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COVALENT BONDING OF PHENOBARBITAL TO PROTEIN FOR THE PRODUCTION OF ANTIBODIES NEUTRALIZING THE TOXIC ACTION OF PHENOBARBITAL

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Recently the possibility of neutralizing the pharmacological effect and toxic action of a number of biologically active compounds with antibodies for these compounds has been demonstrated [1-3]. For the production of such antibodies, conjugated antigens, i.e., chemical compounds covalently bonded to a protein antigenic carrier, must be preliminarily synthesized. When animals are immunized with these conjugates, antibodies that specifically bind the corresponding chemical compounds appear in their blood.

Among drug preparations, barbiturates (especially phenobarbital) are most frequently the cause of poisoning of humans; however, the existing methods of treatment of barbiturate poisoning are as yet rather ineffective [4]. In view of this, we decided to study the possibility of neutralizing the toxic action of phenobarbital by an immunochemical method, based on the use of antibodies for phenobarbital.

The present work is devoted to the development of the synthesis of a conjugated phenobarbital—protein antigen, the production of antibodies to phenobarbital, and the study of their detoxifying activity.

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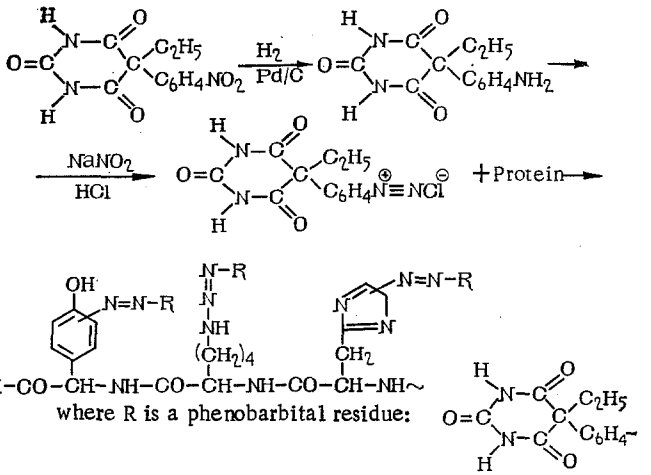
Covalent bonding of proteins to low-molecular-weight compounds is accomplished by various methods when such reactive groups as hydroxy, amino, or carboxyl are present in the molecule of the low-molecular-weight compounds. The phenobarbital molecule does not contain these functional groups; therefore it was necessary to synthesize one of the possible phenobarbital derivatives reactive to protein. It was decided to bond phenobarbital covalently to bovine serum albumin (BSA) by the reaction of the diazonium salt of 5-ethyl-5-(m-aminophenyl)barbituric acid that we synthesized with BSA. The basic advantages of this method are the accessibility and simplicity of the synthesis of the initial diazonium salts as well as the high yields in their reaction with proteins. Covalent addition of phenobarbital is accomplished in this case by means of an azo bond to the phenol ring of tyrosine, the imidazole of histidine, and to the free amino groups of lysine according to the following scheme:



5-Ethyl-5-(m-aminophenyl)barbituric acid was synthesized according to the method of [5] by the nitration of phenobarbital with nitric acid, followed by reduction of the 5-ethyl-5-(m-nitrophenyl)barbituric acid isolated with hydrogen in the presence of Pd/C. The amino derivative obtained was diazotized in acid medium with an equimolar amount of sodium nitrite, and the freshly prepared solution of the diazo salt was reacted with BSA, dissolved in phosphase buffer solution (pH 8.0), under mild conditions at 0°, maintaining pH 8.0-9.0 by the addition of a 20% sodium hydroxide solution. The brightly colored phenobarbital preparation formed, covalently bonded to protein (PB-BSA), was separated from the low-molecular-weight fraction by gel filtration on Sephadex G-25. In repeated gel chromatography of the isolated high-molecular-weight fraction, no separation of low-molecular-weight substances was observed; consequently, the separation and purification are complete. Data on the purification of the synthesized preparations by gel filtration are presented in Fig. 1.

Proteins, especially albumins, are capable of sorbing low-molecular-weight substances under definite conditions; therefore it was necessary to ascertain that under the conditions of the azo-coupling reaction and subsequent purification of the protein fraction by gel filtration, there is no adsorption of low-molecular-weight substances by the protein. Since compounds that might be sorbed on protein are decomposition products of the unreacted diazo salt of 5-ethyl-5-(m-aminophenyl)barbituric acid in the reaction medium, the following control experiment was conducted: 5-ethyl-5-(m-aminophenyl)barbituric acid was diazotized, exposed in phosphate buffer solution at pH 8.0-9.0 in the absence of the protein under conditions analogous to the conditions of the azo-coupling reaction, and the UV spectrum of the solution formed was studied. Then the solution obtained was mixed with a solution of BSA in phosphate buffer solution in various weight ratios, exposed for several hours, separated by gel filtration, and the UV spectra of the high- and low-molecular-weight fractions collected from the column were studied. It was established that the UV spectra of the initial protein and that isolated in the control experiment from a mixture of it with products of the decomposed diazo salt of aminophenobarbital are identical in the case of equal protein concentrations (Fig. 2). Consequently, the sorption of low-molecular-weight substances on protein does not occur under the reaction conditions, and all the phenobarbital in the preparations obtained is covalently bonded to BSA.

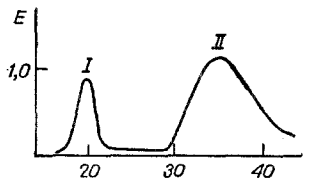


Fig. 1

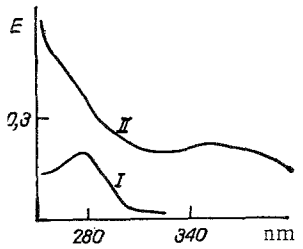


Fig. 2

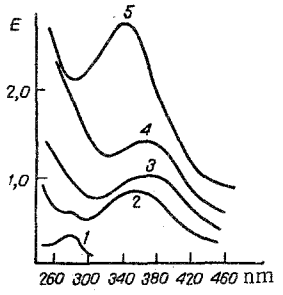


Fig. 3

Fig. 1. Gel filtration of the products of the reaction of the diazo salt of 5-ethyl-5-(m-aminophenyl)barbituric acid with BSA. I) Protein fraction; II) low-molecular-weight fraction. Along y axis: UV absorption at 280 nm; along x axis: fraction number.

Fig. 2. UV spectra at pH 7.0. I) Initial BSA and that isolated from the control experiment in a concentration of 1 mg/4 ml; II) decomposition product of the diazo salt of 5-ethyl-5-(m-aminophenyl)barbituric acid (1 mg/50 ml).

Fig. 3. UV spectra at pH 7.0 and a concentration of 1 mg/2 ml. 1) BSA; 2) conjugate PB–BSA, containing 2.3% phenobarbital; 3) conjugate PB–BSA containing 8,4% phenobarbital; 4) conjugate PB–BSA containing 33,5% phenobarbital; 5) conjugate PB–BSA containing 54.5% phenobarbital.

TABLE 1. Dependence of the Content of Phenobarbital Covalently Bonded to Protein on the Ratio of the Initial Components Introduced into the Reaction

Weight ratio of BSA and 5-ethyl-5-(m-aminophenyl)barbituric acid introduced into the reaction	Content of phenobarbital covalently bonded to BSA in the conjugate PB–BSA, % by weight
18 : 1	2,3
9 : 1	8,4
2 : 1	16,5
1 : 1	33,5
1 : 2	54,5

The phenobarbital content in the synthesized preparations of PB–BSA was determined spectrophotometrically according to the difference of the 5-ethyl-5-(m-aminophenyl)barbituric acid taken for the reaction and the amount unreacted. The amount X of barbituric acid that did not react with BSA (in mg) was calculated according to the formula

$$X = \frac{V \cdot E_v}{E},$$

where V and E_v are the volume and absorption at 280 nm of the low-molecular-weight fraction, isolated by gel filtration after the reaction of azo coupling of aminophenobarbital with BSA.

E is the absorption of decomposition products of the diazonium salt at 280 nm under conditions of the azo-coupling reaction in a concentration of 1 mg/ml, which, as was established in the control experiment, is equal to 14.

It was found that depending on various weight ratios of BSA and the initial aminophenobarbital, products with different percent contents of phenobarbital are formed (see Table 1).

The preparations of PB—BSA isolated and purified by gel chromatography, in comparison with the initial BSA, had a specific absorption in the UV spectrum at 280 nm and contained a new absorption maximum at 360-380 nm, characteristic of azoproteins (Fig. 3).

The synthesized preparation of PB—BSA, containing 2.3% phenobarbital, was used for the immunization of tetrahybrid male mice (A/He × BaLb/c × C57B1/6 × CC57W) weighing 18-20 g. One group of animals received a solution of the conjugate intraperitoneally in physiological solution in a dose of 25 mg/kg six times at 4-day intervals. Another group of mice was analogously given a solution of a mixture of phenobarbital and BSA in doses equivalent to the conjugate; a third group of animals served as the control — they received the corresponding volume of physiological solution. One day after the last injection of the indicated solutions, all the animals, divided into three subgroups, received a single intraperitoneal injection of phenobarbital in doses of 80, 150, or 200 mg/kg. When the preparation was administered in a dose of 80 mg/kg, no death of the animals was observed in any group; when a dose of 150 mg/kg was used, 50% of the animals died in the second and third groups, while all the immunized mice remained alive. Complete protection of the immunized animals was also observed when LD_{100} of phenobarbital (200 mg/kg) was used.

Thus, we synthesized a conjugated antigen PB—BSA, immunization of the animals with which leads to an induction of an extremely strong tolerance in them. No negative phenomena associated with immunization were observed.

EXPERIMENTAL

5-Ethyl-5-(m-aminophenyl)barbituric Acid (I). A total of 2480 ml (0.11 mole) of hydrogen was passed into a suspension containing 10 g (0.037 mole) 5-ethyl-5-(m-nitrophenyl) barbituric acid, 150 ml ethanol, and 1 g of a 10% Pd/C catalyst. The reaction mixture was mixed for 1 h, and the catalyst filtered off. The filtrate was evaporated to dryness, and the precipitate recrystallized from ethanol. Yield 6 g (68%) I with mp 208-209°. Literature data [5]: mp 208-209°.

Conjugated Antigen PB—BSA (II). To a solution of 55.5 mg I in 1 ml concentrated hydrochloric acid, 15.5 mg sodium nitrite, dissolved in the minimum amount of water, was added at 0°. The reaction mixture was exposed at 0° for 1 h, and then the solution of the diazo salt obtained was slowly added dropwise at 0° to a solution of 1 g BSA in 4 ml 0.15 M phosphate buffer solution (pH 8.0) at a pH value of 8.0-9.0, regulated by the addition of a 20% solution of sodium hydroxide. The reaction mixture was exposed for 1 h at 0°, and the conjugate (II) formed was separated from the low-molecular-weight fraction by gel filtration on a column with Sephadex G-25. After lyophilic drying, 1 g of II containing 2.3% by weight phenobarbital according to the data of the UV spectra was obtained.

Gel chromatography was conducted on a column with Sephadex G-25, equilibrated with distilled water at pH 7.0, at a rate of elution of 150 ml/h and a volume of the fraction 5 ml. The UV spectra were obtained on a Spektromom-203 instrument in distilled water or phosphate buffer solution at pH 7.0.

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