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THE PRODUCTION AND CHARACTERIZATION OF ANTIBODIES AGAINST β-ADRENERGIC ANTAGONISTS

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Abstract—Antibodies were raised against β-adrenergic antagonists of the phenoxypropylamine series by the use of an alprenolol-bovine serum albumin conjugate. Binding of [^{3}H]dihydroalprenolol was studied by means of the Farr technique. Inhibition of [^{3}H]dihydroalprenolol binding by several β-adrenergic antagonists revealed the following sequence in binding affinities, propanolol–bromohydrinalprenolol–tazolol–alprenolol–pindolol. Specificity of the antibodies was further shown by their ability to inhibit [^{3}H]dihydroalprenolol binding on the adrenergic receptor from turkey erythrocyte membranes. Specific antibodies were purified by affinity chromatography. Purification from the γ-globulin fraction was 200-fold and recovery of binding capacity was 88 per cent. The heterogeneity index of the purified antibodies was 1 and the association constant 1.4×10^8/mole.

Since the introduction of β-adrenergic blocker [1], the use of these compounds in the treatment of cardiovascular diseases has increased tremendously. The phenoxypropylamine series, the best known example of which is propanolol, has given rise to several therapeutical useful drugs. Recently the introduction of β-adrenergic blockers in the study of the properties of specific receptors from various tissues [2, 3], widened the fundamental importance of this class of drugs.

The isolation of the β-adrenergic receptor by affinity chromatography on an alprenolol–agarose column in our laboratory incited us to elaborate new approaches in the study of those drugs [4]. In this report we present a method to raise antibodies against dihydroalprenolol in rabbits. Their specificity towards several analog drugs was tested, their effect on the binding of alprenolol to β-adrenergic receptors studied and a successful attempt was made to purify the specific anti-alprenolol antibodies by affinity chromatography.

MATERIALS AND METHODS

Chemicals. The following were obtained as gifts: (±)-Alprenolol hydrochloride (Ciba–Geigy Lab.); Propanolol (ICI); Butoxamine hydrochloride (Burroughs Wellcome); Pindolol (Sandoz) and Tazolol (Syntex). [^{3}H]dihydroalprenolol (33 Ci/m-mole) was purchased from New England Nuclear. Bovine serum albumin (BSA) was fraction V from Sigma Chemical Compound, and bovine γ-globulins from Miles. All other reagents were of analytical grade.

Alprenolol-BSA conjugates

Synthesis of bromo-alprenolol. Alprenolol contains an olefin moiety which can readily react with *N*-bromosuccinimide in water to yield the corresponding bromohydrin [5]. *N*-bromosuccinimide (178 mg) was dissolved in 100 ml of 10 mM (±)-alprenolol in water. The mixture was stirred for 2 days at 4° in the dark. Physicochemical data (thin-layer chromatography, u.v. spectra, to be published) indicated the quantitative transformation of alprenolol in its corresponding bromohydrin.

Synthesis of the protein conjugate. Twenty five mg BSA were dissolved in 5 ml of a 7 M guanidium–HCl solution brought to pH 8 with a 0.5 M Tris buffer in order to allow the disulfide bridge to be reduced by the Cleland reagent (dithiothreitol). After addition of 7.25 mg of the reagent the solution was kept under a nitrogen atmosphere for 5 hr at 37°.

The reaction products were sieved through a Sephadex G 25 column (1.6 × 32 cm), equilibrated with 0.1 M sodium carbonate (pH = 8.2), flushed with nitrogen. The fraction eluted in the void volume

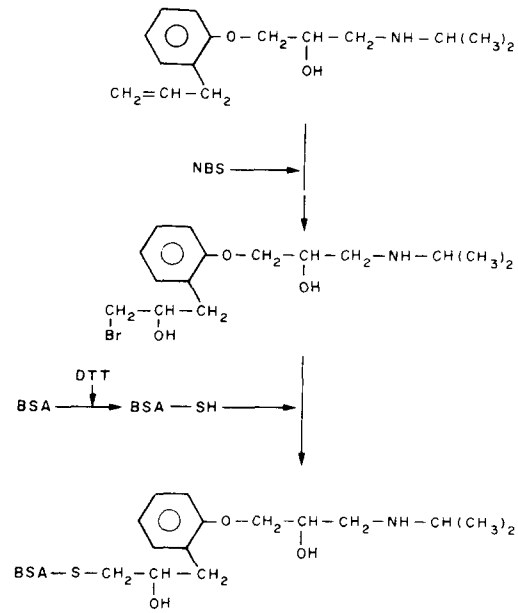


Fig. 1. Reaction scheme for the preparation of the hapten–protein conjugate. NBS = *N*-bromosuccinimide, BSA = Bovine serum albumin, DTT = Dithiothreitol.

of the column was added directly to 20 ml of a solution of 100 mg bromo-alprenolol in the same buffer.

Bromo-alprenolol was coupled to BSA at 4°, under a nitrogen atmosphere, in the dark for 48 hr. After reaction, the mixture was dialyzed against continuously flowing deionized water for 3 days, and lyophilized. The lyophilized product weighed 8.5 mg.

Figure 1 gives a schematic representation of the conjugation reaction.

Immunization. A rabbit was immunized with 1 mg conjugate, dissolved in phosphate-buffered saline (PBS), to which 1 ml of complete Freund's adjuvant (Difco) was added. The emulsion was injected subcutaneously (s.c.) on eight places in the flanks of the animal.

Fourteen, 28 and 42 days later the animal was reinjected with 1 mg of the conjugate dissolved in 1 ml PBS and emulsified in 1 ml incomplete Freund's adjuvant (Difco).

Preparation of the immunoglobulin fraction. Seven days after the booster injections, blood was drawn from the ear vein. After coagulation and centrifugation the serum was collected and an aliquot was used to study the binding of [^{3}H]dihydroalprenolol.

From the remaining serum, the immunoglobulin fraction was purified by precipitating twice with 35% $(NH_4)_2SO_4$. The precipitate was dissolved in a minimal amount PBS to which 0.05% azide was added as preservative. After thorough dialysis against PBS-azide, the immunoglobulin fraction was centrifuged to remove aggregated material and stored at 4° until use. Concentration was determined by measuring the absorbance at 280 nm, taking the value of 1.4 as the absorbance of 1 mg/ml. Concentrated immunoglobulin solutions contained 33 mg ± 0.5 mg/ml.

Binding studies by means of the Farr technique [6]. Different amounts of a [^{3}H]dihydroalprenolol solution in PBS-azide 0.05% were added to 0.100 ml of a 10 mg/ml bovine immunoglobulin.

The mixture was allowed to react overnight at 4° and consequently the immunoglobulins were precipitated by addition of 0.5 ml of cold 100% $(NH_4)_2SO_4$. After 30 min, the suspension was centrifuged for 4 min in an Eppendorf centrifuge (12,000 times/min). After removal of the supernatant, the precipitate was resuspended into 0.5 ml of 50% $(NH_4)_2SO_4$ and centrifuged for 2 min. After removal of the supernatant, the precipitate was dissolved in 0.5 ml PBS-azide 0.05% and transferred into scintillation vials. Five ml of Unisolve was added and radioactivity was measured in a Packard scintillation β-counter.

For inhibition studies, incubations were performed in similar conditions, but in presence of β-adrenergic blockers of different concentrations.

Binding of [^{3}H]dihydroalprenolol to the β-adrenergic receptor of turkey erythrocyte membranes. Aliquots of immunoglobulins were incubated at 30° in 75 mM Tris–HCl buffer pH = 7.4 and 25 mM $MgCl_2$ with 2 nM [^{3}H]dihydroalprenolol for 20 min in a volume of 0.2 ml. Subsequently 0.2 ml of membranes from turkey erythrocytes (8 mg/ml) in the same buffer were added and incubation continued for 10 min. At the end of the incubation, triplicate 0.1 ml aliquots were diluted at 0° in 4 ml of the same buffer. The contents of the tubes were filtered under suction through glass fiber filter discs (25 mm diameter). Filters were washed with 6 ml ice-cold buffer and placed in scintillation vials. One ml HCl

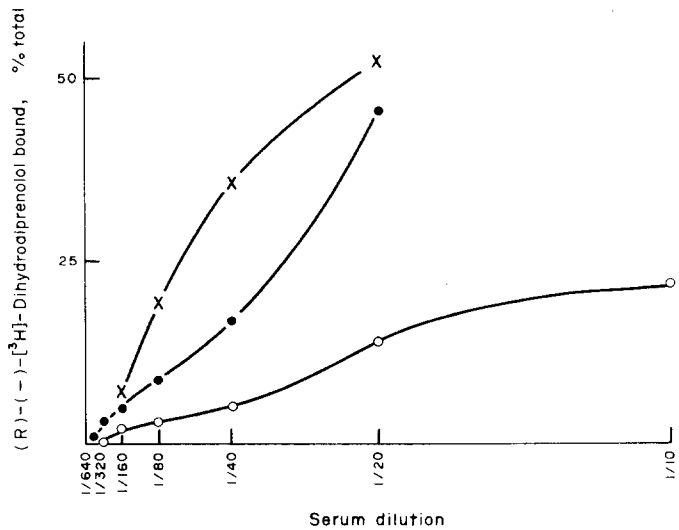


Fig. 2. Binding of [^{3}H]dihydroalprenolol to immune serum. To a final volume of 0.5 ml immune serum dilution, 0.05 ml 10^{-8} M [^{3}H]dihydroalprenolol was added. Bound radioactivity was determined by the Farr technique. Blanks were made up by serum dilutions of pre-immune serum from the immunized animal and subtracted from the radioactivity bound by the immune serum. Points are the mean of at least two independent experiments. O——O Binding after first booster injection (3 weeks after first immunization). ●——● Binding after second booster injection (5 weeks after first immunization). ×——× Binding after third booster injection (7 weeks after first immunization).

1 N and 10 ml scintillation fluid were added and the amount of bound ligand determined.

Affinity chromatography of the anti-alprenolol antibodies. Affinity chromatography was performed on an agarose-alprenolol derivative described elsewhere [4]. Ten ml immunoglobulins (33.6 mg/ml) were passed through a 5 ml column of adsorbent and the column was washed with PBS-azide until the optical density of the effluent returned to the baseline. Elution of the adsorbed antibodies was performed with a 10^{-3} M propanolol solution. Elution fluids were collected, and the immunoglobulins precipitated twice with 50% saturation of $(NH_4)_2SO_4$. The pellet was dissolved in 1 ml PBS-azide and thrice dialyzed in 1 l. of the same buffer. The dialyzed immunoglobulins were brought to 3 ml with PBS-azide and centrifuged to eliminate the aggregates. The obtained solution was tested subsequently for alprenolol-binding capacity.

RESULTS

The binding capacity of the immune serum towards [^{3}H]dihydroalprenolol was measured 1 week after the first, the second and the third booster injections. As shown in Fig. 2, the titer increased thrice after the second immunization and again twice after the third booster. Since the titer after the first booster injection was too low to determine the characteristics of the produced antibodies, the results described were obtained with the immunoglobulins purified 1 week after the second and the third booster. Figure 3 represents the binding characteristics of the antibodies after the second booster of the [^{3}H]dihydroalprenolol concentration as proposed by Nisonoff and Pressman [7]. The curvature of the plot indicates the heterogeneity of the antibody molecules produced. Using the method of Nisonoff [8], we calculated for different values of α, the heterogeneity index, the best fit to a straight line when plotting the reciprocal of alprenolol bound vs the reciprocal of free alprenolol to the power α. Best fit was obtained for $\alpha = 0.8$. The apparent dissociation constant and concentration of specific antibodies was calculated according to following formula:

$$\frac{1}{(AbH)} = \frac{1}{(Ab_t)K_o^{\alpha}[H]^{\alpha}} + \frac{1}{(Ab_t)}$$

where (AbH) = the molar concentration of bound alprenolol, (Ab_t) = the molar concentration of binding antibodies, K_o = the apparent association constant, α = the heterogeneity index, $[H]$ = the molar concentration of free alprenolol.

If one assumes that every antibody has two binding sites, calculations suggest that 0.15 per cent of the immunoglobulin fraction is specific for the dihydroalprenolol and that the apparent association constant is 6×10^6. This value is in accordance with the average antibody affinity obtained for other haptens [9].

To study the specificity of the antibodies, a series of β-adrenergic blockers were tested for their inhibition of the [^{3}H]dihydroalprenolol binding. Table 1 lists the structures of the compounds tested. With the exception of butoxamide all the compounds were able to inhibit binding of [^{3}H]dihydroalprenolol to the antibodies.

Further confirmation of the specificity of the antibodies was given by the inhibition of alprenolol-binding to the β-adrenergic receptors from turkey erythrocyte membranes. As shown in Table 2, the binding of dihydroalprenolol was inhibited 94 and 86 per cent in the presence of respectively 4 mg and 2.5 mg/ml immunoglobulins. The same quantities of bovine immunoglobulins had no effect on the specific β-adrenergic receptor binding.

The increase in titer after the third booster fostered us to purify the anti-alprenolol antibodies by affinity chromatography. Figure 5 shows the binding properties for the γ-globulin fraction, the fraction which was not retained by the affinity-adsorbent and the purified anti-alprenolol antibodies. From the half saturation point it can be deduced that the anti-alprenolol fraction was purified 200-fold. Since from 336 mg γ-globulin 1.5 mg purified anti-alprenolol antibodies were obtained, it means that 83 per cent of the binding capacity could be recovered. Analysis of the binding curve of the purified antibodies by the method of Nisonoff ($r = 0.998$) resulted in a heterogeneity index $\alpha = 1$ and an association constant $K_a = 1.4 \times 10^8$ mole.

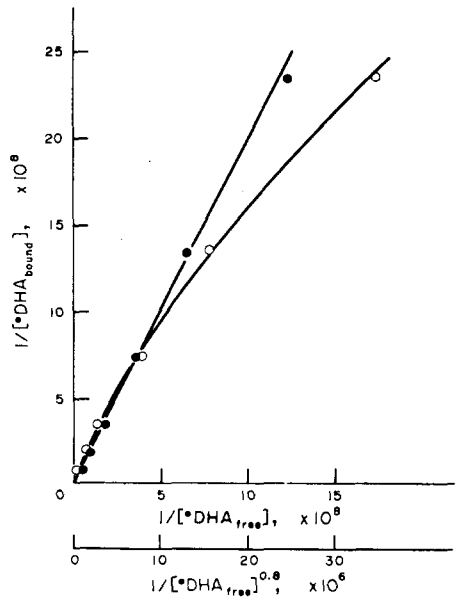
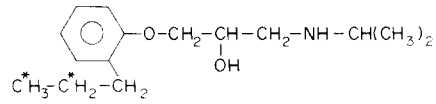
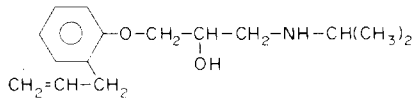
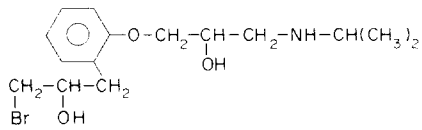
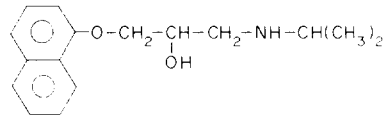
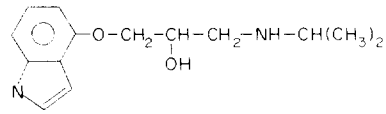
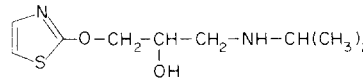
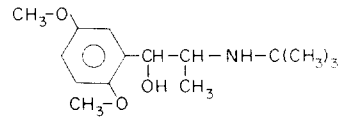


Fig. 3. Reciprocal curve for the binding of [^{3}H]dihydroalprenolol to the immune γ-globulin fraction. Binding studies were performed with different concentrations of [^{3}H]dihydroalprenolol to a 1.33×10^{-5} M solution of immunoglobulins. Blanks, made up of bovine immunoglobulins were subtracted. Results are the mean of two independent experiments. O——O Reciprocal curve. ●——● Best fit calculated by means of the least square method for a heterogeneity index = 0.8 ($r^2 = 0.9985$).

DISCUSSION

The use of radiolabeled β-adrenergic antagonists has permitted direct characterization of the β-adrenergic receptor by means of binding studies [2, 4]. We have recently purified such a receptor from

Table 1. Structure of the different β-adrenergic antagonists studied. ID_{50} doses were calculated from the results of Fig. 4.

Compound	Chemical structure	ID_{50} in μM
Dihydro-alprenolol		—
Alprenolol		0.24
Bromohydrin-alprenolol		0.16
Propanolol		0.039
Pindolol		0.28
Tazolol		0.21
Butoxamine		>100

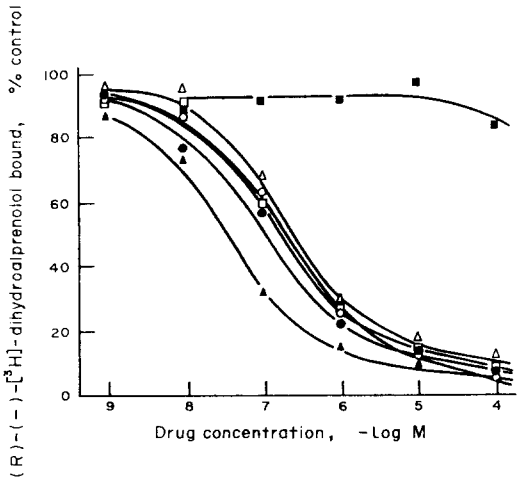


Fig. 4. Inhibition curves for different β-adrenergic antagonists. To 1 mg γ-globulins were added 2×10^{-8} M [^{3}H]dihydroalprenolol and increasing amounts of β-adrenergic antagonists. Blanks were made up with 1 mg bovine immunoglobulins and substracted from the radioactivity bound to the immune globulins. Means of two independent experiments are shown. ○——○ Alprenolol. ▲——▲ Propanolol. □——□ Tazolol. ●——● Bromohydrinalprenolol. △——△ Pindolol. ■——■ Butoxamide.

turkey erythrocyte membranes. It seemed interesting to us to try to induce antibodies against β-adrenergic blockers in order to have specific molecules which could compete with the receptor for the drugs used. Two conditions are necessary for this specific competition. The antibodies raised must interact directly or indirectly with the sites necessary for the binding and the affinity constant of the antibodies should not be too different from that of the receptor. The synthesis of bromoalprenolol in our laboratory and its successful binding on a sulfhydryl-containing spacer-arm [4] incited us to link this compound to reduced serum albumin and obtain in this way an immunogenic hapten–protein conjugate. No analysis of this hapten–protein conjugate was performed in view of the difficulties involved in hapten–protein ratio measurement when no radioactive precursors are available. We may nevertheless infer from the successful induction of antibodies with as little as 1 mg of hapten–protein conjugate that the ratio must be sufficiently high. The low dose of immunogen injected could also account for the relatively high affinity of the antibodies for the hapten [10].

The specificity of the antibodies was assessed by inhibition studies with several β-adrenergic blockers. The absence of inhibition of butoxamide hints towards antibody specificity for compounds

Table 2. Inhibition by anti-alprenolol antibodies of the binding of [^{3}H]dihydroalprenolol to the β-adrenergic receptor of turkey erythrocyte membranes. Inhibition was calculated according to the following formula:

$$\text{Per cent inhibition} = 100 \times \left(1 - \frac{\text{DHA bound to membranes + anti-alprenolol antibodies} - \text{DHA bound to antibodies}}{\text{DHA Bound to membranes}}\right)$$

Controls were performed with non-specific bovine γ-globulins (B.G.G.).

	Per cent inhibition
Membranes + Anti-alprenolol antibodies (4 mg/ml)	94
Membranes + B.G.G. (4 mg/ml)	−13
Membranes + Anti-alprenolol antibodies (2.5 mg/ml)	86
Membranes + B.G.G. (2.5 mg/ml)	

of the phenoxypropylamine class. This contrasts with the specificity of adrenergic receptors for both phenoxypropylamine and phenylethanolamine derivatives [4, 11]. It is worthwhile to mention that dihydroalprenolol and propanolol showed respectively a 10- and 5-fold greater affinity for the antibodies than the other phenoxypropylamine derivatives. The nature of the substituent in the ortho position of the aromatic ring seems linked to the increase in affinity. Study of more analogs is necessary to draw firm conclusions about the role of the groups involved in the binding reaction.

Further assessment of the specificity of the antibodies was made by showing the inhibition of specific alprenolol-binding to the β-adrenergic receptor of turkey erythrocyte membranes. Although the dissociation constant of the antibodies for dihydroalprenolol is ten times higher than that for the β-adrenergic receptor, inhibition was nearly 100 per cent. This is due to the fact that the amount of specific antibodies is much higher than that of the receptor molecules in our experimental conditions.

Finally, a successful purification of the specific anti-alprenolol antibodies was performed with a recovery of 88 per cent of the binding capacity. The slight decrease is due to the binding of residual propanolol used for the elution from the affinity-absorbent.

Indeed, experiments performed in the same conditions but with labeled propanolol showed a residual bound radioactivity of 16 per cent. It is further worthwhile to mention that the association constant after the third booster was increased 23-fold over that of the second booster. At the same time, the heterogeneity index became 1. This means that repeated injection lead to the selection of more homogeneous antibody molecules with a higher affinity for the hapten.

In conclusion, we infer from the association constants as well as from the specificity, that the antibodies can be used in the detection and quantitation of β-adrenergic blockers of the phenoxypropylamino series. The possibility to obtain the specific antibodies in pure form by affinity chromatography will enable us to study more thoroughly the structure-activity relationship between this new hapten and the antibodies.

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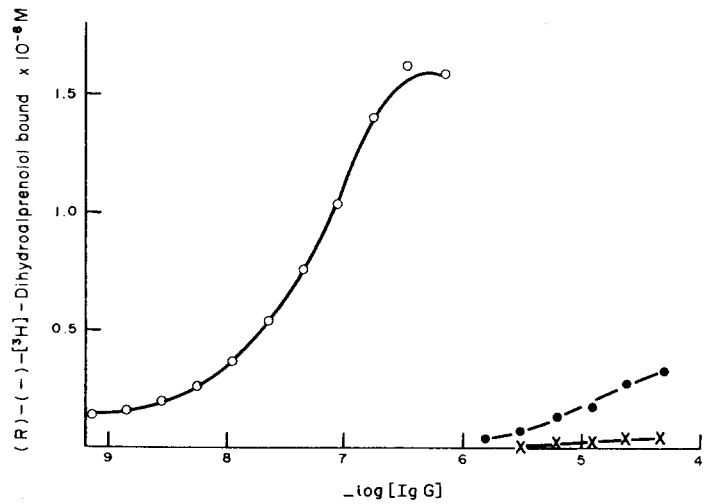

Fig. 5. Affinity chromatography purification of the antibodies. O——O Binding capacity of purified anti-alprenolol antibodies. ●——● Binding capacity of the total γ-globulin fraction. ×——× Bind-ing capacity of the γ-globulin fraction after adsorption. Binding capacity was measured with the Farr technique using 2×10^{-8} M [^{3}H]dihydroalprenolol hapten concentration.

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