tion radiometer. The count efficiency with respect to ^{14}C was 80%; the error of the count did not exceed 5%. The time of half elimination of radioactivity from the blood plasma and organs was determined graphically in semilogarithmic coordinates [4]. In a study of the excretion of the preparation we used a special hermetic chamber with separator of urine and feces [6], in which the animals were placed throughout the experiment. As a result of the special design of the chamber, the exhaled carbon dioxide passed into a special trap. The collected samples of feces and urine were hydrolyzed with formic acid and clarified with a 30% solution of hydrogen peroxide. A correction for quenching was introduced to correct the count, using an internal standard.

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ANTIBODIES TO SECONAL AND THEIR USE FOR THE QUANTITATIVE DETERMINATION OF BARBITURATES

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Earlier we developed various highly sensitive immunochemical methods of quantitative determination of a number of drug preparations (for example, morphine, phenobarbital, catecholamines, and phenothiazines) in biological fluids [1-5]. The present communication is a continuation of these studies, and its goals were to develop a method of synthesis of the conjugated antigen 5-(1-methylbutyl)-5-allylbarbituric acid (seconal, I) with protein, to produce antibodies to I, to study their specificity, and also to develop a method of quantitative determination of low concentrations of barbiturates by the passive hemagglutination inhibition test (PHI test).

EXPERIMENTAL

5-(1-Methylbutyl)-5-(2-hydroxypropyl)-barbituric Acid (II). In 20 ml of concentrated sulfuric acid was dissolved 2 g of I. The solution was mixed at room temperature for 20 min and poured out into ice water. The precipitate formed was removed, dried, and recrystallized from aqueous ethanol. We obtained 1.3 g II. Yield 60%. Mp 204-205°C. According to the literature data [6], mp 205-206°C.

Conjugated Antigen of II-Protein. A solution of 3 g phosgene in 10 ml tetrahydrofuran and 12 ml pyridine was added to a solution of 4 g of the acid II in 60 ml tetrahydrofuran. The reaction mass was mixed for 24 h at room temperature. The precipitate of pyridine hydrochloride formed was filtered off and the filtrate evaporated. The residue was extracted repeatedly with ether. The ether solution was washed with water, dried over magnesium chloride,

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TABLE 1. Content of I Residues Covalently Bonded to One Molecule of Proteins as a Function of the Ratio of the Initial Components of the Conjugation Reaction

Amount of components introduced into reaction, mg	Substitution of I, moles/mole of BSA
5 000/800	2—3
5 000/1 600	10—11
5 000/2 000	14
5 000/15 000	17

Note. In the numerator: BSA; in the denominator: III.

TABLE 2. Dependence of the Titers of Antibodies to I in the PHI Test on the Amount of III Chloroformate Introduced into Reaction with 1 ml of Erythrocyte Precipitate

Concentration of III chloroformate, mg/ml	Dilution of serum, $-\log_2$ of of antibody titer							
	2	3	4	5	6	7	8	9
10	+	+	+	+	+	—	—	—
2	+	+	+	+	+	+	—	—
0,5	+	+	+	+	+	—	—	—
Normal erythrocytes	—	—	—	—	—	—	—	—

Note. Here and in Tables 3 and 4: "+" denotes agglutination; "—" denotes absence of agglutination.

evaporated, and 4.6 g of the chloroformate of 5-(1-methylbutyl)-5-(2-hydroxypropyl)-barbituric acid (III) was obtained. Yield 85%. The chloroformate of III obtained was introduced without further purification into a reaction with bovine serum albumin (BSA). For this purpose 10 g of protein was dissolved in 150 ml of water, and the pH of the solution was adjusted to 8.0-9.0 with a 10% solution of sodium hydroxide. A solution of 4.6 g III in 100 ml of dioxane was added to the solution obtained at 0-10°C dropwise with mixing, maintaining pH 7.0 by the addition of a 10% solution of sodium hydroxide. The reaction mass was mixed for 4 h at room temperature and exposed for 24 h at 4°C. The conjugated antigen obtained was freed of low-molecular-weight impurities by dialysis against distilled water for seven days. After lyophilic drying, 10 g of the conjugated antigen I-BSA (IV) was obtained. The quantitative determination of I in the synthesized conjugated IV was performed spectrophotometrically according to the difference of the free amino groups in the initial BSA and the conjugate obtained, using the method of determination of the free amino groups in protein preparations with trinitrobenzenesulfonic acid [7].

Conjugates IV containing an equal amount of covalently bound I were obtained according to the procedure described above, depending on the weight ratio of the initial components — BSA and II (Table 1).

TABLE 3. Dependence of the Titers of Antibodies to Seconal on the Composition of the Medium in the PHA Test

Composition of medium	Dilution of serum, $-\log_2$ of antibody titer								
	2	3	4	5	6	7	8	9	10
Physiological solution	±	±	±	±	±	±	=	=	=
Medium B	±	±	±	±	±	±	±	±	=

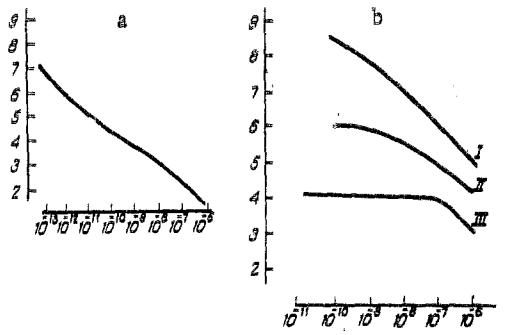


Fig. 1. Calibration curves for the determination of I (a), I, II, and III (b). Along x axes: concentration (in moles/ml); along y axes: $-\log_2$ of antibody titer.

Buffer Solutions and Media for Reactions. Solution A: Tris-buffer: 0.02 M Tris, 0.15M sodium chloride solution, pH 8.2; solution B: medium for the passive hemagglutination test (PHA test). Normal rabbit serum was decomplemented at 56°C for 30 min and then treated with an equal volume of a precipitate of sheep erythrocytes at 37°C for 1 h and at 4°C for 4 h for adsorption of nonspecific agglutins. Then 25 ml of 6% dextran (molecular weight 80,000) and 50 ml of solution A were added to 1 ml of the serum prepared in this way.

Production of Antiserum to I. Rabbits were immunized with a solution of the conjugated antigen I-BSA, containing 10 molecules of I covalently bonded to one molecule of protein, twice a week for six weeks in doses of 25 mg/kg. Blood was collected from the marginal vein of the ear seven days after the last immunization, and the serum was removed. The serum obtained was decomplemented at 56°C for 30 min.

Sensitization of Sheep Erythrocytes by Covalent Bonding of I. To a suspension of 1 ml of erythrocyte precipitate in 20 ml of phosphate buffer pH 7.0, a solution of 10 mg III in the minimum amount of dioxane (2-3 drops) was added with cooling. The reaction mass was incubated at 4°C for 24 h, and the sensitized erythrocytes were washed with physiological solution. Analogously, to obtain erythrocytes with a different degree of substitution, 2 and 0.5 mg III were added to a suspension of 1 ml of the erythrocyte precipitate.

Detection of Antibodies to I by the PHA Test. A series of successive dilutions with immune serum was preparated in a Takachi microtitrator (Japan). For this purpose 0.025-ml portions of solution B were introduced into all the wells of the microtitrator, 0.025 ml of immune serum was introduced into the first well, and then 0.025-ml portions of solution were transferred from the first well into the second and then from each preceding well to each subsequent one. The plates with serum were incubated for 15 min at 37°C. Then 0.025 ml of a 1% solution of I-sensitized erythrocytes in solution A was added to each well. The plates were exposed for 2 h at 37°C. As a control for nonspecific agglutination, decomplemented normal rabbit serum was introduced into the reaction instead of the immune serum, and native cells instead of the sensitized erythrocytes. Hemagglutination was evaluated visually according to the nature of the precipitate in the wells. The last dilution of the serum at which agglutination was still observed was taken as the antibody titer.

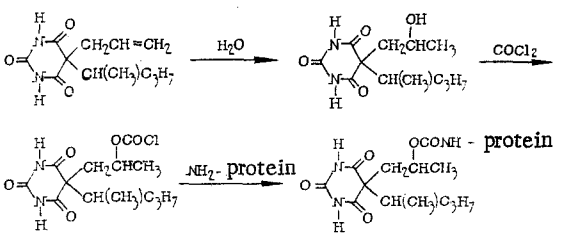
TABLE 4. Determination of Titers of Antibodies to I in Sera of Immunized Rabbits

Serum	Animal No.	Dilution of serum, $-\log_2$ of antibody titer									
		2	3	4	5	6	7	8	9	10	11
Before immunization	1	—	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	—	—
	4	—	—	—	—	—	—	—	—	—	—
Immune	1	+	+	+	+	+	+	+	+	+	—
	2	+	+	+	+	+	+	+	+	+	—
	3	+	+	+	+	+	+	+	+	—	—
	4	+	+	+	+	+	+	+	+	+	—

Procedure of the PHI Test for the Study of Specificity of Antibodies and Construction of Calibration Curves. Dilutions of immune serum were prepared as for the PHA test, then 0.025 ml of a solution of one of the barbiturates of known concentration was added to each well. The mixture was incubated for 15 min at 37°C. Erythrocytes sensitized by I were added to all the wells, the mixture incubated, and hemagglutination evaluated. The operations were repeated with different concentrations of barbiturates, and calibration curves of the dependence of the antibody titer on the barbiturate concentration were constructed (see Fig. 1).

RESULTS AND DISCUSSION

The production of antibodies to I requires preliminary synthesis of conjugated antigen (IV), in which I is covalently bonded to an antigenic protein carrier. A method of production of such a conjugate by the reaction of 5-allyl-5-(3-carboxypropyl)-barbituric acid with bovine γ-globulin has been described in the literature [8]. In this method large amounts of the preparation were introduced into the reaction with protein (500-1000 moles I per mole of protein), and the substitution in the conjugate obtained reached only 2-3 moles of barbiturate per mole of carrier, i.e., a low conversion of the reactive barbituric acid derivatives was observed. To obtain antibodies it was better to use conjugates containing 10-15 hapten groups, covalently bonded to one molecule of the carrier; therefore we decided to develop a different method of bonding I to the protein carrier, permitting the production of conjugates with a larger content of I. For this purpose I was hydrated in the presence of sulfuric acid, producing II, which was introduced into a reaction with phosgene, and the chloroformate III was isolated. This reactive barbituric acid derivative can be covalently bonded to lysine residues in the protein, forming a peptide bond between the I and protein molecules.



We developed optimum conditions of covalent bonding of I to protein. In a study of the dependence of the content of I in the conjugates on the ratio of the chloroformate III and the protein introduced into the reaction, it was established that the content of I is directly proportional to the amount of III introduced into the reaction, so that conjugates of IV with the desired amount of hapten can be obtained (see Table 1). It should be noted that in the method that we developed, in comparison with the well-known method of [8], the conversion of the reactive barbituric acid derivatives is increased by a factor of 50-70, which permits the production of conjugates IV with a larger degree of substitution.

The conjugate IV containing 10 molecules of I covalently bonded to one molecule of BSA was used to immunize rabbits for a period of six weeks. The presence of antibodies to I in

TABLE 5. Specificity of Antibodies to I

Compound used for inhibition of PHA test in 10^{-6} mole/ml conc.	Antibody titer
Without compound (initial titer of PHA test)	1 : 512
I	Entirely inhibited
II	1 : 4
Hexenal	1 : 32
Phenobarbital	1 : 8
Thiopental	1 : 16

the sera of the immunized animals was established by the PHA test, using sheep erythrocytes with I covalently bonded to them as the reagent. We also used the chloroformate III for the bonding of I to the cells. This reactive derivative reacts with the protein components of the erythrocyte membrane. Reaction III with the cells was conducted in isoosmotic physiological saline, adding III to a suspension of erythrocytes dissolved in the minimum amount of dioxane. Then we studied the dependence of the detectable antibody titers in the PHA test on the amount of the reactive derivative I, introduced into the reaction with erythrocytes. Table 2 presents data on the determination of the titers of antibodies in the PHA test, using these erythrocytes as the reagent for the same serum. It was found that when 2 mg I is introduced into the reaction with 1 ml of the erythrocyte precipitate, the largest antibody titer is observed in the PHA test; when this amount is increased or decreased, the sensitivity of the PHA test drops. Therefore we subsequently used erythrocytes with the optimum amount of I bonded to them for the determination of the antibody titers. The sensitized erythrocytes were stable for seven days and ensured good reproducibility of the results of the PHA test.

As is well known, the antibody titer in the PHA test depends not only on the amount of the preparation on the surface of the erythrocytes but also on the composition of the reaction medium; an increase in the sensitivity of the method can be achieved by increasing the pH and the viscosity of the medium. The addition of normal rabbit serum and dextran solution to the reaction medium led to an increase in the antibody titer (Table 3). The antibody titer in rabbits immunized with the conjugate IV ranged from 1:512 to 1:2056 (Table 4).

We studied the specificity of the antibodies obtained by the PHI test. The specificity was determined by comparing the ability of the preparation to which the antibodies were produced, as well as its derivatives and structurally related compounds, to inhibit the agglutination reaction induced by the interaction of the antibodies with erythrocytes sensitized by covalent bonding to I. Solutions of barbiturates in a concentration of 10^{-6} mole/ml were used for inhibition. The data obtained are presented in Table 5. From the data obtained it follows that antibodies to I bind both preparation I and the hydroxyderivative of I, i.e., II (used for conjugation, rather well, and they also react with other barbiturates, i.e., phenobarbital, hexenal, and thiopental. Thus, antibodies universal for drug preparations — barbituric acid derivatives — were obtained.

To develop a method of quantitative determination of barbiturates by the PHI test we studied the dependence of the decrease in the value of the antibody titer on the concentration of added barbiturates. It was established that a significant decrease in the antibody titer is observed even when the liquid to be tested contains 10^{-13} mole/ml I, as well as 10^{-11} mole/ml phenobarbital, thiopental, and hexenal (see Fig. 1). For a quantitative determination of barbiturates in the fluid to be analyzed, this test fluid and I-sensitized erythrocytes must be added to the immune antiserum, the reaction medium incubated, and hemagglutination established, and then the amount of barbiturate calculated according to a calibration curve.

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TOPOGRAPHY OF α- AND β-ADRENORECEPTORS

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The experimental material now accumulated permits molecular modeling of the structure of the binding sites of certain types of the best-studied receptors of pharmacologically active substances. The method of construction of the structure of the adrenoreceptors (AR) used in the present work includes comparative estimates of the energies of interaction of a large group of substances with α_1- and β-AR, the detection of the nature of the physicochemical bonds of individual fragments of the molecules to the corresponding portions of the receptor, and three-dimensional modeling of the arrangement and orientation of the functional groups of the binding site, using the Covey–Pauling–Koulton molecular model.

Approximation and Assumptions Used in the Modeling of the Topography of the Binding Sites of Receptors

1. The energy of binding of a substance to a receptor represents the sum of the partial energy contributions of the interactions among the individual fragments of molecules of the compound with the corresponding groups of the binding site. The energies of the bonds encountered in receptor–ligand interactions are cited in [1].

2. Calculation of the change in the free energy of formation of the receptor–ligand complex was performed from the function $\Delta G = -RT \cdot \ln K_{bond}$, where $K_{bond} = 1/K_\partial \cdot K_\partial$ is the apparent dissociation constant of the complex, equal to $IC_{50}/(1 + [RL]/K_\partial^{RL})$, where IC_{50} is the concentration of the substance producing 50% substitution of the radioligand in the complex with the receptor; [RL] is the concentration of the radioligand; K_∂^{RL} is the apparent dissociation constant of the complex of the radioligand with the receptor.

3. To estimate the degree of hydrophobic interaction (rearrangement of the solvate shell when two hydrophobic regions are in contact), we used the distribution coefficients of individual lipophilic fragments of the molecules in the octanol/water system [2, 3]. The distribution coefficients of whole molecules evidently do not reflect the true processes of resolvation occurring when the substance is deposited on the receptor and are unsuitable for the investigation of structure-specific interactions.

4. Receptors labeled by a radioligand are structurally homogeneous; the functional groups of their binding sites are rigidly fixed and do not change their arrangement in the interaction with compounds of different chemical natures.

5. The topography of the active groups of the binding sites was modeled according to the principle of minimization of the number of functional groups providing for the corresponding energies of interaction for the maximum number of compounds.

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