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BINDING OF BENACTYZIN TO BOVINE SERUM ALBUMIN

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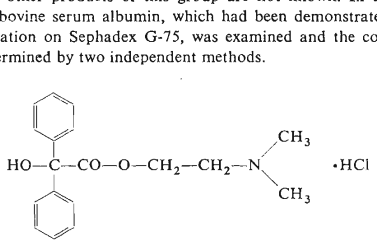
Many of the compounds used as drugs are bound in the organism to proteins, predominantly to serum albumin[1]. The character of this binding affects their transport, distribution, and elimination. Simultaneously the binding of these compounds to proteins can be utilized as a model system representing the interaction of the drug with the receptor.

In their review, Mayer and Guttman[1] discussed methods, interpretation of experimental results, and compounds which have been studied with regard to their binding to proteins. This review shows that the group of parasympaticolytics has been studied relatively very little, actually only one of its members – atropine. Oroszlan and Maengwyn-Davies[2–4] have demonstrated the binding of atropine to bovine serum albumin, determined the pharmacologic effect of the atropine–albumin complex, and some of the constants characterizing this binding.

Similar studies on other products of this group are not known. In this study the binding of benactyzin (*I*) to bovine serum albumin, which had been demonstrated[5] in preliminary experiments by gel filtration on Sephadex G-75, was examined and the constants characterizing this binding were determined by two independent methods.



I

EXPERIMENTAL

Gel filtration on Sephadex G-75 (Pharmacia, Uppsala). Solution of benactyzin (Léčiva, Prague), bovine serum albumin (Man Research Lab., mol. wt. 69000) or their mixtures (10 min after mixing) in 0·15M phosphate buffer at pH 7·0 were fractionated on a column of Sephadex G-75 (2 × 20 cm). The benactyzin content of fractions was determined by the method described earlier[6] which is based on the formation of hydroxamic acid from the ester by the action of alkaline solution of hydroxylamine. The arising hydroxamic acid forms with Fe^{3+}-ions a colored complex (λ_{max} 500 nm) in acid media. The protein content was determined by absorbance measurement at 280 nm. The quantity of the drug bound to albumin was calculated from the difference between the quantity of benactyzin applied to the column and the quantity of free benactyzin which had passed through the column. These experiments were carried out with seven different amounts of benactyzin and a constant quantity of bovine serum albumin ($2{\cdot}9 \cdot 10^{-6}$ mol). The same experiments were performed with human γ-globulin (Institute for Sera and Vaccines, Prague).

Direct measurement of benactyzine concentration in the presence and absence of $3 \cdot 10^{-4}$M bovine serum albumin in 0·15M phosphate buffer at two different pH-values (5·0 and 7·0). The quantity of benactyzin bound to albumin was calculated from the difference between the quantity of benactyzin detected in the presence and absence of bovine serum albumin. The same experiments were performed with human γ-globulin.

RESULTS AND DISCUSSION

The quantity of bound benactyzin was established from the difference between the quantity of benactyzin applied and the quantity of free detected benactyzin. The results of the measurement were processed by the graphical method of Klotz[8]. The interpolation of the lines into experimental

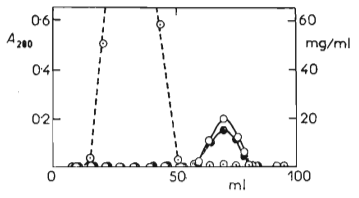


FIG. 1

Fractionation of Benactyzin and Bovine Serum Albumin on Sephadex G-75

The experimental conditions are described in the text. Applied to the column was 200 mg of benactyzin (○ detected benactyzin), 200 mg of bovine serum albumin (⊙ detected by absorbance measurement at 280 nm), and 200 mg of benactyzin and 200 mg of bovine serum albumin (● detected benactyzin).

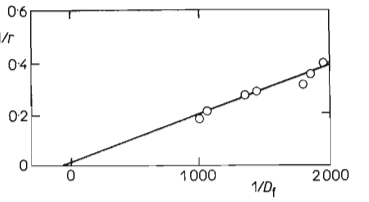


FIG. 2

Klotz Plot of Binding of Benactyzin to Bovine Serum Albumin at pH 7·0 as Measured by Gel Filtration on Sephadex G-75

r, mol of benactyzin bound to mol of bovine serum albumin; D_f molar concentration of free benactyzin.

TABLE I

Constants Characterizing the Binding of Benactyzin to Bovine Serum Albumin
K_{dis}, Dissociation constant; k_1 association constant; n number of binding sites; ΔF change in free energy.

Method	pH	K_{dis}	k_1	n	ΔF
Spectrophotometry	5·0	$6{\cdot}3 \pm 1{\cdot}00 \,.\, 10^{-3}$	$2{\cdot}06 \,.\, 10^{3}$	13 ± 2	4·44
Gel filtration	7·0	$1{\cdot}8 \pm 0{\cdot}20 \,.\, 10^{-2}$	$1{\cdot}94 \,.\, 10^{3}$	35 ± 3	4·40
	7·0	$1{\cdot}5 \pm 0{\cdot}15 \,.\, 10^{-2}$	$2{\cdot}07 \,.\, 10^{3}$	31 ± 4	4·44

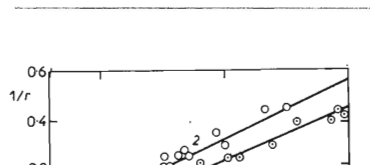


FIG. 3

Klotz Plot of Binding of Benactyzin to Bovine Serum Albumin at pH 7·0 (*1*) and 5·0 (*2*)
Designation of coordinates the same as in Fig. 2.

points and the statistical evaluation were carried out by the method[7] of regression analysis on Minsk 22 computer.

The resolution of gel filtration of albumin, benactyzin, or of their mixtures can be seen in Fig. 1. The quantity of benactyzin in the mixture is smaller. The difference represents benactyzin bound to albumin. This effect was not observed in experiments with γ-globulin and the quantity of benactyzin after gel filtration was the same in the presence or absence of globulin. Fig. 2 shows the binding of benactyzin to bovine serum albumin in the Klotz[8] plot.

The quantity of benactyzin detectable in the presence of bovine serum albumin (second method) is decreased by the amount of benactyzin bound to albumin. The processing of the results of benactyzin calibration in the presence and absence of albumin in the Klotz[8] plot at two different pH-values is shown in Fig. 3. The detectable quantity of benactyzin in the presence and absence of γ-globulin was the same. The constants characterizing the binding of benactyzin to bovine serum albumin are given in Table I.

As can be seen, the number of binding sites in the molecule of albumin increases with pH between 5 and 7 and so does the dissociation constant. This increase has been demonstrated[2-4] to occur during the binding of atropine to the same protein. The size of the constants characterizing the binding of atropine to bovine serum albumin is different from the size of constants of the same binding of benactyzin. The number of binding sites for benactyzin in the molecule of albumin at pH 7·0 is in agreement with the number determined by biological titration by Fusek and coworkers[9]. As follows from the linear Klotz plot, the binding sites are of only one class. The determined constants permit the calculation of the change in free energy (ΔF) of the bond studied.

The variations in ΔF with pH are negligible. The size of ΔF for the binding of benactyzin to bovine serum albumin is a little smaller than ΔF for the binding of atropine to the same protein ($\Delta F =$ $= 4{\cdot}65 - 4{\cdot}75$ kcal/mol). These differences, however, are minimum.

The biological activity of both parasympaticolytics is decreased in the presence of bovine serum albumin[2,4-9]. It is probable that during therapeutical administration of benactyzin or atropine a part of the drug is bound to albumin and its biological role is that of a reservoir regulating the level of the drug in the organism.

The binding of benactyzin to bovine serum albumin is most likely selective since human γ-globulin does not bind this drug under identical conditions. So far we are lacking detailed knowledge of the character of the binding site in the molecule of the albumin and this problem will require specification. Most likely – as in the case of atropine[2-4] – the binding sites constitute free amino groups of certain amino acids.

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