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ON THE CONTEMPORANEOUS, REVERSIBLE INTERACTION OF DIFFERENT LIGANDS WITH SERUM ALBUMINS IN DILUTE AQUEOUS SOLUTIONS. FLUORESCEIN AND PHENYLBUTAZONE

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The binding affinity of fluorescein and of phenylbutazone to human serum albumin (HSA) and to bovine serum albumin (BSA), respectively, as well as of the two drugs together to each protein in dilute aqueous solution has been studied by means of gel permeation chromatography, circular dichroism, U.V. absorption and fluorescence spectroscopy. Identity of and/or interdependence between primary binding sites for the two ligands considered on HSA and BSA are evidenced and correlated with a simple theoretical approach to mixed drugs binding.

1. Introduction

There is a vast literature on the physico-chemical aspects of drug binding by serum albumins in dilute aqueous solution [1–3]. Seldom, however, the contemporaneous binding of two or more drugs (or, generally speaking, ligands) by such macromolecules is illustrated in sufficient detail.

In fact, this aspect is of particular relevance considering that the carrier proteins do usually interact in vivo with more than one ligand and that a better understanding of possible cooperativity effects in such mixed bindings is of pharmacological relevance as well as of interest in probing the alleged conformational ductility of these proteins. We wish to report here gel permeation chromatography and spectroscopic data on the binding of fluorescein, phenylbutazone and both drugs contemporaneously onto human serum albumin (HSA) and bovine serum albumin (BSA) respectively. Our experimental data, interpreted on the basis of a simplified theoretical scheme apt to the description of multiple binding equilibria, do point out a quite different behaviour between BSA and HSA towards ligands considered in terms of both intrinsic binding constants and of cooperativity (in the case of course of mixed ligands binding).

2. Experimental

Samples used were described elsewhere [4]. Gel permeation chromatography measurements were performed according to the method of Hummel and Dreyer [5], using a column of about 1.0 cm inner diameter, 50 cm length, filled with Sephadex G25 medium, with thermostatic jacket maintained at 37°C. All data were plotted as Scatchard plots (figs. 1 and 2). In the two ligand species measurements the bound fraction of fluorescein only was determined by spectrophotometry and the related data were plotted as $r_F = [F]_b/[P]_T = n_{F,b}/n_{P,T}$ versus $r_F/[F]$

each plot being obtained from a series of data at various concentrations of free fluorescein [F] and constant free phenylbutazone concentration ($n_{F,b}$ is the number of moles of bound fluorescein and $n_{P,T}$ that of total protein).

Spectrophotometric data were obtained at 25°C by means of a Coleman-Hitachi EPS 3T spectrophotometer, using thermostated rectangular cuvettes 1 to 10 mm path length.

Difference spectra were recorded using as reference a solution of fluorescein in water: the sample cell containing initially the same fluorescein solution was added of incremental amounts of a solution of albumin and fluorescein at constant stoichiometric fluorescein concentration (the results are reported in the figs. 3 and 4). Another set of difference spectra was recorded starting with a sample solution containing both fluorescein and albumin to which a solution of albumin, fluorescein and phenylbutazone was added dropwise, keeping the stoichiometric concentrations of both fluorescein and albumin constant (the results are reported in the figs. 5 and 6).

Circular dichroic spectra were performed by means of a Roussel Jouan dichrograph II provided with thermostated cell housing; the results are reported in the figs. 7, 8, 9, 10.

3. Theoretical considerations

Before discussing our experimental data, we believe that an excursus on the mathematical aspects involved in the use of the popular Scatchard function [6] (i.e. $r/[A]$ versus r, being $r = [A]_b/[P]_T$ the ratio between bound ligand concentration and total protein concentration and where [A] is the concentration of free ligand) when cooperativity between binding sites exists may be helpful.

A theoretical treatment for the reversible binding of a ligand onto a protein is that due to Klotz and Hounston [7,8]. Another recent theoretical approach to the cooperative binding of ligands onto linear biopolymers is due to Schwarz [9–11]. The general purposes and deductions of these two approaches are similar, nevertheless some difference appears. The Schwarz's treatment seems to us very appropriate in the case of linear biopolymers characterized by several classes of identical binding sites with cooperativity in the interaction with ligands generally limited to nearest neighbours; the Klotz's treatment seems more suitable for cases of proteins characterized by a limited set of different binding sites between which several cooperative interactions are explicated by one or more ligand species.

After these premises and starting from the theoretical treatment of Klotz and Hounston [7,8] we will give the analytical expressions for $r/[A]$ versus r and for its derivative in the case of one ligand species; then we will extend the treatment to the more general case of more than one ligand species.

3.1. The case of one ligand species, A

According to the cited treatment [8], and with a change in the symbology for the sake of simplicity in its successive extension (sect. 3.2), let us define:

$$[P]_T/[P] = Z = \sum_{\alpha=0,A} \sum_{\beta=0,A} \cdots \sum_{\mu=0,A} \sum_{\nu=0,A} k_\alpha k_{\alpha,\beta} \cdots k_{\alpha,\beta,\ldots,\mu} k_{\alpha,\beta,\ldots,\mu,\nu} [\alpha][\beta]\cdots[\mu][\nu] , \quad (1)$$

and consequently

$$r = \frac{[A]}{Z}\frac{dZ}{d[A]} , \qquad \frac{r}{[A]} = \frac{1}{Z}\frac{dZ}{d[A]} , \quad (2,3)$$

provided that $[0] = 1$, $k_0 = k_{\alpha,0} = \cdots = k_{\alpha,\beta,\ldots 0} = 1$ and with [P] the completely free protein concentration and $k_{\alpha,\beta\ldots}$ the intrinsic binding constants. For instance, $k_{0,A,0,A}$ is the intrinsic binding constant between the ligand A and the fourth site on the protein when the second site is already occupied by another A molecule while the first, third... sites are void. It is then possible in principle for every total number of protein sites n and after correctly expressing Z and $dZ/d[A]$, to express [A] from eq. (2) as a function of r and to obtain the analytical expression

(3) for the Scatchard function. In practive, expliciting [A] from eq. (2) implies finding the physically significant solution of an equation of degree n, which is rather tedious for $n = 3$ or 4 and is not actually feasible for higher values of n. However, if $n = 2$ the result is very simple:

$$Z = 1 + k_A[A] + k_{0,A}[A] + k_A k_{A,A}[A]^2 , \tag{4}$$

$$r = (k_A[A] + k_{0,A}[A] + 2k_A k_{A,A}[A]^2)/Z , \tag{5}$$

from which

$$[A] = \frac{(k_A + k_{0,A})(1 - r) - \{(k_A + k_{0,A})^2(1 - r)^2 - 4k_A k_{A,A}(r - 2)r\}^{1/2}}{2k_A k_{A,A}(r - 2)} \tag{6}$$

and then

$$\frac{r}{[A]} = \frac{2k_A k_{A,A}(r - 2)r}{(k_A + k_{0,A})(1 - r) - \{(k_A + k_{0,A})^2(1 - r)^2 - 4k_A k_{A,A}(r - 2)r\}^{1/2}} . \tag{7}$$

In this case it is not difficult to obtain also the expression for the first derivative of eq. (7), i.e.:

$$\frac{d(r/[A])}{dr} = \frac{1}{[A]}\left(1 - \frac{r}{[A]}\frac{d[A]}{dr}\right)$$

and considered that:

$$\frac{1}{d[A]/dr} = \frac{dr}{d[A]} = \frac{Z(k_A + k_{0,A} + 4k_A k_{A,A}[A]) - [A](k_A + k_{0,A} + 2k_A k_{A,A}[A])^2}{Z}$$

one can see that:

$$\frac{d(r/[A])}{dr} = -\frac{(k_A + k_{0,A})^2 - 2k_A k_{A,A} + 2k_A k_{A,A}(k_A + k_{0,A})[A] + 2k_A^2 k_{A,A}^2[A]^2}{(k_A + k_{0,A}) + 4k_A k_{A,A}[A] + k_A k_{A,A}(k_A + k_{0,A})[A]^2} \tag{8}$$

or, from direct derivation of eq. (7) with respect to r:

$$\frac{dr/[A]}{dr} = -\frac{(k_A + k_{0,A})k_A k_{A,A}(r^2 - 2r + 2)\{(k_A + k_{0,A})^2(r - 1)^2 - 4k_A k_{A,A}(r - 2)r\}^{1/2} +}{\{[(k_A + k_{0,A})^2 - 2k_A k_{A,A}](r - 2)r + (k_A + k_{0,A})^2\}\{(k_A + k_{0,A})^2(r - 1)^2 - 4k_A k_{A,A}(r - 2)r\}^{1/2} +}$$

$$\frac{+ k_A k_{A,A}(r - 1)\{[(k_A + k_{0,A})^2 - 4k_A k_{A,A}](r - 2)r + 2(k_A + k_{0,A})^2\}}{+ (r - 1)\{[(k_A + k_{0,A})^2 - 4k_A k_{A,A}](r - 2)r + (k_A + k_{0,A})^2\}(k_A + k_{0,A})} . \tag{9}$$

From these expressions some particular cases may be easily obtained:

1) Two identical independent sites: $k_A = k_{0,A} = k_{A,A} = k$. Expressions (5) and (7) reduce to the well known forms:

$$r = 2k[A]/(1 + k[A]), \qquad r/[A] = 2r - kr$$

2) Two different, independent sites: $k_A \neq k_{0,A} = k_{A,A}$. Expression (5) becomes:

$$r = k_A[A]/(1 + k_A[A]) + k_{0,A}[A]/(1 + k_{0,A}[A])$$

3) Two identical cooperative sites: $k_A = k_{0,A} \neq k_{A,A}$. Expression (5) becomes:

$$r = 2k_A[A](1 + k_{A,A}[A])/\{1 + k_A[A](2 + k_{A,A}[A])\} .$$

In this case if $k_{A,A} > k_{0,A}$ the Scatchard plot is convex upward, and if $k_{A,A} < k_{0,A}$ the plot is concave upward

and then can simulate the presence of two different and independent sites.

4) Two different and cooperative sites with $k_{0,A}$ negligible with respect to k_A and $k_{A,A}$:

$$r = \frac{k_A[A](1 + 2k_{A,A}[A])}{1 + k_A[A](1 + k_{A,A}[A])}, \qquad \frac{d(r/[A])}{dr} = -\frac{k_A^2 - 2k_A k_{A,A}(1 - k_A[A] - k_{A,A}[A]^2)}{k_A(1 + 4k_{A,A}[A] + k_A k_{A,A}[A]^2)} \tag{10,11}$$

In this case it is of some interest to notice that if $k_{A,A} = k_A/4$ expressions (10) and (11) become:

$$r/[A] = k_A - \tfrac{1}{2} k_A r \quad \text{and} \quad d(r/[A])/dr = -\tfrac{1}{2} k_A ,$$

so that the Scatchard plot would simulate the case of two identical independent sites.

3.2. The case of more than one ligand species

In this case, and with the above definitions,

$$Z = \sum_{\alpha=0,A,B} \sum_{\beta=0,A,B} \cdots \sum_{\nu=0,A,B} k_\alpha k_{\alpha,\beta} \ldots k_{\alpha,\beta,\ldots,\nu}[\alpha][\beta]\cdots[\nu] \tag{12}$$

always, provided that $[0] = 1$ and $k_0 = k_{\alpha,0} = \cdots = k_{\alpha,\beta..0} = 1$. So, for instance, $k_{0,B,A,0,B}$ is the intrinsic binding constant between the ligand B and the fifth protein site, being the second site already occupied by another B molecule, the third one by an A molecule and the first, fourth... sites unoccupied. With more than one ligand species it is possible to define a ratio r for each species so that

$$r_A = ([A]/Z)(\partial Z/\partial[A]) \qquad r_B = ([B]/Z)(\partial Z/\partial[B]) \tag{13}$$

and so on, with the other species (e.g. C, D, ... etc.) free concentrations and held constant. Maintaining the free concentrations of B, C, etc. constant at the desired value in a series of measurements at various [A] values (as it is easy to obtain in practice with the Hummel and Dreyer technique [5]) it is possible to construct Scatchard plots of $r_A/[A]$ versus [A] and then to deal with these data in terms of the analytical expressions derived from (12) as we will show here for the case of two sites and two ligand species.

With these last restrictions only, expression (12) becomes:

$$Z = 1 + k_A[A] + k_{0,A}[A] + k_B[B] + k_{0,B}[B] + k_A k_{A,A}[A]^2 + k_B k_{B,B}[B]^2 + k_A k_{A,B}[A][B] + k_B k_{B,A}[A][B] ,$$

and then

$$r_4 = \frac{(k_A + k_{0,A} + k_A k_{A,B}[B] + k_B k_{B,A}[B])[A] + 2k_A k_{A,A}[A]^2}{Z} \tag{14}$$

from which:

$$[A] = \{(r_A - 1)\phi + \theta\}/\{2(2 - r_A)\psi\}$$

with

$$\psi = k_A k_{A,A} , \quad \phi = k_A + k_{0,A} + k_A k_{A,B}[B] + k_B k_{B,A}[B] ,$$

$$\theta = \{\phi^2 - (2 - r_A) r_A(\phi^2 - 4\psi\chi)\}^{1/2} = \{\phi^2(r_A - 1)^2 + 4(2 - r_A) r_A \psi\chi\}^{1/2}$$

$$\chi = 1 + k_B[B] + k_{0,B}[B] + k_B k_{B,B}[B]^2 ;$$

and then

$$r_A/[A] = \{2r_A(2 - r_A)\psi\}/\{(r_A - 1)\phi + \theta\} \tag{15}$$

the first derivative in this case is, respectively as function of [A] and of r_A

$$\frac{\partial(r_A/[A])}{\partial r_A} = -\frac{\phi^2 - 2\psi\chi + 2\psi\phi[A] + 2\psi^2[A]^2}{\phi\chi + 4\psi\chi[A] + \psi\phi[A]^2} \tag{16}$$

$$= -\frac{2\psi\{\phi\theta[(r_A - 1)^2 + 1] + (r_A - 1)(\phi^2 + \theta^2)\}}{[\phi(r_A - 1) + \theta]^2\theta}. \tag{17}$$

Let us briefly consider some particular cases:

1) Identical and independent sites:

$$k_A = k_{0,A} = k_{A,A} = k_{B,A}, \qquad k_B = k_{0,B} = k_{B,B} = k_{A,B}.$$

It is easy to see that expressions (15) and (17) reduce to:

$$\frac{r_A}{[A]} = \frac{2k_A - r_A k_A}{1 + k_B[B]}, \qquad \frac{\partial(r_A/[A])}{\partial r_A} = -\frac{k_A}{1 + k_B[B]},$$

the only difference due to the presence of species B (in addition to A) is its competition at the two sites and this fact reflects in the term at the denominator. In the case of different and independent sites (i.e.: $k_A \neq k_{0,A} = k_{A,A} = k_{B,A}$; $k_B \neq k_{0,B} = k_{B,B} = k_{A,B}$) no relevant simplification occurs in the more general expressions (15) and (17).

2) Identical and cooperative sites:

$$k_A = k_{0,A} \neq k_{A,A} \neq k_{B,A} \quad \text{and/or} \quad k_B = k_{0,B} \neq k_{B,B} \neq k_{A,B}$$

with also $k_{B,A} = (k_A/k_B)k_{A,B}$, owing to symmetry considerations. In this case a somewhat relevant simplification occurs if cooperativity exists only between two A molecules bound to the protein (i.e. $k_{A,B} = k_{B,B} = k_B$), for it is then:

$$\frac{r_A}{[A]} = \frac{(2 - r_A)r_A k_A k_{A,A}}{(1 + k_B[B])\{(r_A - 1)k_A + \{k_A^2(r_A - 1)^2 - k_A k_{A,A}(2 - r_A)r_A\}^{1/2}\}}$$

3) Different and cooperative sites with $k_A \gg k_{0,A} \approx k_{A,A} \approx k_{B,A} \approx 0$ and $k_{A,B} \neq k_{B,B} \approx k_{0,B} \gg k_B$: in this case direct substitution in (15) brings to an expression of the type 0/0. But developing θ in series:

$$\theta = \{\phi^2(1 - r_A)^2 - 4r_A(2 - r_A)\psi\chi\}^{1/2} = \phi(1 - r_A) + \frac{4r_A(2 - r_A)\psi\chi}{2(1 - r_A)\phi} + \cdots,$$

inasmuch as the omitted terms are negligible, the final result may be written:

$$\frac{r_A}{[A]} = \frac{2(2 - r_A)r_A\psi}{2(2 - r_A)r_A\psi\chi/(1 - r_A)\phi} = \frac{\phi}{\chi}(1 - r_A) = \frac{(1 + k_{A,B}[B])}{(1 + k_{0,B}[B])}k_A(1 - r_A).$$

4. Results and discussion

4.1. The interaction between fluorescein and serum albumin

The affinity measurements, performed by the GPC technique, have shown the presence of essentially two or possibly three binding sites for fluorescein on both HSA and BSA with quite similar affinities in the two cases, being the related Scatchard plots nearly linear (see figs. 1 and 2). There is substantial agreement with literature data [12], once differences in temperature and ionic strength are accounted for. In addition, microcalorimetric measurements [4] have not shown, within experimental errors, any differences in the enthalpies of interaction. To shed more light on the possible differences (not shown but not excluded by thermodynamic data alone) between

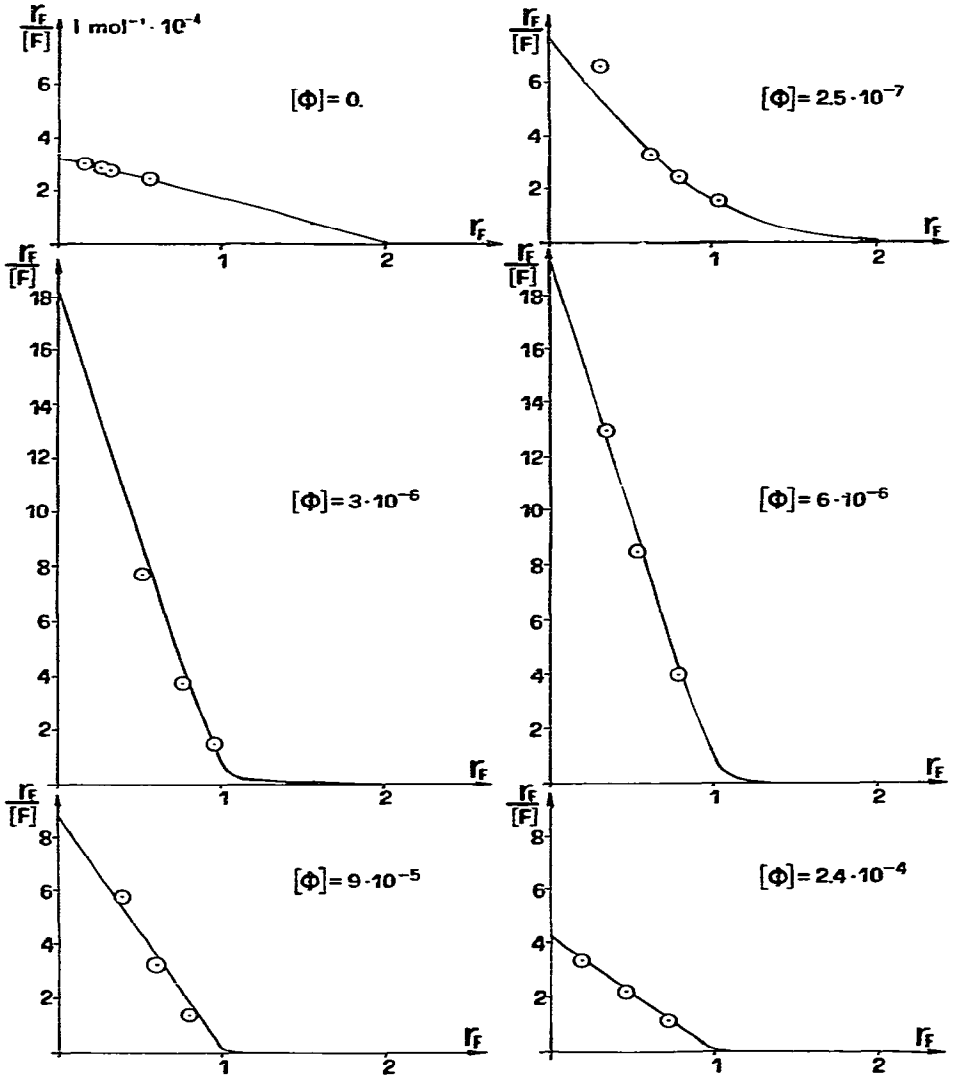

Fig. 1. Scatchard plots for the system BSA-fluorescein-phenylbutazone: [F]: fluorescein free concentration; [ϕ]: phenylbutazone free concentration; o: experimental data; the curves are calculated as described in the text.

the binding sites of each protein, spectrophotometric and circular dichroic measurements were performed in addition to the spectrofluorimetric ones already reported [4].

The results of the spectrophotometric titrations are given in figs. 3 and 4. The fluorescein absorption band at 489 nm is shifted to longer wavelengths as the ligand binds onto the protein (the effect is more marked in the BSA case). Particularly significant is the absence of a clear isosbestic point (as it also happens in the CD spectra): this finding implies the non identity of the sites of binding also with regard to the binding constant. Moreover, as outlined in the theoretical considerations presented in section 3 above, a nearly linear Scatchard plot (as in the present

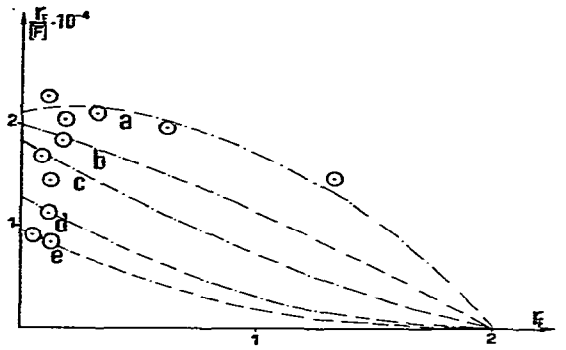

Fig. 2. Scatchard plots for the system HSA-fluorescein-phenylbutazone at various phenylbutazone free concentrations: a: $[\phi] = 0$. b: $[\phi] = 3 \times 10^{-6}$ M; c: $[\phi] = 10^{-5}$ M; d: $[\phi] = 5 \times 10^{-5}$ M; e: $[\phi] = 10^{-4}$ M. The curves are tentatively calculated, as an example, using the following set of constants: $k_F = 2 \times 10^4$, $k_{0,F} = 10^3$, $k_{F,F} = k_{\phi,F} = 1.5 \times 10^4$, $k_\phi = 1.2 \times 10^4$, $k_{0,\phi} = k_{\phi,\phi} = k_{F,\phi} = 4 \times 10^5$.

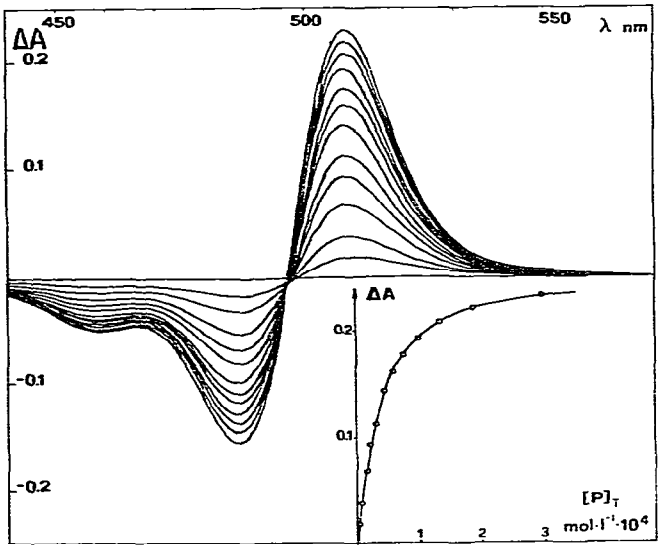

Fig. 3. Difference absorption spectra of fluorescein 2×10^{-5} M at various BSA concentrations $[P]_T$, as evidenced in the insert for $\lambda = 508$ nm.

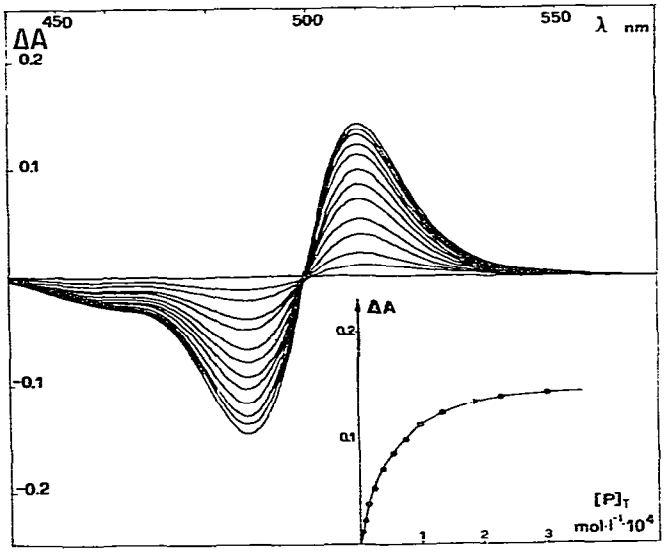

Fig. 4. Difference absorption spectra of fluorescein 2×10^{-5} M at various HSA concentrations $[P]_T$, as evidenced in the insert for $\lambda = 510$ nm.

case) is compatible only with the presence of identical and non-cooperative sites (and this is excluded by spectral evidences) or with different but (positively) cooperative sites.

For what concerns the CD spectra a noticeable difference emerged between the BSA-fluorescein and the HSA-fluorescein systems. Only in this last case in fact interaction with the protein induces a strong extrinsic dichroic band of fluorescein at 489 nm; this phenomenon is of course related to the asymmetry induced in the ligand upon binding onto the protein. In the BSA-fluorescein complex this effect practically does not appear; nevertheless this evidence alone is not sufficient to decide if (at least) one site of binding is only conformationally or even topologically different in the two proteins. In this regard it is interesting to notice also that, as it may be inferred from the titration diagrams of figs. 7 and 8, the CD extrinsic band is not exhibited by the more tightly bound ligand molecule (see the sygmoid shape of titration curve of fig. 8 and the decrease of the band on increasing C_P/C_F ratio beyond unity in fig. 7).

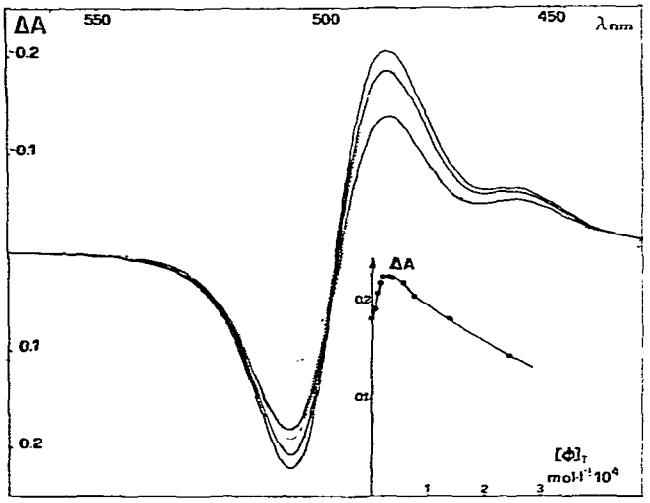

Fig. 5. Difference absorption spectra of fluorescein 2×10^{-5} M in presence of BSA 2×10^{-5} M at various phenylbutazone concentrations [ϕ], as evidenced in the insert for $\lambda = 508$ nm.

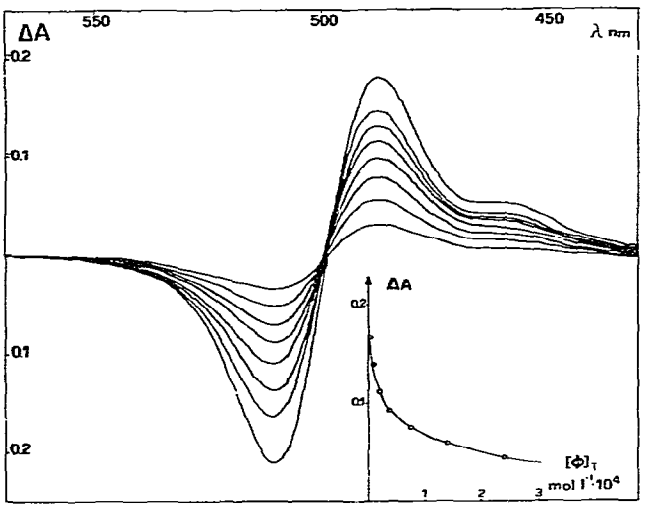

Fig. 6. Difference absorption spectra of fluorescein 2×10^{-5} M in presence of HSA 2×10^{-5} M at various phenylbutazone concentrations [ϕ], as evidenced in the insert for $\lambda = 510$ nm.

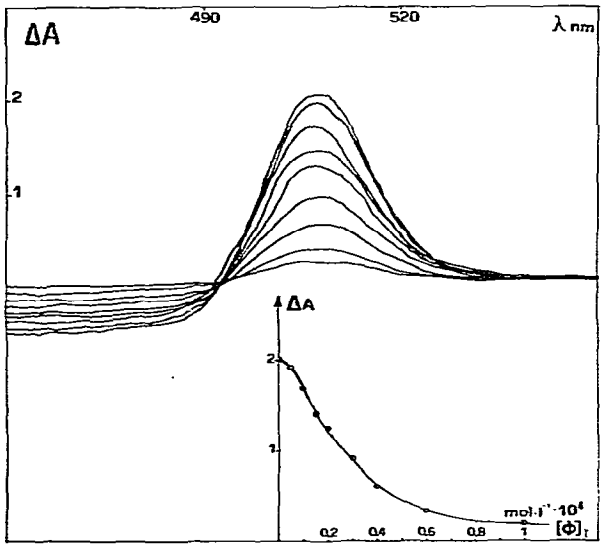

Fig. 7. CD spectra of fluorescein 5×10^{-5} M at various HSA concentrations $[P]_T$, as evidenced in the insert for $\lambda = 508$ nm.

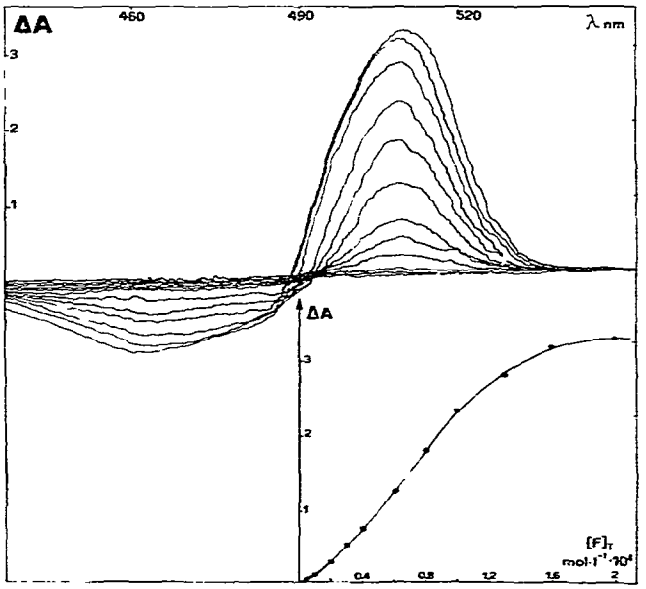

Fig. 8. CD spectra of fluorescein at various concentrations [F], as evidenced in the insert for $\lambda = 508$ nm, in presence of HSA 5×10^{-5} M.

4.2. The interaction between phenylbutazone and serum albumin

The GPC data relative to the interaction of phenylbutazone with HSA and BSA have shown the presence of one high affinity binding site and of some other sites of lower affinity [4] in substantial agreement with literature data [13]. In the hypothesis of non cooperativity, the values for the intrinsic binding constants relative to the primary sites are: $K \approx 1 \times 10^6$ for BSA and; $K \approx 4 \times 10^5$ for HSA. From microcalorimetric data, on the contrary, quite different ΔH_b values are obtained for the two cases [4] ($\Delta H_b \approx -9$ kcal/mol for BSA and $\Delta H_b \approx -1$ kcal/mol for HSA). This rather strong difference in binding enthalpy may suggest that the primary site for phenylbutazone is topologically different in the two albumins.

For these systems no spectral data were collected, mainly because of the overlapping of albumin and phenylbutazone absorption bands in the UV region, and of the subsequent difficulties in the interpretation of spectral data.

4.3. The simultaneous interaction of fluorescein and phenylbutazone with BSA

The GPC technique, according to the method of Hummel and Dreyer [5], revealed particularly proper in the study of the simultaneous interaction of phenylbutazone and fluorescein with BSA and with HSA, since it enables to fix "a priori" the free concentrations of the ligands in the measurements. In this way the experimental data are easily dealt with the theoretical scheme presented above (section 3). In the case of interaction with BSA a set of series of determinations were performed, each series with a fixed phenylbutazone free concentration and various fluorescein free concentration values; and each series of data was diagrammed in the form of a Scatchard plot relative to fluorescein binding (fig. 1). These graphs reveal by inspection that the presence of phenylbutazone at relatively low free concentrations has a double effect on the binding of fluorescein onto the protein: it induces the release of one of the two more tightly bound fluorescein molecules from the protein (probably by mere competition) but simultaneously it promotes a stronger binding of the other (graphs c and d of fig. 1). At higher concentrations, phenylbutazone then provokes the release of this tightly bound fluorescein too, possibly by competition according to mass action law. Indeed, once these qualitative considerations are translated in terms of intrinsic binding constants on the basis of the theoretical scheme presented above, it is seen that the experimental data of fig. 1 may be quite satisfactorily reproduced by eq. (15) using the appropriate intrinsic binding constants, according to a two sites-two ligand species model. Having assumed for fluorescein alone $k_F \approx 3 \times 10^4$, $k_{0,F} \approx 2 \times 10^3$, $k_{F,F} \approx 1 \times 10^4$ l/mol on the basis of the data of graph a in fig. 1, and previous considerations, and similarly for phenylbutazone alone $k_\phi \approx 1 \times 10^6$, $k_{0,\phi} \approx k_{\phi,\phi} \approx 2 \times 10^4$, our experimental data could be reproduced assuming that $k_{F,\phi} \approx k_{0,\phi}$ (i.e. no relevant cooperative effect between fluorescein bound at site 1 and phenylbutazone at site 2) and imposing $k_{\phi,F} \approx 2.2 \times 10^5$ l/mol, as shown by calculated curves in fig. 1. This model is qualitatively supported also by spectral data, even if in this kind of measurements total (and not free) fluorescein concentration is held constant: in this respect spectral variations of fluorescein-BSA solutions induced by adding phenylbutazone were followed by means of spectrofluorimetry, difference absorption spectrophotometry and circular dichroism. The fluorescence data were considered in a preceding paper [4]. The difference absorption spectra are reported in fig. 5, and show an initial increase of the difference spectrum at low added phenylbutazone concentrations (i.e. increasing of bound fluorescein) followed by a decrease at high phenylbutazone concentrations (decrease of bound fluorescein). Similar considerations stem from our CD data (fig. 9) even if in this case the spectra are rather scanty (bound fluorescein develops a very low CD extrinsic band which moderately rises on phenylbutazone initial addition, and then slightly decreases).

We believe that the possibility of extending the quantitative treatment on the basis of the above theoretical scheme to spectral data would deserve some consideration.

4.4. The simultaneous interaction of fluorescein and phenylbutazone with HSA

The influence of phenylbutazone on the binding of fluorescein onto HSA revealed markedly different with respect to the BSA case. Indeed, even if experimental GPC data appeared less accurate as a consequence of the low quantity of fluorescein retained by the protein in this case, it is evident from the data of fig. 2 that the only effect of phenylbutazone addition on fluorescein binding is to promote a displacement of this ligand from the protein. If this behaviour has to be ascribed to mere competition or to a negatively cooperative effect cannot be deduced from these data, although it could in principle be possible on the basis of the theoretical treatment presented above once more accurate data were available. However spectral data confirm this effect, as can be inferred from our fluorescence results reported in a preceding paper [4] and from spectrophotometric and CD data illustrated in figs. 6 and 10. In all cases, addition of phenylbutazone to a HSA-fluorescein solution gradually changes the optical properties of fluorescein from those typical of the bound form to those typical of the free one. Of some structural interest is the fact that from the sygmoid aspect of the CD titration curve it may be inferred that low phenylbutazone concentrations preferably displace fluorescein bound to the tighter site, i.e. the non CD extrinsic band producing one.

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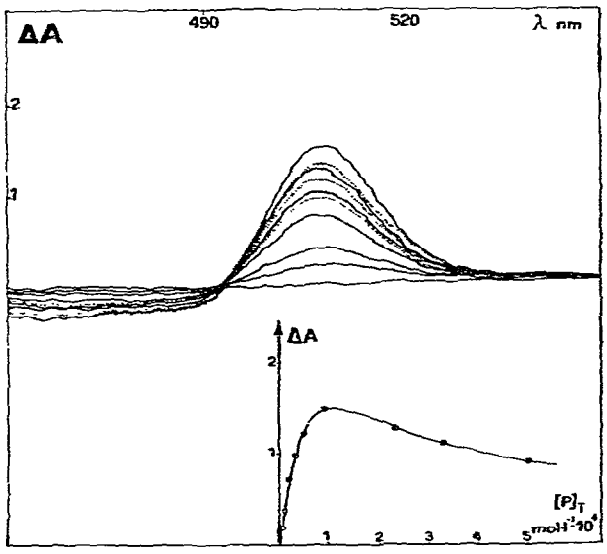

Fig. 9. CD spectra of fluorescein 5×10^{-5} M in presence of BSA 5×10^{-5} M at various phenylbutazone concentrations [ϕ] as evidenced in the insert for $\lambda = 500$ nm.

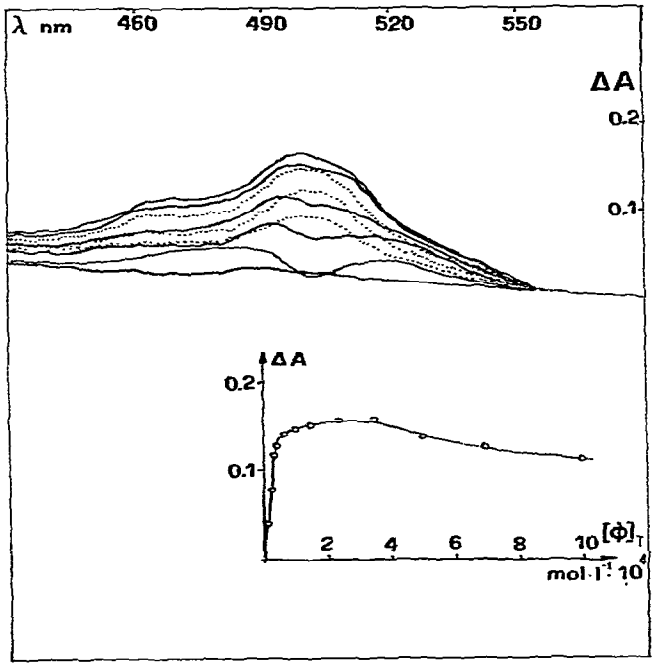

Fig. 10. CD spectra of fluorescein 5×10^{-5} M in presence of HSA 5×10^{-5} M at various phenylbutazone concentrations [ϕ], as evidenced in the insert for $\lambda = 508$ nm.

References

[1] J. Steinhardt and J.A. Reynolds, Multiple equilibria in proteins (Academic Press, New York, N.Y., 1969).
[2] M.C. Meyer and D.E. Guttmann, J. Pharm. Sci. 57 (1968) 895.
[3] J.J. Vallner, J. Pharm. Sci. 66 (1977) 447.
[4] G. Manzini, V. Crescenzi and A. Ciana, Biophys. Chem. 10 (1979) 389.
[5] J.P. Hummel and M.J. Dreyer, Biochim. Biophys. Acta 63 (1962) 530.
[6] G. Scatchard, Ann. N.Y. Acad. Sci. 51 (1949) 660.
[7] C.J. Thompson and I.M. Klotz, Arch. Biochem. Biophys. 147 (1971) 178.
[8] I.M. Klotz and D.L. Hunston, J. Biol. Chem. 250 (1975) 3001.
[9] G. Schwarz, Eur. J. Biochem. 12 (1970) 442.
[10] G. Schwarz, Biophys. Chem. 6 (1977) 65.
[11] G. Schwarz, Biophys. Struct. Mechanism 2 (1976) 1.
[12] L.O. Anderson, A. Rehnström and D.L. Eaker, Eur. J. Biochem. 20 (1971) 371.
[13] P. Aussel, M. Audran, J.L. Chanal and R. Marignan, Annales pharm. france 31 (1973) 489.