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THE MEASUREMENT OF BINDING CONSTANTS USING CIRCULAR DICHROISM

BINDING OF PHENYLBUTAZONE AND OXYPHENBUTAZONE

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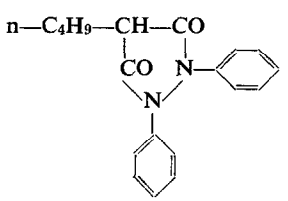
Abstract—The binding of drugs to protein can produce a change in circular dichroic effect. This provides a rapid method for estimating association constants which has the added advantage that some indication of binding site may also be inferred. The method has been applied to the binding of phenylbutazone and oxyphenbutazone to human serum albumin. Association constants for these systems have been determined and it has been shown that oxyphenbutazone has an extra binding site possibly involving a hydrogen bond to imidazolium ion in the protein.

SEVERAL instances of the induction of circular dichroism in symmetrical molecules which are co-solutes with disymmetrical molecules have been reported.[1–3] The binding of small symmetrical molecules to protein may also occur in a disymmetrical environment, and the subsequent disymmetry of the system may be revealed by a change in the circular dichroic spectrum.[4, 5] The present paper deals with the binding constants obtained for phenylbutazone (I) and oxyphenbutazone (II) using circular dichroic measurements.



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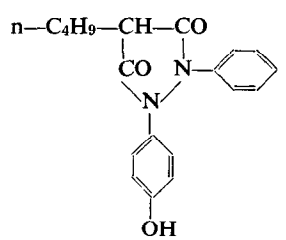

II

METHODS

Human serum albumin (HSA; electrophoretic purity 100%) was obtained from Behringwerke A.G. Phenylbutazone and oxyphenbutazone were from Geigy Chemicals.

Circular dichroism measurements were carried out on a Roussel–Jouan Model A dichrograph modified[6] to give a ten times greater sensitivity. Unless otherwise noted solutions were of 0·1% w/v HSA in M/15 phosphate buffer pH 7·4 and contained from 1 to 100 μg/ml of either substrate. Path lengths from 0·1 to 5·0 cm were used.

RESULTS

(a) *Binding of phenylbutazone*

The circular dichroic spectrum of HSA alone and the difference spectrum of phenylbutazone bound HSA are shown in Fig. 1. The difference spectrum shows a maximum at 285 mμ, but it was found preferable to measure the change in spectrum with concentration at 290 mμ rather than at the maximum, since a wider range of concentrations could be used at the latter wavelength before total blacking out of the transmitted light occurred.

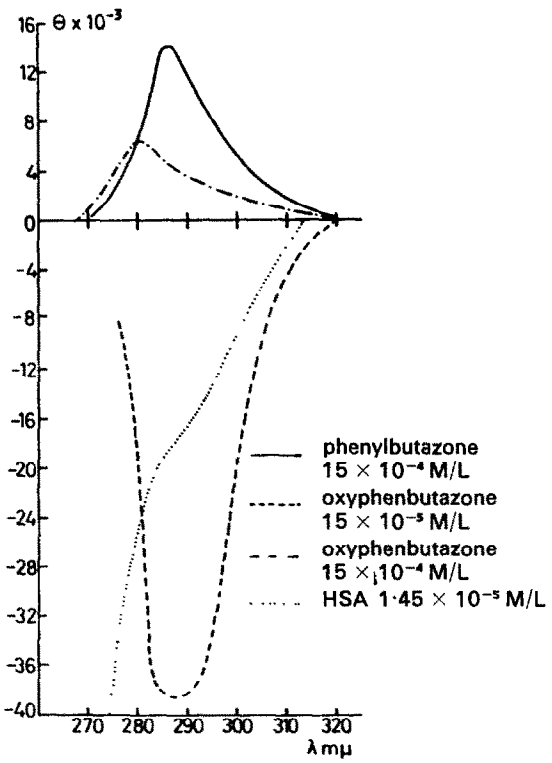

FIG. 1. Circular dichroic spectrum of human serum albumin and difference spectra of albumin in the presence of phenylbutazone and oxyphenbutazone. Molar ellipticities [θ] (deg. cm^2 d mole^{-1}) were calculated with respect to the protein concentration for HSA alone and to the drug concentrations for the difference spectra. All solutions were M/15 in the phosphate buffer pH 7·4. Difference spectra were obtained using solutions of 0·1% HSA. ——— 1·5 × 10^{-4} M phenylbutazone; - - - - - - - 1·5 × 10^{-5} × 10^{-5} M oxyphenbutazone; -.-.-.-. 1·5 × 10^{-4} M oxyphenbutazone; . . . . . 1·45 × 10^{-5} M HSA.

Figure 2 shows the change in the circular dichroic difference spectrum at 290 mμ as HSA is titrated with phenylbutazone. Binding constants were calculated from this and similar curves in the following way.

If the tangent to the curve at the origin is drawn then it can be shown (see Appendix) that the distance from the ordinate to the tangent at a given value of $\triangle\theta$ is equivalent to the concentration of bound drug, while that from the tangent to the curve gives the

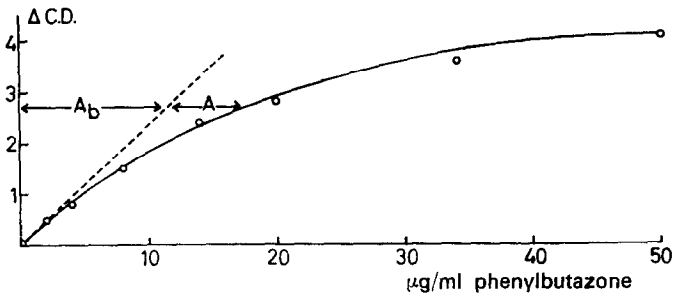

FIG. 2. Change in circular dichroism obtained on titrating 0·1% HSA with phenylbutazone. The change, shown in arbitrary units, was measured at 290 mμ. A_b and A refer to bound and free drug respectively (see text). Measurements were made in M/15 phosphate buffer pH 7·4

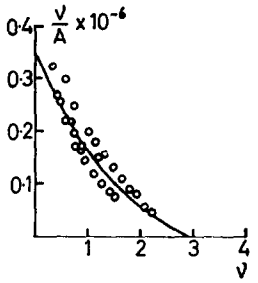

FIG. 3. Scatchard plot of binding of phenylbutazone to human serum albumin.

free drug present. The simultaneous values of bound and free drug concentrations can then be used to estimate n and K.

Values of bound and free substrate obtained in this way were plotted by the method of Scatchard[7] (Fig. 3). Good reproducibility between the results of different experiments was observed. In view of the curvature of the plot it was analysed on the assumption that there were two types of site available owing to electrostatic interaction but that the interaction was small.[8] The average association constant and numbers of sites of each type per protein molecule is then $K_1 = 2{\cdot}37 \times 10^5$ l./mole, $n_1 = 1{\cdot}14$; $K_2 = 4{\cdot}56 \times 10^4$ l./mole, $n_2 = 1{\cdot}86$.

(b) *Binding of oxyphenbutazone*

Titration of HSA with oxyphenbutazone shows first a small negative change in the CD spectrum of the mixture and then, at higher concentrations a positive change (Figs. 1 and 4).

The initial negative change in spectrum is linear up to a concentration of substrate corresponding to that of protein, suggesting that a very strong 1:1 binding of drug to protein occurs. Thereafter the spectral change gradually increases, following a normal curve. Association constants were calculated on the assumption that K_1 is very large and $n_1 = 1$; i.e. that the first molar equivalent of substrate is so strongly bound as to be virtually removed from solution. Drawing the tangent to the initial portion of the rising curve, the horizontal distance from axis to tangent was then taken as equal to the amount of drug bound + 1 molar equivalent, the distance from tangent to curve

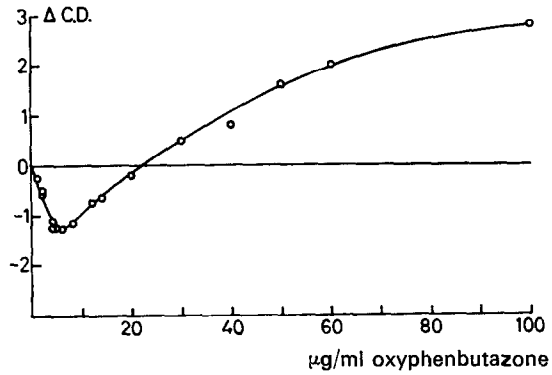

FIG. 4. Change in circular dichroism obtained on titrating 0·1% HSA with oxyphenbutazone. The change, shown in arbitrary units, was measured at 290 mμ. Measurements were made in M/15 phosphate buffer pH 7·4.

remaining a measure of the free substrate. Calculation on this basis showed $K_2 = 2{\cdot}32 \times 10^5$ l./mole, $n_2 = 3{\cdot}55$ and $K_3 = 1{\cdot}16 \times 10^4$ l./mole, $n_3 = 8{\cdot}45$.

(c) *Effect of pH*

The effect of changing pH on the maxima of the difference CD spectra generated by the two drugs is shown in Fig. 5. Measurements were made at a concentration equimolar in drug and protein. The values obtained for phenylbutazone show only a slight variation over the pH range studied. HSA alone, not shown in the figure, also showed only a slight variation at 290 mμ. Oxyphenbutazone showed a change from a marked negative effective at pH 5·8 to a slight positive effect at pH 8·4.

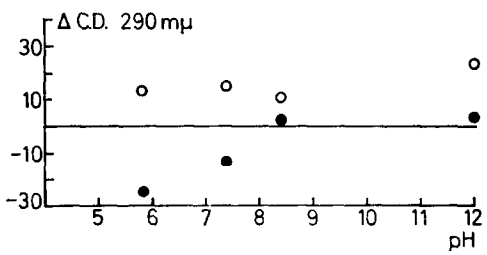

FIG. 5. Change with pH of the circular dichroic difference spectra of human serum albumin in the presence of phenylbutazone or oxyphenbutazone. Measurements, in arbitrary units, were made at 290 mμ. Difference spectra were obtained between solutions containing $1{\cdot}45 \times 10^{-5}$ M HSA and $1{\cdot}5 \times 10^{-5}$ M drug at a given pH and $1{\cdot}45 \times 10^{-5}$ M HSA alone at the same pH. Solutions were M/15 in phosphate buffer or M/100 in NaOH. O phenylbutazone ● oxyphenbutazone.

DISCUSSION

The method of estimating association constants outlined above can be applied to systems where binding of the drug alters the conformation, and hence the ellipticity, of the protein; or to systems where binding occurs within a disymmetrical environment. The disadvantages of the method lie (i) in that only those binding sites causing a change in ellipticity are revealed and (ii) in the inaccuracies inherent in drawing a tangent to the origin. The advantages lie in the rapidity with which measurements can be made on

the undisturbed system; and in that extra structural information can be obtained from the spectra themselves.

The assumption made in this method is that the change in circular dichroic spectrum is directly proportional to the amount of material bound. While this may be true in the case where only one site per molecule of protein is involved it needs more justification when more than one site with more than one association constant is implicated. In the latter case the sites may be heterogenous, or they may be similar with electrostatic interactions occurring between them. If they are heterogenous there is no *a priori* reason why the proportionality between C.D. change and amount bound should be maintained. There may be large differences between the environments of the bound molecules at the different sites, and the disymmetrical field of the weaker bound site could be much greater than that at the stronger bound site, or vice versa. In such cases discontinuities in the C.D.–concentration plot might be expected.

If they are similar, with differences caused by changes in electrostatic interaction then the diminution in overall electrostatic charge which produces a lowering of association constant will also cause a decrease in the perturbing field of the chromophore and hence a concurrent lowering of the induced C.D. effect. The first association constant would then be more accurately determined than the second.

In the case of phenylbutazone the results for the association constants compare favourably with those obtained by equilibrium dialysis methods, suggesting that the C.D. effect is indeed proportional to the bound substrate. This has also been shown by Chignall,[5] who found a linear relationship between C.D. effect and the calculated amount bound. We have also investigated some other systems and found good agreement between results from equilibrium dialysis or ultra-filtration methods and the present method using C.D. change.

The initial linear change in C.D. spectrum occurring when HSA is titrated with oxyphenbutazone (Fig. 4) indicates that a strong one to one binding of drug to protein takes place over this concentration range. There is no evidence for a comparable binding between phenylbutazone and HSA. In view of the close structural similarity of the two drugs, and the accompanying similarity in pK_a,[9] it is reasonable to ascribe this difference in binding to the hydroxyl group of oxyphenbutazone.

From the wavelength maxima it appears that the changes in dichroic spectra obtained on addition of either drug to HSA are caused by asymmetric perturbations of the $n \rightarrow \pi^*$ carbonyl transitions. If phenylbutazone is protein bound via the C4 atom then binding of oxyphenbutazone via the hydroxyl group would cause a change in the spacial relationship of the protein to the carbonyl group. It is not difficult to construct models in which a section of protein chain which is in, say, a right hand quadrant with respect to a carbonyl of a C4 bound phenylbutazone molecule moves to a left hand quadrant relative to the 'same' carbonyl in an —OH bound oxyphenbutazone, thus accounting for the reversal of sign in the dichroic spectrum.

The effect of changing pH on the difference spectrum of oxyphenbutazone (Fig. 5) showed that no change occurred between pH 8·4 and pH 12, corresponding to the second ionisation of oxyphenbutazone.[10] The change occurring between pH 5·8 and 8·4 corresponds to the ionisation range of the imidazolium ion in HSA[11] and suggests a binding to the protein via such a group. Formation of a hydrogen bond between imidazolium ion and the hydroxyl group of oxyphenbutazone could account for all the measured effects.

The similarity between the first binding constant for phenylbutazone and the second for oxyphenbutazone suggest that binding is to the same site for both drugs. The initial interaction of oxyphenbutazone with the protein presumably makes two or three sites more accessible. Several investigations have indicated that anionic compounds have a common binding site to serum albumin.[12–14] The binding of fatty acids[15, 16] and alkyl sulphates[17] increases with increasing size of the non-polar substituent, and it has been suggested that some form of hydrophobic bonding is involved as a secondary requirement to the ionic interaction.[5, 12, 15] Alternatively, this secondary process could be more stereospecific than is implied by a simple hydrophobic bonding.[16] Certainly this alternative would see more likely in the present instance where, after the initial effect, oxyphenbutazone is bound as strongly as phenylbutazone, despite the fact that partition coefficients for the two substances[5, 9] show it to be much the more hydrophilic.

Appendix: Method for the calculation of association constants

The relationship between the number of molecules of ligand bound per mole of protein, ν, the concentration of free ligand, A, and the association constant, K, is given by

$$\nu = \frac{n\,KA}{1 + KA} \tag{i}$$

where n = number of equivalent sites per protein molecules.

If the concentrations of bound drug and protein are A_b and S respectively, then substituting in (i) (i)

$$A = \frac{A_b}{(nS\text{-}A_b)\,K.} \tag{ii}$$

The total concentration of drug $T = A + A_b$ (iii)

and eliminating A from equations (ii) and (iii)

$$KA_b^2 - A_b\,(1 + nSK + TK) + nSTK = \mathrm{O}. \tag{iv}$$

When A_b tends to zero A_b^2 does so more rapidly, so that, from (iv)

$$A_b \longrightarrow \frac{nSTK}{nSK + TK + 1.} \tag{v}$$

Further, as $T \longrightarrow \mathrm{O}$

$$A_b \longrightarrow \frac{nSKT}{nSK + 1} \tag{vi}$$

i.e. at low concentrations a plot of A_b versus T is linear.

If the change in circular dichroism is proportional to the amount of drug bound, then a plot of the ellipticity change $\Delta\,\theta$ at a given wavelength against the total amount of ligand added will give a curve proportional to the curve A_b versus T of equation (iv) throughout and of equation (v) initially.

At the origin the tangent to this curve is the line given by (vi). But this tangent is also the line $\Delta\,\theta = \text{const} \times A_b$ and this is true at all values of $\Delta\,\theta$ at a given wavelength. Hence throughout the initial part of the curve the distance from the ordinate to

the tangent at a given value of $\Delta\theta$ is equivalent to the concentration of bound drug, while that from the tangent to the curve gives the free drug present. Equation (i) may then be used to find n and K.

Dixon has proposed a graphical solution of equilibrium data when only the total concentration of the added reagent is known experimentally[18] and this has been extended by Kilroe-Smith.[19] The final treatment of results by Dixon's method is somewhat different from that outlined above. Both methods are based on the same fundamental equation for equilibrium, but diverge according to the assumptions made as the concentration tends to zero. Using the present notation Dixon assumes that as $T \to 0$, $nS\text{-}A^2 \to nS$. The error in this is $\frac{A_b}{nS}$.

In the present method the assumption is that

$$A_b(1 + nSK + TK) - KA_b^2 \to A_b(1 + nSK)$$

with an error of $\frac{T - A_b}{nS + \frac{1}{K}}$.

This error is always smaller than that of Dixon if $A_b > \frac{1}{2}T$, a condition which is satisfied at low values of T if $nS > \frac{1}{K}$.

The present method has the further advantage that it does not depend on results at high concentrations to establish a limiting value. Such results are often difficult to obtain by spectroscopic means without total extinction occurring.

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