to the inhibitory effects of amino acid chloramine derivatives (biogenic chloramines) compared to erythrocytes and neutrophils. The antiaggregant effects of biobenic amines, as covalent platelet inhibitors, in platelet-rich plasma are characterized by their increased reaction capacity with molecular targets in cells. Quantitative parameter of this initial selectivity (ratio of rate constant of inactivation of platelet receptors to rate constant of side reaction with plasma proteins) far surpasses 1. N,N-Dichlorotaurine is a perspective antiaggreant among the studied biogenic chloramines. This agent is stable and exhibits specific pharmacological activity in all test systems, including animal model of thrombosis.

Key Words: *chloramine; sodium hypochlorite; amino acid; taurine; N,N-dichlorotaurine; platelet aggregation; erythrocyte hemolysis; leukocyte*

Phagocytizing cells generate hypochlorite anions (hypochlorous acid) in the myeloperoxidase-catalyzed reaction and chloramine derivatives of animo acids and related compounds (biogenic chloramines) as the secondary products of this reaction [19-21]. Hypochlorite produces a bactericidal effect [19] and modifies other cells [10,13,23]. Experiments an erythrocytes and neutrophils showed that chloramine derivatives of biogenic compounds are less cytotoxic than hypochlorite [15,24]. This is why the compounds containing amino groups, *e.g.* amino acids, taurine, *etc.*, in some cases can be considered as traps of "active chlorine" and used for cell protection from oxidative damage caused by hypochlorite [15,19]. At the same time, there are data on modifying effects of chloramines on some cells [17,23]. We showed that chloramine derivatives of amino acids inhibit aggregation capacity of rabbit and human platelets [2,3,5,6,9-11].

Here we compared the effects of chloramine derivatives of amino acids and taurine on platelets, erythrocytes, and leukocytes, studied the mechanism of the antiaggregant effect of chloramine derivatives on platelets, and initial selectivity of the antiaggregant effect of biogenic chloramines.

MATERIALS AND METHODS

Amino acids, taurine, ADP, luminol, phorbol-12-myristate-13-acetate (PMA, Sigma), and sodium hypochlorite (NaOCl; Aldrich) were used. The concentration of NaOCl was measured by the method of iodometric titration. Chloramine derivatives of amino acids were produced in the reaction with NaOCl, molar concentration of amino acids in the reaction mixture by 10% surpassed that of NaOCl. N,N-Dichlorotaurine was obtained by adding taurine into NaOCl solution (1:2 final molar ratio).

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The formation of N-chloramino acids was monitored spectrophotometrically by the presence of a maximum (at 253-255 nm for monochloro derivatives and at 300 nm for N,N-dichlorotaurine) in the adsorption spectrum. The concentration of chloramine derivatives of amino acids and taurine was measured by the method of iodometric titration.

Experiments were performed on rabbit and human platelets in the whole blood, platelet-rich plasma (PRP), and isolated blood cells.

For obtaining PRP, rabbit blood was stabilized with 3.8% sodium citrate (9:1 v/v) and centrifuged at 460*g* for 15 min. For isolation of platelets, EDTA (1 mM) was added to PRP followed by centrifugation at 1850*g* for 7 min. The pellet was resuspended in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 6 mM Na_2HPO_4, 1.47 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM glucose, 0.5 mM EDTA, pH 7.4) and recentrifuged under the same conditions. The pellet was resuspended in the same buffer without EDTA.

The blood from healthy donors drawn from the ulnar vein was mixed 6:1 with acid cictrate (85 mM triple-substituted sodium citrate, 71 mM citric acid, 11 mM D-glucose). PRP was prepared by centrifugation of the blood at 210*g* for 15 min.

Polymorphonuclear leukocytes were isolated from the peripheral rabbit blood stabilized with sodium citrate. Erythrocytes were precipitated by adding 500 kDa dextran to the blood. Cells in the supernatant were fractionated by differential centrifugation in a density gradient. The cells were suspended in phosphate buffered saline and stored at the temperature of melting ice. The obtained fraction of polymorphonuclear leukocytes consisted primarily of neutrophils (90-93%). Cell viability evaluated by trypan blue exclusion test was 95-97%. During chemiluminescence (CL) recording, luminol was added to neutrophil suspension (10^6 cell/ml) to a final concentration of 0.02 mM. Chloramine derivatives of amino acids and taurine and NaOCl were added to the cell suspension 5 min before PMA and luminol.

For isolation of erythrocytes, the pellet after isolation of PRP was twice washed with buffered physiological saline (10 min at 460*g*).

Platelet aggregation (PA) in the whole blood was recorded by the impedance method on a Whole Blood Aggregometer-591 (Chrono-log Corporation). The method of PA measuring in the blood consists in the follows: two electrodes are placed into the blood; when electric current flows in the circuit, platelet monolayer covers the electrodes. Addition of an agonist to the blood leads to platelet aggregation, the aggregates adhere to platelets covering the electrodes and the impedance increases. Changes in the impedance are proportional to PA degree. The quantitative parameter of PA was the change in blood impedance recorded 8 min after addition of the agonist (10 μM ADP) and $CaCl_2$ (1 mM). The results are presented as $\Delta A/\Delta A_0$, where ΔA_0 and ΔA are changes in impedance in control and experimental samples, respectively.

Human PA in the plasma was induced by adding 0.4-2.0 μM ADP and recorded on a turbidimetric Platelet Ionized Calcium Aggregometer (Chrono-log Corporation). The quantitative measure of PA was maximum change in light transmission of the suspension recorded after attaining a plateau on the light transmission curve. The aniaggregant effect of the test compounds was quantitatively characterized by the magnitude of aggregation inhibition (R) standardized to the control: $R=(\Delta T_0-\Delta T)/\Delta T_0$, where ΔT_0 and ΔT are changes in light transmission in control and experimental samples, respectively. The ΔT value characterizes (approximate proportionality) the number of cells in aggregates.

Aggregation of isolated rabbit platelets resulting in the formation of only small aggregates was studied by kinetic method based on recording of small-angle light scattering [12]. A beam of He-Ne laser (632.8 nm) passed through a sample of platelet suspension in a rectangular cuvette. Light scatter within 0.5-7.0° was recorded using a combination of diaphragms. PA was induced by adding 0.2 μM ADP.

PA in rabbit plasma was induced by adding ADP (10 μM) and studied by the tirbodimetric method on an aggregation analyzer (Biola).

Hemolysis of isolated erythrocytes was recorded on a Biola aggregation analyzer. The degree of hemolysis was evaluated by the increase in light transmission in erythrocyte suspension in phosphate buffer (5×10^6 cell/ml) recorded over few hours.

Luminol CL in neutrophil suspension was recorded on a lumiaggregometer (P.I.C.A., CL channel, Chrono-log Corporation).

RESULTS

At first we studied possible protective effect of taurine and alanine during inhibition of human and rabbit PA with NaOCl. NaOCl (final concentration 0.5 mM) or physiological saline (control) was added to PRP samples containing 10 mM taurine or 5-10 mM alanine or without additives. PA was analyzed after 5 min using agonist ADP (10 μM). Alanine and taurine were used in relatively high concentrations sufficient for trapping considerable amount

of NaOCl against the background of competition with other active chemical groups in the plasma. A surprising result was obtained. Alanine and taurine did not protect platelets and even potentiated the antiaggregant effect of NaOCl by ~1.7 times (Table 1). In the absence of NaOCl, alanine and taurine had no effect on PA. These findings suggest that platelets, in contrast to other blood cells, are very sensitive to the modifying effect of biochloramines.

In further experiments we studied the effect of chroramines on isolated rabbit platelets, erythrocytes, and neutrophils. Aggregation capacity of isolated platelets was evaluated by changes in the initial aggregation (formation of small aggregates), in particular, changes in small-angle light scatter over 1-2 min. We found that ADP-induced aggregation of isolated platelets (2.4×10^8 cell/ml) was suppressed by N,N-dichlorotaurine in a concentration of 10 μM by 50% (Table 1). Chloramine derivatives of amino acids produced similar effects.

Damage to erythrocytes (5×10^6 cell/ml) evaluated by the degree of hemolysis after 4-5 h (sharp increase in light transmission of erythrocyte suspension) was observed at far higher concentration of chloralanine (1 mM, Fig. 1). At the same time, erythrocytes were easily hemolyzed under the effect of 0.01 mM NaOCl (Fig. 1) as was shown in previous studies [13-15].

Then, we studied the effects of biochloramines on leukocytes. The processes of phagocyte activation accompanied by the formation of reactive oxygen forms are usually studied by the CL method using chemical activators. Here we measured CL in the neutrophils—luminol system on non-activated cells and under conditions of their stimulation with PMA. It was found that N-chlorophenylalanine and other chloramines sharply increase luminol CL in the suspension of non-activated neutrophils. The CL intensity in this case was higher than CL of luminol solutions induced by N-chlorophenylalanine, but is similar to CL of a mixture of luminol, N-chlorophenylalanine, and H_2O_2 in a concentration of 20-30 nM. Hence, intensification of CL in the neutrophils—luminol system in the presence of N-chlorophenylalanine does not depend on cell activation, but is determined by direct co-oxidation of luminol by chloramine and H_2O_2 [1].

N,N-dichlorotaurine in a final concentration of 0.010-0.025 mM did not change luminol luminescence in the suspension of PMA-stimulated neutrophils. After increasing the concentration of N,N-dichlorotaurine to 0.05 mM, the intensity of CL measured in maximum increased by 1.5 times (Fig. 1). Similar results were obtained for N-chlorotaurine and N-chlorophenylalanine. At the same time, NaOCl (0.01-0.04 mM) sharply inhibited luminol luminescence caused by activated cells (Fig. 1). It can be hypothesized that similarly to nonactivated cells, in PMA-activated neutrophils the increase of CL in the presence of N,N-dichlorotaurine (0.05 mM) was determined by its direct action on luminol, but not on cells. Thus, chloramine derivatives of amino acids and taurine in the studied concentrations had no effect on the production of reactive oxygen species recorded by luminol CL in leukocyte suspension.

For more precise comparison of the sensitivity of platelets and other blood cells we should standardize the amount of the acting compound for equal volumes of the cell substances, because biochloramines cause irreversible chemical modification of cells. The erythrocyte volume 20-fold surpasses the volume of a single platelet [16], *i.e.* the total volumes of 20 mln platelets and 1 mln erythrocytes are similar. The above data suggest that aggregation activity of 20 mln platelets decreases 2-fold under the effect of ~1.7 nmol chloramine, while damage to 1 mln. erythrocytes leading to hemolysis requires 200 nmol chloramine. If the effect of chloramines is standardized for equal sur-

TABLE 1. Inhibition of PA with NaOCl and N,N-Dichlorotaurine (*M*±*m*)

Platelet sample	Additives	Inhibition, %
Rabbit PRP	Alanine, 5-10 mM	0±10
	NaOCl, 0.5 mM	39±2
	Alanine, 5-10 mM, then NaOCl, 0.5 mM	68.0±2.5
Human PRP	Taurine, 10 mM	0±5
	NaOCl, 1 mM	41±4
	Taurine, 10-10 mM, then NaOCl, 1 mM	71±4
	N,N-dichlorotaurine, 0.25 mM	40±7
Isolated rabbit platelets	N,N-dichlorotaurine, 10 μM	53±12
	NaOCl, 20 μM	52±9

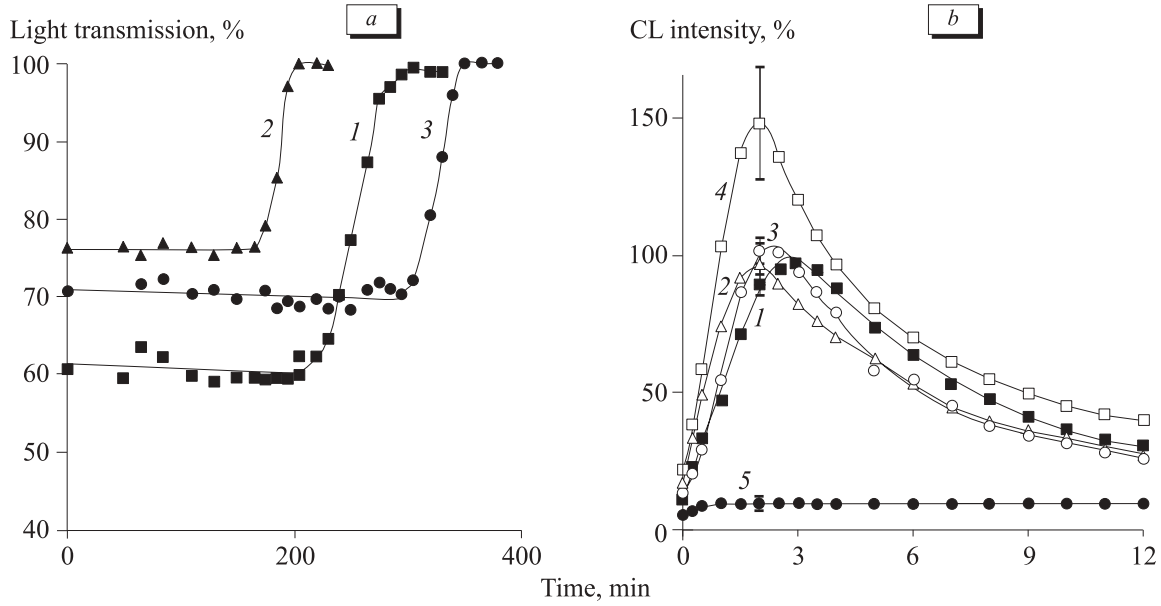

Fig. 1. Kinetic curve of erythrocyte hemolysis (*a*) and intensity of luminol-enhanced CL in neutrophil suspension (*b*) under the effect of chloramines and NaOCl. *a*: *1*) N-chloralanine, 1 mM; *2*) N-chloralanine, 1.6 mM; *3*) NaOCl, 0.01 mM. Concentration of erythrocytes in the sample 5×10^6 cell/ml. *b*: *1*) control; *2-4*) N,N-dichlorotaurine in concentrations of 0.005, 0.025, and 0.050 mM, respectively; *5*) addition of NaOCl, 0.04 mM Chemiluminescence maximum in the control is taken as 100%. Concentration of neutrophils in the sample, 10^6 cell/ml. Neutrophils were stimulated with PMA, 0.1 μg/ml.

face of cells, theproportion of the acting amount of chloramines for platelets and erythrocytes is 3-fold lower. Thus, platelets are characterized by 100-fold higher sensitivity to the modifying effects of chloramines than erythrocytes.

It is known that chloramine derivatives of amino acids are polar water-soluble compounds, which cannot penetrate into cells. Hence, their inhibitory effect on PA is a result of interaction with surface structures. Extracellular domains of receptors for agonists and(or) fibrinogen can be molecular targets for chloramines on the platelet surface. Indeed, analysis of published data on the structure of most important aggregation receptors on platelet surface (ADP receptors $P2Y_1$ and $P2Y_{12}$, collagen receptors, receptors for von Willebrand factor (glycoprotein Ib), fibrinogen (glycoprotein IIb/IIIa), and thrombin) showed that they contain a considerable number of reactive sulfur-containing atomic groups.

In biochloramines active chloramine group oxidizes thiol and thioester groups of amino acid residues in proteins. The most possible result of covalent modification of proteins by biochloramines is conversion of these groups into a sulfinic acid residue.

ADP-induced PA in PRP is inhibited by a number of chloramine derivatives of amino acids, the antiaggregant effect of these compounds depends on their structure [2,6]. First, comparison of antiaggregant activity of biochloramines with different location of chloramine group in the molecule showed that the compounds where the chloramine group is located at a distance of 3-5 carbon atoms from the carboxyl group are most effective. The existence of a negatively charged group at the site of biochloramine interaction with the plasma membrane responsible for this structural feature was hypothesized [6]. Second, it was found that antiaggregant effect of chloramine derivatives of amino acids on platelets in PRP after addition of ADP depends on their molecular weight (size of molecule) [2] (Fig. 2). Some compounds with low molecular weight (N-chloroglycine, N-chloralanine, N-chloroserine, van der Waals volumes within 0.083-0.100 nm^3) produce more pronounced antiaggregant effect, which increased with decreasing the molecular weight (Fig. 2). It is quite possible that these compounds unlike others (N-chloroleucine, N-chlorophenylalanine) can interact with chemical groups of platelets located in cavities of the plasma membrane and surface proteins inaccessible for chloramines with higher molecular weight.

Pronounced specific pharmacological activity against the background of weak (permissible) side effect is an important characteristic of drugs. This can be attained, if the preparation selectively modifies the targets, platelet proteins in our case. Blood plasma contains a considerable number of sulfur-containing chemical groups, which can be attacked

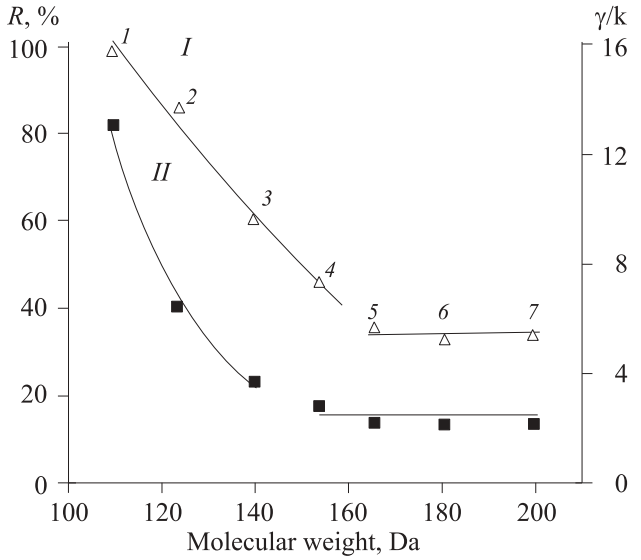

Fig. 2. Inhibition of PA (*I*; left ordinate) and initial selectivity (*II*, right ordinate) in rabbit PRP after addition of chloramine derivatives of amino acids, 1 mM. Platelets were activated by adding 10 μM ADP. *1*) glycine; *2*) alanine; *3*) serine; *4*) treonine; *5*) leucine; *6*) glutamine; *7*) phenylalanine.

by the chloramine group [22]. Therefore, the antiaggregant effect of chloramine derivatives of amino acids in PRP reflects the balance of their interaction with platelets and other components.

It can be expected that in PRP diluted with autologous plasma the antiaggregant effects of chloramine derivatives of amino acids will decrease due to their enhanced utilization in the reaction with plasma components. Indeed, preliminary dilution of PRP by 1.6 times with autologous plasma reduced the antiaggregant effect of N-chloramino acids by 15-20% compared to non-diluted PRP (final concentration of the test compounds in both series was 1 mM). The effect of low-molecular-weight N-chloramino acids under these conditions remained unchanged, probably because the number of platelets also decreased after dilution in parallel with increasing the content of reactive chemical groups of the plasma. Thus, the differences between the studied compounds were preserved after dilution of PRP with autologous plasma. The compounds for which the antiaggregation effect did not depend on their molecular weight were more intensively utilized in reactions with plasma components and weaker inhibited PA than in case of non-diluted PRP.

Platelet inhibitors are widely used for the prevention of arterial thromboses. Of these agents, covalent inhibitors are more effective. They suppress platelet functions via chemical modification of molecular targets. The efficiency of covalent inhibitors depends on their two main properties. The first property is long-term (ideally, permanent, for the whole cell life-time) inhibition of platelet function. The second property stems from the first one and consists in cumulative effect of low doses of the inhibitor in the body. This allows administration of low single doses thus reducing side effects.

Covalent inhibitors include acetylsalicylic acid (aspirin) and thienopyridines [18,25]. Among thienopyridines, clopidogrel, (2-chlorophenyl)-(6,7-dihydro-4*H* thieno[3,2-*c*]pyridine-5-yl)-acetic acid, is most widely used [18]. The effect of acetylsalicylic acid as a covalent inhibitor consists in translocation of the acetyl group to the hydroxyl group of serine-530 of platelet cyclooxygenase I (prostaglandin H_2-1 synthase) with the formation of O-acetylserine [25]. In case of thienopyridines, the antithrombotic effect is produced by a metabolite formed in the liver and carrying an active sulfhydryl group. This metabolite covalently modifies ADP receptor ($P2Y_{12}$ purine receptor) by reacting with the thiol group of cisteine with the formation of mixed disulfide [18].

The search for new platelet inhibitors are in progress all over the world. We propose biogenic chloramines as new covalent inhibitors of platelets [2-11].

Pronounced specific pharmacological activity against the background of weak (permissible) side effect is an important characteristic of drugs. This can be attained, if the preparation selectively modifies the targets, certain platelet proteins in our case. In case of reversible noncovalent inhibitors, the selectivity at the level of molecular targets is well known and is determined by the formation of a specific complex with active center of the target molecule.

At least two different mechanisms can determine the selectivity of covalent inhibitors. It can result from physiological processes (secondary selectivity), when chemical modifications of blood proteins are eliminated. Another mechanism is initial selectivity, when covalent inhibitor exhibits increased affinity for molecular targets on the platelet. Addressed effect of the inhibitor consists in inactivation of platelet receptor; let γ is the rate constant of this reaction. In PRP, the targeted reaction is paralleled by side reactions of the inhibitor with reactive chemical groups of plasma proteins; let k is the integral rate constant of these reactions. It follows that γ/k ratio can be used as a qualitative parameter of initial selectivity of chloramine inhibitors. Theoretical analysis of the kinetics of these reactions and data of turbidimetry analysis of PA in PRP showed that this ratio can be determined from the degree of aggregation and concentrations of reactive chemical groups of plasma proteins [4]. The inhibitory effect of the studied biochloramines on PA in PRP is characterized by pronounced ini-

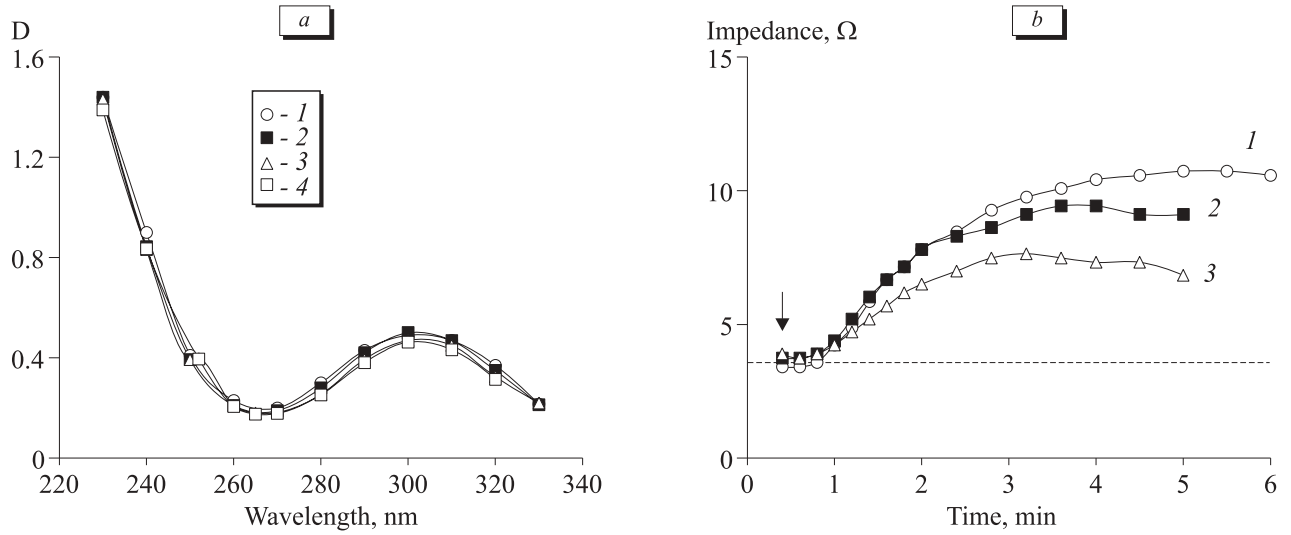

Fig. 3. Adsorption spectra of N,N-dichlorotaurine (*a*) and its effect on platelets in the whole blood (*b*). *a*: adsorption spectrum of N,N-dichlorotaurine (0.75 mM) immediately (*1*) and 1 (*2*), 2 (*3*), and 4 (*4*) months after synthesis. N,N-dichlorotaurine solution was stored in a concentration of 150 mM at 7°C. D is optical density of solution in a 1-cm cuvette. *b*: typical kinetic curves of ADP-induced PA in whole rabbit blood. *1*) control; *2* and *3*) addition of N,N-dichlorotaurine in concentrations of 0.005 and 0.125 mM, respectively. Arrow: addition of ADP (10 μM) and $CaCl_2$ (1 mM).

tial selectivity, because $\gamma/k>1$ (Fig. 2). The value of initial selectivity is useful during testing of potential covalent inhibitors of platelets as the criterion of their efficiency.

The observed regularities of the action of chloramine derivatives of amino acids on platelets allowed us to start the development of a new antithrombotic drug [8]. N,N-Dichlorotaurine (N,N-dichlor-2-aminoethansulfonic acid) is the most promising agent in this respect. N,N-Dichlorotaurine solution is characterized by high stability during long-term storage (4 months), which is seen from the absence of changes in adsorption spectra (Fig. 3) and iodometric titration data. We were first who obtained N,N-dichlorotaurine in a solid state [7]. This agent exhibits pronounced antithrombitic effect. After addition of N,N-dichlorotaurine in moderate concentrations (~ tens millimoles per liter) to whole blood, ADP-induced PA recorded by the impedance method was markedly suppressed (Fig. 3). These data suggest that platelets are highly sensitive to N,N-dichlorotaurine and selectively interact with this agent in the blood. Experiments on intact rabbits showed that ADP-induced PA in PRP decreased by 81±11% one hour after intravenous injection of N,N-dichlorotaurine (estimated concentration in the blood was ~1 nM). No toxic effects were noted after intravenous injection of N,N-dichlorotaurine to rabbits.

We compared antithrombotic activity of N,N-dichlortaurine and some other biochloramines on the mouse model of thrombosis. Intravenous injection of chloramine derivatives of amino acids and N,N-dichlortaurine reduced mouse mortality from thombosis induced by intravenous injection of ADP [5]. After intravenous injection of a mixture containing 15 μg collagen and 7 μg epinephrine, mouse survival in the control group was 24%. Preliminary injection of N,N-dichlortaurine (estimated concentration in the blood ~0.25 mM) improved mouse survival to 71% ($p<0.01$). The therapeutic dose of N,N-dichlortaurine in the studied model was by one order of magnitude lower than the dose producing toxic effect. Thus, N,N-dichlortaurine exhibits pronounced specific pharmacological antiaggregant activity and is a potential drug substance.

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