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Abstract. The relevance of studying the regulation of protein-ligand interactions is due to the emergence of new views on the role of metabolites and their key importance in vital processes. To study the protein-ligand interaction, the AB0 antigen-antibody blood system and the enzyme-substrate system of dehydrogenases were used as a test system, and ethanol was used as an influencing factor. In experiments performed with A and B blood erythrocyte antigens, natural AB0 system antibodies and monoclonal antibodies under the influence of ethanol performed change of the degree of agglutination and the time to onset of erythrocyte agglutination. It was found that ethanol can regulate the enzyme-substrate interactions of dehydrogenases: lactate dehydrogenase (EC 1.1.1.27), glyceraldehyde phosphate dehydrogenase (EC 1.1.1.12), and α-glycerol phosphate dehydrogenase (EC 1.1.1.8). The increase in the activity of studied enzymes under the influence of ethanol in the whole blood hemolysate was 2.5 - 3 times higher than in the isolated medium (with pure enzyme preparations).

1. Introduction

Protein-ligand interaction is crucial in almost of basic biological processes, such as cell regulation, biosynthesis and degradation of organic compounds, signaling by hormones, initiation of DNA replication, transcription and translation, formation of multimolecular complexes, virus packaging and immune response [1]. Recent technological advances have made it possible to identify previously unknown effects of metabolites that may affect functioning of proteins. Inter-cell signaling is widespread to support cell proliferation, differentiation and adaptation. The interaction network in a cell often manifests how protein function is regulated by other components, including low-molecular-weight metabolites.

Ethanol has a wide range of physiological effects in the body. For most of the ethanol effects, it is not known whether ethanol will interact with protein or indirectly influence on the protein functioning. A theory of ethanol binding with proteins prevails. There appeared publications on discrete, identified binding sites of ethanol with proteins, such as alcohol dehydrogenase, adenylate cyclase, GABA receptor, N-methyl-D-aspartate and glycine receptors [2, 3]. As is known, metabolites serve not only as an object of enzyme action, but also play the most important role as information molecules, regulate the activity of biomolecules in various cell compartments, in the extracellular space, and in biological fluids [4]. Being products of enzymatic transformation, metabolites create a microenvironment in which

proteins perform their function, and thereby become information carriers, forming a kind of memory of a past molecular event.

2. Materials and methods

We studied veinous blood of 90 almost healthy individuals, whose average age was 26.8± 1.5 years. Clinical characteristics of health were confirmed by the following criteria: the absence of chronic somatic diseases and viral infections (viral hepatitis B, C and HIV infection). The following study materials were used: standard red blood cells from ReversCell (USA), monoclonal antibodies from BIO-Rad (USA), ETOH2 reagent kit to determine ethanol concentration by Roche-Diagnostics (Germany), Precinorm and Precipat control sera by Roche-Diagnostics (Germany), homogeneous enzyme preparations GAPDH, α-GPD and LDH (ICN Biomedicals, USA), buffer solutions: 0.05 M Tris-HCl buffer (pH 8.6), 0.05 M triethanolamine hydrochloric acid buffer (pH 7.5), 0.1 M phosphate buffer, (pH 7.4), and ethanol solution.

The ethanol concentration in the blood serum was determined using COBAS INTEGRA 400 plus biochemical analyzer. Blood typing was performed using Chemos SP II immunohematological analyzer (BIO-Rad). A test system was developed to evaluate the effect of ethanol on the antigen–antibody interaction of the AB0 blood system. Before performing the hemagglutination reaction, we preliminarily incubated red blood cells of different AB0 blood groups, anti-A and anti-B antibodies of blood plasma and anti-A and anti-B monoclonal antibodies with ethanol (table 1).

Table 1. An algorithm for studying the effect of ethanol on the antigen-antibody interaction of the AB0 system.

100 ml of blood was incubated for 5 min with 20 ml of 0.3 mM ethanol Agglutination reaction was performed with standard monoclonal antibodies	100 ml of plasma was incubated for 5 min with 20 ml of 0.3 mM ethanol Agglutination reaction was performed with standard red blood cells of A(II)-AB(IV) blood groups	100 ml of monoclonal antibodies was incubated for 5 min with 20 ml of Agglutination reaction was performed with standard red blood cells of A(II)-AB(IV) blood groups
Parameters of AB0 antigen-antibody agglutination reaction were evaluated: agglutination degree (pt) and time to agglutination onset (sec)		

The agglutination degree was calculated according to W. Marsh with an indication of the agglutination degree and the agglutination intensity score from 0 to 12. Time to agglutination onset was measured using a stopwatch in seconds, from the moment the components were mixed until the first signs of agglutination appeared. In an experiment with plasma antibodies, the whole blood was preincubated with ethanol, then it was separated by centrifugation into plasma and formed elements, and the plasma antibodies were agglutinated with standard red blood cells.

Enzyme activity was determined using LAMBDA 20 spectrometer. Assay quality control was performed using Precinorm and Precipat control sera (Roche-Diagnostics, Germany). The activity of dehydrogenases GAPDH, α-GPD and LDH was determined using the Warburg test. Whole blood hemolysate was used for the experiment. To obtain the hemolysate, 100 µl of whole blood was added to 0.9 ml of bidistilled water cooled down to 0 °C. All procedures were performed in cold conditions. Enzyme preparations were diluted as follows: GAPDH - using 0.05 M Tris-HCl buffer (pH 8.6), α-GPD - using 0.05 M triethanolamine hydrochloric acid buffer (pH 7.5), and LDH - using 0.1 M phosphate buffer, (pH 7.4), ethanol solution. To calculate the specific activity of enzymes (E/mg), protein concentration in the hemolysate was measured by the Lowry assay; the protein content in the commercial preparation was determined by measuring absorbance at 280 nm. The algorithm for studying the effect of ethanol on the activity of dehydrogenases is shown in table 2.

Table 2. The algorithm for studying the effect of ethanol on the activity of dehydrogenases.

10 ml of hemolysate	10 ml of enzyme preparation
GAPDH, α-GPD, LDH in buffer was incubated for 20 min with 0.3 mM ethanol at 25 °C	

Statistical processing of the obtained results was performed using the program Statistical Package for the Social Science (SPSS) 10.0.

3. Results

As an object of studying the effect of ethanol on the protein-ligand interaction, antigen-antibody interactions of the AB0 blood system and enzymes interacting with substrates were chosen. Previously, we showed that the ethanol content in the blood of clinically healthy individuals is virtually the same, and the blood group did not affect its concentration. The reaction medium pH was within the control values, namely, 7.11±0.04. The effect of ethanol on the antigen–antibody interaction of the AB0 system was evaluated by two parameters: agglutination degree and time to onset of agglutination of antigen-antibody of the blood AB0 system. In the first series of model experiments, the effect of ethanol on AB0 system antigens was studied. The effect of ethanol on the degree and time to onset of A(II), B(III) and AB(IV) blood groups antigens agglutination with standard monoclonal antibodies is shown in tables 3 and 4.

Table 3. The effect of ethanol on the agglutination degree of red blood cells of A(II), B(III) and AB(IV) blood groups (pt).

Statistical parameter (n=30)	Control Antigen A A(II)	Experiment Antigen A A(II)	Control Antigen B B(III)	Experiment Antigen B B(III)
M±SD	12	11.0±0.21	12	12
Δ%		-8.4		-
p		p<0.05		
Statistical parameter (n=30)	Control Antigen A AB(IV)	Experiment Antigen A AB(IV)	Control Antigen B AB(IV)	Experiment Antigen B AB(IV)
M±SD	12	11.0±0.19	12	11.0±0.19
Δ%		-8.4		-8.4
p		p<0.05		p<0.05

Table 4. The effect of ethanol on the onset of agglutination of red blood cells of A(II), B(III) and AB(IV) blood groups (sec).

Statistical parameter (n=30)	Control Antigen A A(II)	Experiment Antigen A A(II)	Control Antigen B B(III)	Experiment Antigen B B(III)
M±SD	6.0±0.03	5.0±0.07	6.0±0.03	5.0±0.06
Δ%		-16.6		-16.6
p		p<0.1		p<0.1
Statistical parameter (n=30)	Control Antigen A AB(IV)	Experiment Antigen A AB(IV)	Control Antigen B AB(IV)	Experiment Antigen B AB(IV)
M±SD	6.0±0.05	6.0±0.06	6.0±0.03	6.0±0.05
Δ%		-		-
p		-		-

It was found that after incubation with ethanol, the degree of agglutination of A(II) and AB(IV) blood group red cells decreased by 8.4%. The presence of ethanol in the medium reduced the affinity of antigens of A(II) and AB(IV) blood group RBCs to antibodies, but did not change the agglutination degree of B(III) blood group RBCs. Time to onset of agglutination of A(II) and B(III) blood group RBCs deviated from the control values more significantly than the agglutination degree, namely, by 16.6%.

The effect of ethanol on natural blood plasma antibodies and monoclonal antibodies of the AB0 blood system were studied in the next experiments in order to test their ability to bind with A and B antigens (tables 5, 6).

Table 5. The effect of ethanol on the agglutination degree of plasma antibodies of the AB0 system (pt).

Statistical parameter	Control Anti-A antibodies	Experiment Anti-A antibodies	Control Anti-B antibodies	Experiment Anti-B antibodies
M±SD	10±0.33	12.0±0.30	8.0±0.34	10±0.31
Δ%	-	+20	-	+25
p		p<0.1		p<0.1

Table 6. The effect of ethanol on the onset of monoclonal antibody agglutination (sec).

Statistical parameter	Control Anti-A antibodies	Experiment Anti-B antibodies	Control Anti-A antibodies	Control Anti-A antibodies
M±SD	6.0±0.03	5.0±0.04	6.0±0.03	5.0±0.04
Δ%		-16.6		-16.6
p		p<0.1		p<0.1
Statistical parameter	Control Anti-A antibodies	Experiment Anti-A antibodies	Control Anti-B antibodies	Control Anti-B antibodies
M±SD	6.0±0.03	7.0±0.05	6.0±0.04	6.0±0.04
Δ%		+16.6		-
p		p<0.1		-

The agglutination process changes: when interacting with non-incubated anti-A and anti-B antibodies, the degree of erythrocyte agglutination was below the maximum value of 12 pt, which was observed in previous experiments with group-specific erythrocyte antibodies. After incubation with ethanol, the agglutination degree of anti-A and anti-B plasma immunoglobulins increased by 20% and 25%, respectively.

Pre-incubation of anti-A and anti-B monoclonal antibodies with ethanol promotes faster agglutination with blood group A(II) and B(III) RBCs, and slower agglutination with blood group AB(IV) RBCs in the experiment with A-antibodies (time to agglutination onset decreases). It is important to note that under the influence of ethanol the rate of agglutination of red blood cells of AB(IV) blood group (a combination of two antigens A and B) with monoclonal anti A-antibody slow down, but does not change monoclonal anti-B antibody AB(IV) blood groups.

The effect of ethanol on enzyme-substrate interactions of dehydrogenases in hemolysate and in an isolated environment (enzyme preparations) was studied. The activity of GAPDH, α-GPD and LDH did not change during incubation without ethanol. After incubation with ethanol, it was found that the activity of dehydrogenases increased both in the hemolysate and in an isolated environment (tables 7, 8).

Table 7. The effect of ethanol on the activity of GAPDH, α-GPD and LDH (enzyme preparations) (E/mg).

Statistical parameter	GAPDH	α-GPD	LDH
Control	0.251±0.003	0.192±0.003	0.317±0.004
	Incubation without ethanol		
M±SD	0.250±0.004	0.195±0.007	0.314±0.007
Δ%	Δ% -0.4	Δ% +1.5	Δ% -1.0
p	p>0.05	p>0.05	p>0.05
	Incubation with ethanol		
M±SD	0.440±0.005	0.244±0.011	0.517±0.006
Δ%	Δ% +75	Δ% +27	Δ% +63
p	p<0.001	p<0.1	p<0.001

Table 8. The effect of ethanol on the activity of GAPDH, α-GPD and LDH (enzyme preparations) (E/mg).

Statistical parameter	GAPDH	α-GPD	LDH
Control	23.19±0.87	2.94±0.02	493.4±17.1
	Incubation without ethanol		
M±SD	22.72±0.88	2.98±0.04	485.8±19.1
Δ%	Δ% -2	Δ% +1.3	Δ% -1.6
p	p>0.05	p>0.05	p>0.05
	Incubation with ethanol		
M±SD	30.17±0.77	3.26±0.04	625.1±16.5
Δ%	Δ%+30	Δ%+11	Δ%+27
p	p<0.01	p<0.05	p<0.01

The stimulating effect of ethanol on the activity of the studied dehydrogenases in the hemolysate and in the isolated medium was found (figure 1).

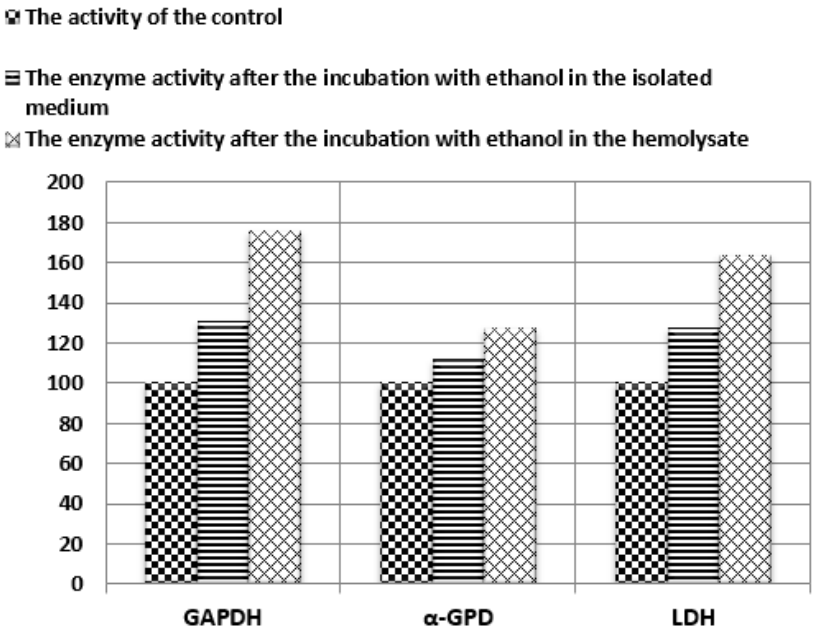

Figure 1. Comparative evaluation of the enzyme activity in the hemolysate and in the isolated medium (100% is the activity of enzymes in the control samples).

4. Discussion

The effect of ethanol on the degree of agglutination of red blood cells A(II), B(III) and AB(IV) blood groups shows that the A(II) blood group antigen with terminal N-acetylgalactosamine and the A and B, AB (IV) blood group antigens react to the presence of ethanol in the environment. Literature contains data on the effect of various compounds on antigen–antibody interactions [5]. According to their chemical structure, A and B antigens differ by the fact that N-acetylgalactosamine (A-antigen) has NHCOCH3 in C2 position, while D-galactose (B-antigen) has OH group in this position. A and B antigens have different epitopes, and their binding with the antibody paratope will differ.

The interaction of the antigen epitope and the antibody paratope involves a variety of non-covalent bonds, namely, hydrogen bonds, hydrophobic interactions and van der Waals interactions. The specificity of antigen-antibody reaction is particularly determined by the action of these forces. Of course, antibodies recognize not only individual chemical groups, but also the spatial configuration of antigen determinants. Computational studies of N.Sinha showed that antigen binding with an antibody is due to electrostatic optimization of the antigen-binding site resulting in higher specificity of antibodies. Hydrophobic interactions contribute to the conformational flexibility and reactivity of immunoglobulins [6].

The time of agglutination onset decreased both in experiments with blood group A(II) antigen A and in experiments with blood group B(III) antigen B. Ethanol did not affect the time of onset of agglutination of AB(IV) blood group red blood cells. Apparently, the presence of two antigens A and B on the membrane of AB(IV) blood group red blood cells neutralizes the effect of ethanol. When studying the effect of adrenaline on the time to onset of erythrocyte agglutination, a decrease in this erythrocyte agglutination parameter in people with A(II), B(III) and AB(IV) blood groups has been observed, explained by the activation of α-adrenoreceptors [7]. The ability of acetylcholine to reduce the time to onset of erythrocyte agglutination is associated with the activation of M-cholinergic receptors and is explained by the decrease in the negative surface charge due to the increase in K+ output from erythrocytes and the increase in micro-viscosity of their membranes [8]. The time to agglutination onset mainly depends on the magnitude of negative charge on the RBC surface: when it decreases, the time to agglutination onset decreases, since the forces contributing to the repulsion of RBCs from each other decrease, and the likelihood of their interaction increases. The change in erythrocyte agglutination parameters under the influence of ethanol is likely associated with the conformational modification of antigens due to the changed microenvironment, which may also determine their reactivity.

The obtained results show that ethanol changes the antigen-antibody interaction due to direct and indirect effects, which is proved by fluctuations in two indicators of the agglutination reaction-the degree and time of the beginning of agglutination of the AB0 system. The blood group belonging to the AB0 system determines the specificity of the response of red blood cell antigens and antibodies to them to the action of ethanol, forming individual characteristics of the body.

It was found that ethanol has different effects on the start time of agglutination of monoclonal antibodies with AB0 system antigens. It is possible that ethanol may have a direct influence on the antigen-binding sites of the antibody paratope resulting in higher interaction specificity, or optimize the conditions for binding of antibodies with antigens, affecting the environment of these molecules. AB0 system antibodies belong to class M immunoglobulins. For example, in a study by Jaśkiewicz E. et.al. it was found that anti-M monoclonal antibodies have a specific peptide paratope in which methionine at the 8th position and valine at the 6th position play a key role in the formation of immune complexes. Glycosylation of key amino acid residues and amino acid residues adjacent to the most specific part of the anti-M antibody paratope modulates the paratope structure and changes the properties of amino acid residues present in the antigen binding site resulting in changes in the specificity of anti-M monoclonal antibodies [9]. Thus, when studying the interaction of lysozyme and anti-lysozyme antibody, it is shown that the surfaces of the epitope of lysozyme and paratope of the antibody are complementary even outside the binding site. It is also shown that water molecules form hydrogen bonds between the lysozyme epitope and the antibody paratope [10].

The results show that ethanol, due to its direct and indirect effects, alters the antigen-antibody interaction, which is proved by the variation of two agglutination reaction parameters: the degree and time to onset of agglutination of the antigen-antibody of the AB0 system. The AB0 blood group determines the specificity of the response of erythrocyte antigens and antibodies to the action of ethanol, forming the individual characteristics of the body.

Ethanol can regulate the enzyme-substrate interactions glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase, α-glycerol phosphate dehydrogenase. A comparative evaluation the effect of ethanol on the activity of dehydrogenases in the polyenzyme, polysubstrate medium (hemolysate) and in the isolated medium showed that the shift in the GAPDH, α-GPD and LDH activity occurred upwards in both media, but in quantitative terms, the enzyme activity in the isolated medium increased significantly less than in the hemolysate.

It is possible that direct and indirect effects of ethanol are summed up in the hemolysate, i.e. the interaction of ethyl alcohol with other reactive compounds is mediated, which creates an optimal environment for the course of the enzymatic reaction.

The studies of Y. Y. Schuckaya established that hydrogen peroxide is capable to oxidize SH-groups of cysteine in the catalytic center GAPDH [11]. The results obtained in a number of studies show the modulating effect of various compounds on the activity of α-GPD. Thus, ethanol-induced liver damage leads to increased activity of α-GPD in hepatocytes [12]. In the works of M. Wallner et al. ethanol in concentrations up to 1 mm was found to enhance the function of GABA receptors [13]. It is possible that ethanol modifies the structure of dehydrogenases, has a steric effect on the reacting atoms in the active center, and thus changes the activity of enzymes.

The increase in LDH activity in hemolysate is commensurate with the increase in HAFD activity and is significantly higher than the increase in α-HFD activity. Apparently, the effect of ethanol on the molecules of enzymes that have a tetrameric structure has similar mechanisms. The effects of ethyl alcohol on various proteins are largely determined by their subunit composition.

The systemic effect of ethanol on the enzyme-substrate interactions of the studied dehydrogenases has been established. The main goal of such enzyme sensitivity is to respond to environmental changes, adapt the cell to other conditions and give a proper response to the stimulus. The identified effect of ethanol on the activation of dehydrogenases in the erythrocyte lysate allows extrapolating the results to the possible effect of ethanol on the function of intracellular metabolism, as well as suggesting the presence of multiple effects of ethanol through modifying the conformation of proteins that subsequently enter into protein-ligand interactions. Thus, ethanol can play the role of a molecular metabolic process regulator by modulating protein-ligand interactions. Our data allows us to supplement the idea of protein-ligand interactions under certain conditions.

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