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Binding of organic ions by proteins—1. Interaction of 1-benzyl-3-indazoleoxyacetate with serum albumin

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Interaction of acidic non-steroid drugs with proteins even though not in itself sufficient to insure biological activity, apparently reflects some pertinent property of these compounds [1]. It has been observed that anti-inflammatory drugs of a variety of structures (aspirin, indomethacin, phenilbutazone) inhibited the thermal denaturation of serum albumin [2, 3]. Mizushima first proposed a relationship between clinical efficacy and the ability of drugs to stabilize plasma proteins [2]. However it seems that this antidenaturating property is connected with the antirheumatic and not with the anti-inflammatory effect. Thus acetylsalicylic acid is active as an antirheumatic agent only at doses which bring about hematic concentrations capable of stabilizing proteins but not at anti-inflammatory doses, and benzydamine, an effective anti-inflammatory agent devoid of antirheumatic activity, does not have any stabilizing effect on proteins [3, 4]. Reports concerning 1-benzyl-3-indazoleoxyacetate* (BZ) showed a good correlation between its biological activity and the ability of BZ to stabilize certain proteins [5–7]. BZ appears to be a good agent to check the theoretical and practical significance of the pathogenetic role of the altered properties of extracellular protein systems [8]. Thus to have a reasonably adequate understanding of the functional properties of the drug, the interaction of BZ with serum albumin has been studied. The work reported in the present paper involves determination of the binding isotherms for BZ of native human (HSA) and bovine (BSA) serum albumin and the kinetics of competitive reaction between BZ and 1-anilino-8-naphtalene sulfonate (ANS) with albumin.

HSA (fraction V) and BSA were obtained from Sigma Chem. Co. (U.S.A.). Solutions of approx. 1% of protein were deionized on a mixed-bed ionic exchange column (AG 501-X8, Biorad Lab.); these solutions were stored at 4° and discarded after 6 days if not used earlier. Concentrations were determined from optical density measurements at 279 nm by a Cary 14 spectrophotometer, using $E^{1\%}_{1\,cm} = 6.67$ for BSA [9] and 5.8 for HSA; all calculations were carried out assuming a mol. wt of 69,000.

BZ was obtained from Angelini Laboratories, Italy; its extinction coefficient is $E^{1\%}_{1\,cm} = 195$ at 310 nm.

The extent of binding of BZ by HSA and BSA was measured by the equilibrium dialysis technique. Visking tubing (the Scientific Instrument Center, England) was treated by boiling 1 hr in distilled water, followed by repeated washing. Dialysis bags (with 3 ml of 25 μM protein) were suspended in 9 ml of buffer solution containing BZ and shaken for 18 hr. Binding of BZ by protein was calculated by comparing the absorbance at 310 nm of the dialysate and of the content of the dialysis tube. Reversibility of binding was checked. The precision of the method was determined by repeated experiments; the standard deviation for affinity constants was about 0.09.

The equilibrium data have been evaluated by the Hill equation, commonly used in work with heme proteins [10].

$$\bar{Y}/(1 - \bar{Y}) = (BSA \,.\, BZ)/(BSA) = K(BZ)^n$$

where $\bar{Y}$ is the fractional saturation, K is the overall affinity constant, n an empirical parameter, and the terms in parenthesis are the concentrations of the protein bound to ligand (BSA . BZ), of the free protein (BSA) and of the free ligand (BZ). The middle term of the equation is valid even for more than one binding site to BZ, when they are equivalent and independent [11] (see below). A plot of log $(\bar{Y}/(1 - \bar{Y}))$ vs log (BZ) should give a straight line with a slope equal to n, the apparent order of reaction; a value of n equal to 1 corresponds to an hyperbolic curve [12]. A least squares analysis was employed for fitting experimental data.

Binding of BZ to serum albumin is not associated with significant optical density or fluorescence changes. Therefore the kinetics of the reaction between BZ and serum albumin could be followed only indirectly, using the competition in binding to serum albumin between BZ and the fluorescence probe ANS.

Fluorescence spectra were determined with the use of a Turner spectrofluorimeter Model 210. The excitation wavelength was 370 nm and the fluorescent emission was recorded between 400 and 600 nm.

The rate of displacement of ANS by BZ (and vice versa) was determined with a Gibson–Durrum apparatus, measuring the fluorescence of ANS.

The reactions involved in the replacement on the protein (P) of one ligand (X) by another (Y) can be represented by the scheme:

$$PX + Y \rightleftharpoons PY + X,$$

which may be considered to involve the reactions:

$$PX \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P + X$$

and

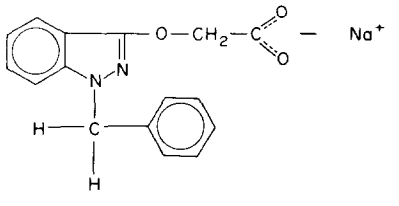
$$P + Y \underset{\tau_2}{\overset{\tau_1}{\rightleftharpoons}} Y.$$

Therefore, R, the first order rate constant for the approach to equilibrium, will be a function of the rate constants for the reaction of P with X and Y [10]. The individual R values obtained in several experiments under the same conditions showed a reproducibility measured by a standard deviation of about 0.1.

Binding of BZ to serum albumin in 0.1 M phosphate buffer pH 7 and 17° is illustrated in Fig. 1, in which it is shown that the behaviour of human and bovine albumin is identical. Moreover the data indicate (by the best fit) that under these conditions 3 molecules of the organic anion may be bound by a single protein molecule with a value of the apparent affinity constant of $7.6 \pm 0.7 \times 10^4\,M^{-1}$.

The addition of NaCl to the system does not produce a change in the stoichiometry (Fig. 2). Under these condi-

* Structural formula of 1-benzyl-3-indazoleoxyacetate (BZ).



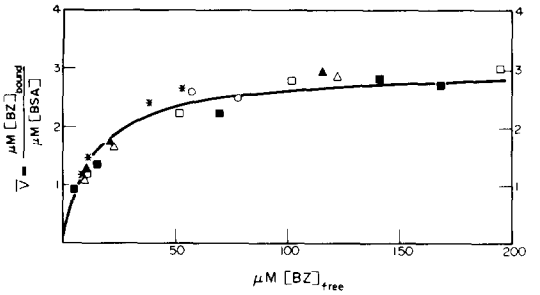


Fig. 1. Binding of BZ to human (■, ▲, *) and bovine (□, △, ○) serum albumin (25 μM) in 0.1 M phosphate pH 7 and 17°. The line is a rectangular hyperbole calculated according to the Hill equation (see Materials and Methods).

tions the binding constant decreases by a factor of 3, suggesting the importance of charge effects in the interaction; however this effect tends to reverse at a high salt concentration (KCl 3 M).

In order to get further insight into the interaction between the aromatic anion and serum albumin, the effects of pH and temperature have been investigated. Binding of BZ by BSA between pH 5.3 and 10, is almost constant. At pH 4.5 however there is a decrease in the association constant and an increase in the number of BZ molecules bound. This may be related to the known conformation change of the protein under these conditions [13]. Temperature changes also do not affect the binding of BZ by BSA.

Interaction studies, at equilibrium, between BZ and the complex HSA.ANS have been carried out. It is well known that the quantum yield of ANS, which is very small in water solution, increases dramatically upon a binding to serum albumin [14, 15]. On addition of BZ to this system a significant fall (about 50 per cent) in fluorescence of ANS (at a molar ratio of ANS to HSA of 1.5) occurs, which is interpreted as due to direct binding of BZ to the protein.

The kinetic behaviour of the competition reaction between BZ and ANS for binding sites on HSA is shown in Figs. 3 and 4. The reaction involved in the displacement of ANS by BZ (Fig. 3) may be written:

$$HSA.ANS^* + BZ \rightleftharpoons HSA + ANS + BZ \rightleftharpoons HSA.BZ + ANS$$

where the star indicates the fluorescent species. The time course of the reaction, over the concentration range

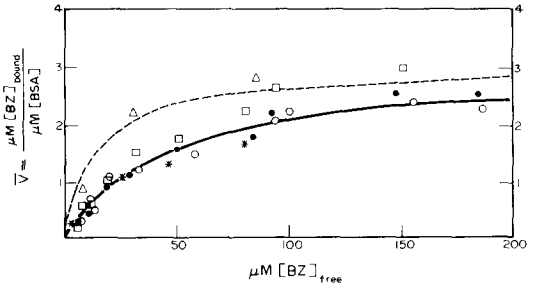


Fig. 2. Effect of ionic strength on the binding of BZ to bovine serum albumin (25 μM) in 0.1 M phosphate at two different pH's and 17°. pH 7: ○—1 M KCl; ●—2 M KCl; □—3 M KCl; pH 6: * —2 M KCl. Dashed line refers to the control experiment (△) in low salt concentration and pH 7. Full and dotted lines are rectangular hyperbole calculated according to the Hill equation, with three binding sites per molecule of serum albumin.

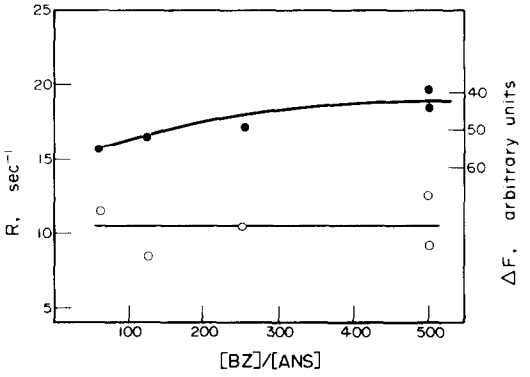


Fig. 3. Replacement of ANS (25 μM) bound to human serum albumin (6.25 μM) by BZ in 0.1 M phosphate pH 7 and 20°. Open symbols refer to the rate constant of replacement (R); closed symbols to relative fluorescence change (ΔF).

explored, was pseudo first order. The rate of approach to equilibrium is independent from BZ concentration (Fig. 3), at least at a concentration ratio of the two ligands above 50; this indicates that under the conditions of the experiment the rate limiting step is the dissociation of ANS from the protein.

The replacement of BZ by ANS is shown in Fig. 4. The pseudo first order velocity constant for the fluorescent change increases initially with increasing concentration of ANS but then tends to remain constant. The direct binding of ANS to the protein is probably lost in the dead time of the apparatus. The observed pseudo first order process should then reflect the replacement of BZ by ANS. The change in rate with ANS concentration is consistent with a replacement reaction, the limiting value at high ANS concentration reflecting the dissociation velocity constant of BZ from the protein.

Therefore the results show that a constant value of 3 moles of BZ bound/mole of protein is found over the different experimental conditions studied. This number of high affinity binding sites on serum albumin for BZ appears to be less than that for other organic ions, which is usually between 4 and 10 [16]. In particular it is the same as that for flufenamic acid [17], for phenylbutazone [18] as well as for nitroderivatives of aromatic anions [19] and is one half of that for sodium salicylate [20, 21].

Some ligands, such as ANS, show deviation from statistical binding equilibria when bound to serum albumin and

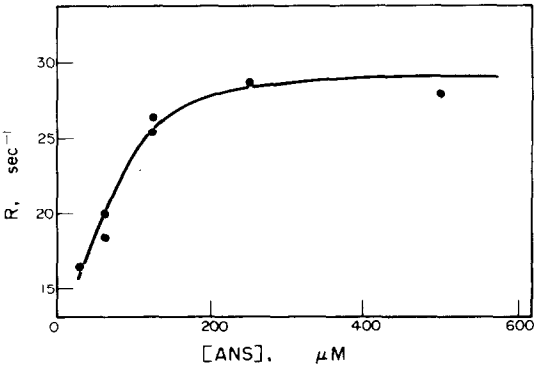


Fig. 4. Rate constant of replacement (R) of BZ (6.25 μM) bound to human serum albumin (6.25 μM) as a function of ANS concentration in 0.1 M phosphate pH 7 and 20°.

the data have been interpreted in terms of both heterogeneity of sites [22] and sites equivalence with cooperative interaction [23]. The reported results, instead, indicate that the binding of BZ by serum albumin is of simple type and statistical; in fact the system appears to obey the law of mass action with equivalent and independent sites: this is shown by the value of n near unity in the Hill plot (Fig. 5).

The strength of interaction between serum albumin and BZ is high and of the same order of magnitude of the high affinity site of phenylbutazone [17], of indole analogues [24–26] and some derivatives (nitro and chloro) of benzene-sulphonate [16]; moreover the affinity of BZ for serum albumin is about 4 times stronger than that reported for the salicylate binding sites [21] and more than twice that of the two lower affinity sites of phenylbutazone [18]. These latter results could explain the data which indicate that BZ possesses a stabilizing effect on proteins higher than those of phenylbutazone and of salicylic acid [8].

Moreover it must be recalled that blood levels obtained in volunteers, following administration of BZ [8], correspond to the order of magnitude of concentration used in these experiments.

It has been suggested [27, 28] that the binding sites on serum albumin for organic anions are constituted by nonpolar and positively charged aminoacid residues, the latter being arginines. In fact, chemical modifications on serum albumin show a marked decrease in the affinity for large anions only when arginine residues (not lysine) are blocked [27].

The absence of pH dependence of the BZ binding, together with the ionic strength effect, agree with such a hypothesis on the structural conformation of the interaction sites. As far as temperature effect is concerned, the results indicate that the affinity of BZ for serum albumin is due primarily to positive entropy changes ($\Delta F = -6.7$ kcal/mole; $\Delta H \simeq 0$ kcal/mole; $\Delta S = +22$ e.u.): this is in line with the general conclusions on combination of serum albumin with aromatic anions [16, 29], even though some exceptions exist, such as ANS, for which ΔH is about half of ΔF° at room temperature.

The constancy of the number of BZ's sites in serum albumin with increasing the ionic strength suggests that purely electrostatic factors do not have major importance in the complex formation, even though there is a decrease in the affinity constant.

Changed and unchanged derivatives of serum albumin prepared by partial chemical modification of the native protein show a difference in binding affinity for ANS and indicate that about 1 kcal of free energy is derived from electrostatic interactions and the rest from van der Waals forces [27]; it would appear that a similar deduction may be made here.

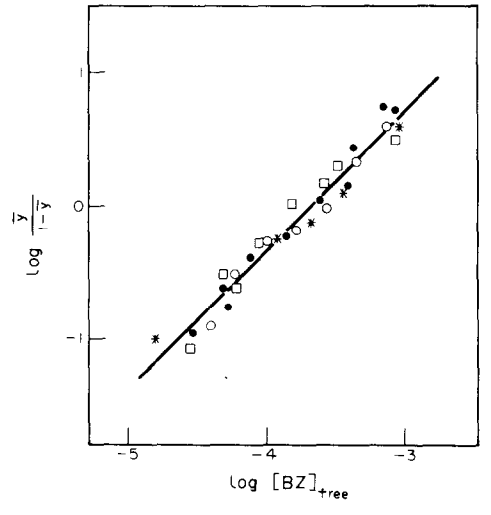


Fig. 5. Hill plot of the data at high ionic strength reported in Fig. 2. The slope is equal to unity.

The case of BZ is similar to that of flufenamic acid [17], but contrary to the interaction of phenylbutazone with serum albumin, for which the strength of electrostatic binding of the drug with a lysine-amino group of the protein [30] seems to be the principal determinant of the magnitude of the association constant [1]. The effect of salt concentration on the interaction between BZ and serum albumin may be explained in terms of: (a) changes in ionic atmosphere of the associating molecules, (b) competitive inhibition by the chloride ions, (c) a 'salting out' effect.

Both the first two mechanisms may be involved in the weakening of the binding when the ionic strength is increased; the direct competition for the same site between the carboxylate group and chloride ion has previously been noted for the indole analogues containing the carboxylate group [31]. On the other hand, the reversing of the inhibition effect at very high salt concentration (3 M KCl) is reminiscent of the stabilization of unchanged indole analogues with serum albumin; the increase in the ionic strength over a certain salt concentration favours their association to serum albumin [31].

The competition experiments with ANS suggests that the high affinity sites for BZ might be common with some of those for ANS, which are 4 [32].

In fact, it has been suggested the existence of two main classes for binding of high affinity ligands, the one comprising phenolsulphonphtalein dyes, ANS and bilirubin, and the other fatty acids and aromatic aminoacids [33].

The equilibrium and kinetic data, obtained by fluorescent measurement, are compatible with a simple competitive binding mechanism, but the possibility that the BZ binding produces structural changes in protein, which alter the interaction of the remaining sites with ANS, cannot be excluded.

The kinetic experiments indicate that the rate of dissociation of BZ from the protein is about $30\,sec^{-1}$.

Assuming the simple scheme:

$$BZ + HSA \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} (HSA.BZ)$$

the combination velocity constant can be calculated to be $2 \times 10^6\,M^{-1}\,sec^{-1}$.

In conclusion the present results on the interaction of BZ with serum albumin may be summarized as follows. (i) The interaction occurs at three binding sites with high affinity. (ii) The sites behave as if they were equivalent and independent. (iii) Charge effects are present but not of primary importance in the binding: thermodynamically, entropy effects dominate the reaction. (iv) Rates of association and dissociation are high and in line with those found in general for interaction of proteins with specific ligands.

From another point of view the present results may help to rationalize the antirheumatic power of this organic anion [5], on the basis of the proposed relationship between clinical efficacy and the ability of this molecule to interact with plasma proteins [7, 8].

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