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BINDING OF BROMOSULPHTHALEIN TO SERUM ALBUMINS

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SUMMARY

The binding of bromosulphthalein to human and bovine serum albumin was studied by infrared spectroscopy, laser-Raman spectroscopy, visible spectroscopy and pH measurements in order to obtain information on the binding forces involved. No conformational change of the proteins was observed during the tight binding of the first three bromosulphthalein molecules as indicated by the kinetics of the H–^{2}H exchange and infrared spectroscopy in ^2H_2O. Subsequent occupation of the low affinity binding sites causes a partial unfolding of the proteins. Binding of the dye at the high affinity sites is accompanied by a change in intensity and a shift of the lactone carbonyl band in infrared and laser-Raman spectra as well as a decrease of the visible absorption at 580 nm suggesting a hydrophobic environment. Binding at these sites is caused by Van der Waal or hydrophobic forces since the charge of the proteins remains unchanged during this process. It may be concluded that the main binding forces at the 14 low affinity binding sites consist of electrostatic interactions as indicated by pH shift studies and model studies for the bathochromic shift of the quinoic dye.

INTRODUCTION

One up to now not often used tool for the study of intermolecular forces that bind small molecules to macromolecules is infrared spectroscopy. Using the special technique of "Frustrated Multiple Internal Reflection" and optimized conditions for the recording of spectra in aqueous protein solution [1] we have attempted in the present study to obtain information on the binding processes with proteins.

As a model protein we used albumin which is known to bind a large variety of molecules with different affinities. Binding may occur with apolar compounds such as hydrocarbons [2] or polar molecules like anionic azo- or triphenylmethane dyes [3–6]. For our studies we took bromosulphthalein, which has been reported to bind tightly to albumin at three sites and to losely associate at about 14 additional sites [6–8].

We shall show that in combination with other methods infrared spectroscopy can give valuable information on the binding sites and the nature of their interaction with smaller molecules.

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

MATERIALS AND METHODS

Materials

Bromosulphthalein (4,5,6,7-tetrabromo-3,3-disulphophenolphthalein disodium salt) was purchased from Fluka AG, Buchs, Switzerland, ^2H_2O (99%) from Merck, Darmstadt, Germany. DEAE-Sephadex A-25 from Pharmacia, Uppsala, Sweden. The following proteins were used without further purification: human serum albumin (Fraction V, lyophilized), human serum albumin (crystallized), bovine serum albumin (Fraction V, lyophilized), bovine serum albumin (crystallized), all albumins obtained from Serva, Heidelberg, Germany. The lyophilized preparations after dissolving in water showed a pH of 7.0 and were used for infrared spectroscopy, crystallized preparations showed a pH around 5.2 in water and were used for measurements of the pH shift.

3-Carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl and 2,2,6,6-tetramethylpiperidine were obtained from SYNVAR-Company, Palo Alto, Calif., U.S.A. 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) was prepared by oxidation of 2,2,6,6-tetramethylpiperidine with H_2O_2 at pH 8 [9].

Methods

Absorbance measurements were made with an Aminco DW-2 spectrophotometer in 1-cm cuvettes. Wavelengths were controlled by the aid of a didymium standard. EPR measurements were done with a Varian E-9 spectrometer in the X-band. All pH measurements were performed on a digital pH meter (model 801, Orion, Cambridge, U.S.A.) with a pH combination electrode (N6O, Schott GmbH, Mainz, Germany).

Infrared spectra were recorded with a Perkin–Elmer 457 spectrophotometer with a reflection attachment of the Wilks Corporation, South Norwalk, Conn., U.S.A. We used the technique of frustrated multiple internal reflection, independently developed by Fahrenfort [10] and Harrick [11]. One drop of the protein solution (about 0.03 ml) was spread on the germanium crystal (25 reflections at an angle of 45°) which was fixed in a vertical position on the reflection attachment. A second germanium plate served as a blank in the reference beam. Solution spectra could also be studied by this method as capillary films between the germanium plate and a covering glass.

Infrared H–^{2}H exchange studies have been performed to detect structural changes in proteins (cf. refs 12 and 13). In our studies we followed the method of Blout et al. [13] and took the decrease of the absorbance ratio $A_{\text{amide II}}/A_{\text{amide I}}$ with time as a measurement of exchange. This ratio varied between 0.45 and 0.48, when calculated from completely dried films cast from albumin solutions in water. These results are in agreement with those in solution obtained by Blout et al. [13]. For each measurement we took a drop of the exchanging albumin solution and spread it on the germanium crystal. Reproducible spectra were recorded as soon as interference colours developed. In the H–^{2}H exchange studies we could neglect the back exchange from the water vapour because we were only comparing spectra performed under identical conditions. Therefore, we made no attempt to calculate the absolute values of exchange.

RESULTS

Albumin Infrared Spectra and H–^{2}H exchange

Infrared spectra of human serum albumin are shown in Fig. 1. Thin films were cast from 1 mM solutions in $^{2}H_2O$ after 30 min of exchange and solutions containing in addition 12 mM bromosulphthalein.

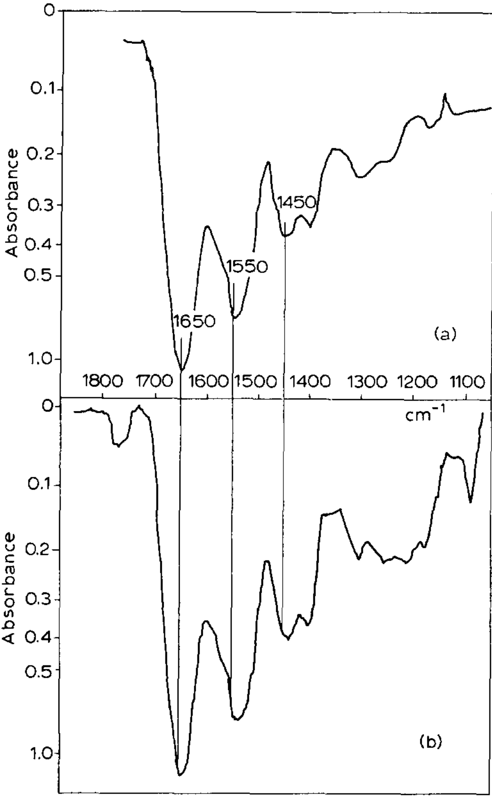

Fig. 1. Infrared spectra cast from $^{2}H_2O$ solutions: (a) 1 mM human serum albumin; (b) 1 mM human serum albumin, 12 mM bromosulphthalein. Both recorded with the thin film technique after 30 min of exchange.

The amide frequencies of the protein remained unchanged by small concentrations of the dye. The amide I band is located at 1649 cm^{-1}, the amide II band at 1542 cm^{-1} and the amide II′ band at 1445 cm^{-1}.

Higher concentrations of the dye, however, caused slight modifications of the spectrum. All amide bands of the protein are broadened and shifted to lower wavenumbers (Fig. 1, Table I). As described in the literature [14, 15] unordered protein structures in $^{2}H_2O$ show an amide I band at 1643 cm^{-1}. It can be concluded therefore from our observations that addition of high concentrations of bromosulphthalein to albumin induces a transition of a certain part of the α-helix to the solvated random-coil conformation.

TABLE I

AMIDE I AND AMIDE II FREQUENCIES (cm^{-1}) OF 1 mM HUMAN SERUM ALBUMIN CAST FROM ^2H_2O SOLUTIONS BEFORE AND AFTER ADDITION OF VARIOUS CONCENTRATIONS OF BROMOSULPHTHALEIN

	Frequencies (cm^{-1})	Bromosulphthalein	
		3 mM	18 mM
Amide I	1649	1649	1647 1643
Amide II	1542	1542	1542 1538
Amide II′	1445	1445	1445 1435

These findings were confirmed by our H–^{2}H exchange experiments. The decrease in absorbance of the amide II band relative to the amide I band in deuterated water is taken as a measure for the exchange rate. This parameter is shown in Fig. 2 for human serum albumin and three different concentrations of bromosulphthalein.

The protein, in the first minutes shows such a rapid exchange of hydrogens that the exact kinetics could not be measured. Within the next 24 h further exchange takes place until a value $A_{\text{amide II}}/A_{\text{amide I}}$ of 0.22 is reached. From these data no direct conclusion on the conformation of the protein can be drawn, although it is known [13], that the rapid H–^{2}H exchange is presumably due to the deuteration of the random-coil regions of the polypeptide chains. In the time interval of slower exchange (10 min to 3 h) the exchange of the helical portions is assumed and the non-exchangeable hydrogens are attributed to the highly hydrophobic regions [13]. When bromosulph-

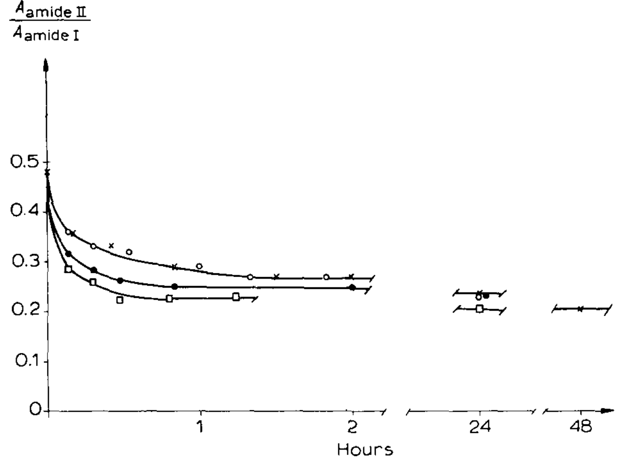

Fig. 2. $A_{\text{amide II}}/A_{\text{amide I}}$ as a function of time for human serum albumin and for three different dye concentrations (pH = 7.0; $T = 21$ °C). ×—×, 1 mM albumin; ○—○, 1 mM albumin, 3 mM bromosulphthalein; ●—●, 1 mM albumin, 5 mM bromosulphthalein; □—□, 1 mM albumin, 12 mM bromosulphthalein.

thalein is added in up to 3-fold higher molar concentrations than human serim albumin no effect on the hydrogen exchange is observed. With increasing concentrations of the dye, however, an acceleration of the exchange for the fast and slow phase is seen without affecting the number of non-exchanging hydrogens. With very high ratios of bromosulphthalein/human serum albumin (12 and higher) also the number of the non-exchanging hydrogens decreases indicating that strongly hydrophobic areas are now partly exposed to the surrounding solvent. Similar data were derived with bovine serum albumin.

Effect of binding to albumin on infrared and laser-Raman spectra of bromosulphthalein

In aqueous solution the infrared spectrum of the dye shows a pattern of sharp and characteristic bands. A complete assignment of the bands to the corresponding vibrations is difficult. But by comparison with data in the literature [16, 17] and laser-Raman spectra we come to the following interpretation (Table II). After binding of bromosulphthalein to human serum albumin a change in the intensity and in the position of the carbonyl group of the lactone ring is noted. The decrease in the intensity of the band can be observed by comparing spectra of the ligand with those of the dye plus albumin (Figs 3 and 4). The expanded infrared spectra show, that the absorbance at 1765 cm^{-1} is accompanied by a second absorbance around 1775 cm^{-1}

TABLE II

CHARACTERISTIC INFRARED AND RAMAN BANDS OF BROMOSULPHTHALEIN IN ACID SOLUTION (cm^{-1})*

Infrared	Raman	Vibrating groups
1765 s	1763 w	>C=O
1610 m	1610 s	(benzene ring)
1510 sh 1490 m	1535 w	(benzene ring)
1415 m.br	—	(substituted benzene ring)
1350 m.br	1358 w	>C–OH
1300 m.br 1260 sh 1200 b.vr 1175 sh	1290 w 1260 w 1211 v.w 1160 m	$-SO_3^-$ >C–O–H
1130 m 1090 s 1030 s	— 1095 w 1030 s	$-SO_3^-$

* s, strong; m, medium; br, broad; w, weak; v.br, very broad; sh, shoulder.

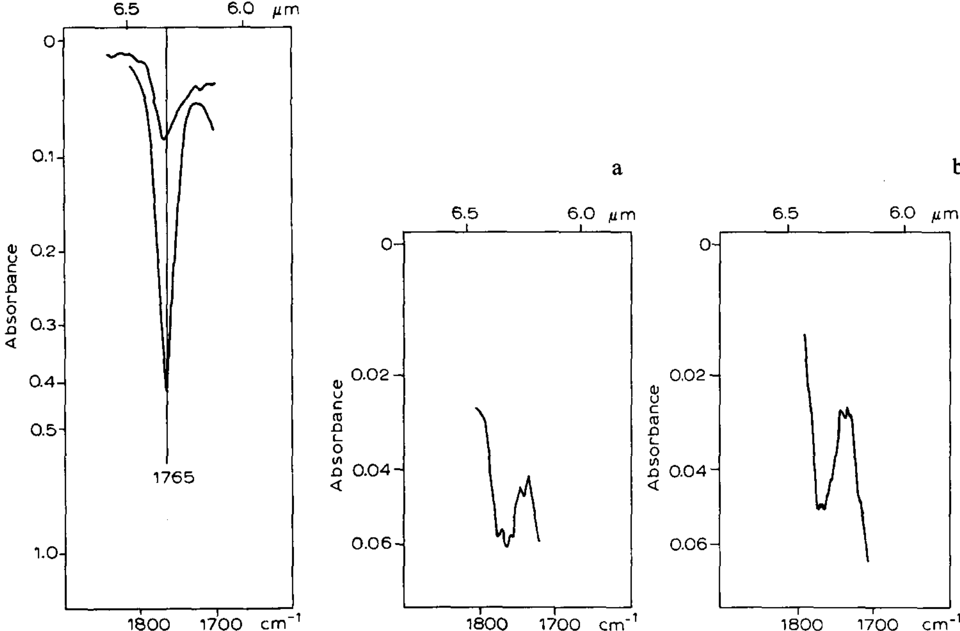

Fig. 3. Carbonyl band of 50 mM bromosulphthalein in 25% dioxane, 75% ^2H_2O (top) and of 50 mM bromosulphthalein in ^2H_2O (glass covered film).

Fig. 4. Infrared spectra of the carbonyl band of bromosulphthalein in the presence of human serum albumin in ^2H_2O (glass covered film). a, 10 mM albumin, 30 mM bromosulphthalein; b, 10 mM albumin, 50 mM bromosulphthalein.

after binding (Fig. 4). With increasing concentrations of bromosulphthalein (Fig. 4b) the band between 1765 and 1775 cm^{-1} is broadened and increases. The decrease in intensity of the carbonyl band and its slight shift to higher wavenumbers during binding can be simulated by transferring the dye in solutions of lower polarity (Fig. 3, top curve), which suggests that the carbonyl group of bound ligand at low ratios of dye/ human serum albumin is in a hydrophobic environment.

These findings could be confirmed by laser-Raman spectra. Free bromosulphthalein shows a small carbonyl band at 1763 cm^{-1}. During binding the carbonyl band of the dye increases and its maximum shifts to 1810 cm^{-1} as theoretically expected (Fig. 5).

Visible spectra

In order to obtain more information on the binding of bromosulphthalein to albumin and to support the infrared data we looked for spectra changes in the visible region. At pH 8.0 about 7% of the bromosulphthalein is present as a quinoic dye, which has an absorption maximum at 580 nm. At lower pH values the colourless lactone is formed [16]. Addition of human serum albumin to the dye resulted in a decrease of absorbance at 580 nm with a concomitant formation of a band at 591 nm (Fig. 6). To decide what type of interaction between the albumin molecules and the dye is responsible for the spectral changes during binding we studied models.

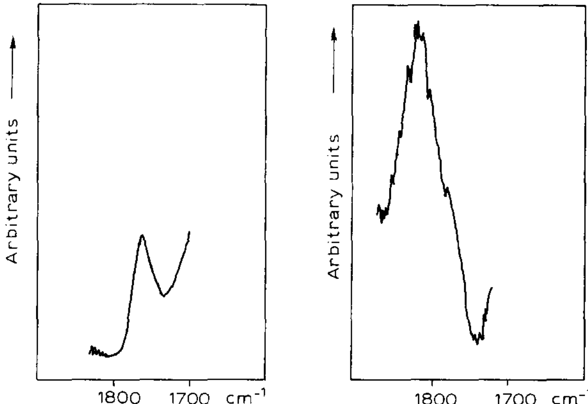

Fig. 5. Laser-Raman spectra of the carbonyl band of bromosulphthalein in water. a, 10 mM bromosulphthalein; b, 10 mM bromosulphthalein, 1 mM bovine serum albumin.

A decrease in absorbance of bromosulphthalein can also be observed by replacing water as a solvent by dioxane. During this process the maximum of absorbance is shifted to longer wavelengths (Fig. 7). However, a shift of 11 nm as observed during binding to albumin could not be obtained. In order to determine the polarity of the dioxane–water mixtures we used the hyperfine splitting of the TEMPO spinlabel

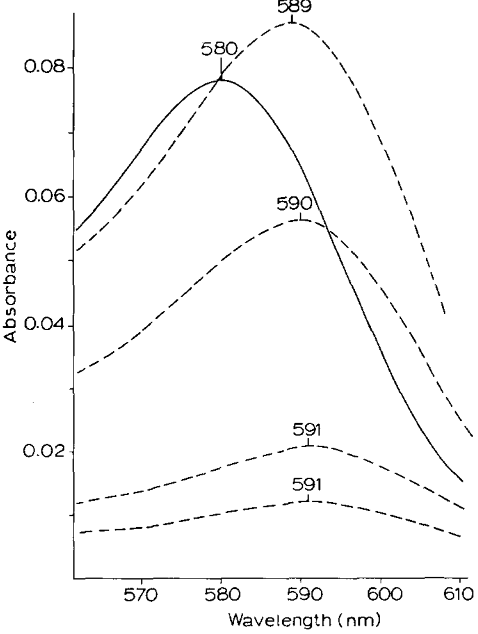

Fig. 6. Spectra of bromosulphthalein in the presence and in the absence of albumin. The solid line represents bromosulphthalein (15 μm), the dashed line human serum albumin (15 μm) plus bromosulphthalein in increasing concentrations (10, 15, 30 and 40 μM). Buffer: 0.1 M Tris–HCl, pH 8.0 (20 °C).

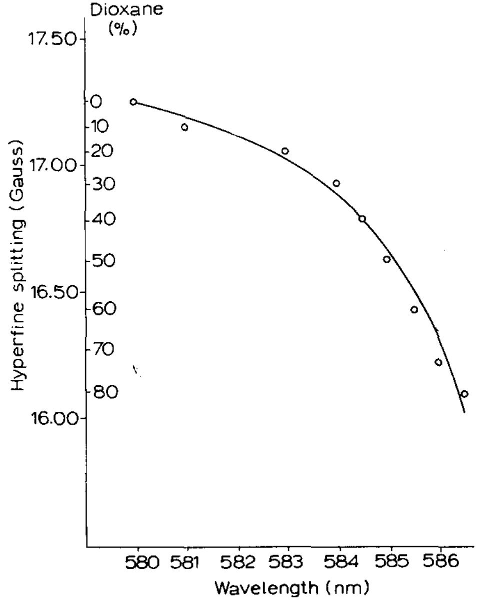

Fig. 7. Correlation between the hyperfine splitting of the TEMPO spinlabel and the absorption maximum of bromosulphathlein in mixtures of dioxane (0–80%) and 0.1 M NaOH.

according to Brière et al. [18]. This scale corresponds to the *z*-scale introduced by Kosower [19].

A model that mimicks the effect of binding by electrostatic forces was found in DEAE-Sephadex A-25 which upon binding of the dye shifts the absorption maximum to 590 nm. This cannot be due to a decrease in polarity since bound 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl spinlabel shows the same hyperfine splitting as in water.

The spectral properties of the small quantity of bromosulphthalein bound to albumin having an absorption maximum at 591, therefore can be explained by electrostatic interaction, while the observed decrease in absorbance at 580 nm can be caused by hydrophobic forces.

pH changes associated with binding

After addition of a neutral salt to a protein at its isoelectric point Scatchard and co-workers [20, 21] observed a pH shift of the solution. Differences between the pH shifts for various salts were found to be due to differences in affinity and amount of the binding of their anions. Those which bind strongly produce a large upward displacement of the pH value. As shown in Table III addition of bromosulphthalein to an unbuffered solution of bovine serum albumin (6.3 mg/ml) causes a successive shift of the pH from 5.16 to 6.41. Similar results were derived from our experiments with human serum albumin.

An explanation of the electrostatic interactions of the bound anion with albumin is based on the assumption that albumin is a spherical macro-ion with a radius of 3 nm and a collision diameter of 3.2 nm. This would represent the closest distance

TABLE III

DISPLACEMENT OF pH, THE VALUES FOR THE ELECTROSTATIC FACTOR w AND THE NUMBER OF BOUND ANIONS $\bar{\nu}$ AFTER ADDITION OF INCREASING AMOUNTS OF BROMOSULPHTHALEIN

w	ΔpH	$\bar{\nu}$	Bromosulphthalein ($10^4 \times$ molarity)
0.1065	0.045	0.24	0.38
0.1036	0.080	0.45	0.76
0.1006	0.125	0.72	1.14
0.0983	0.265	1.55	2.28
0.0907	0.435	2.76	3.42
0.0875	0.590	3.88	4.56
0.0848	0.720	4.89	5.70
0.0824	0.820	5.73	6.86
0.0814	0.885	6.26	7.60
0.0789	0.990	7.23	9.50
0.0761	1.060	7.74	11.40
0.0741	1.115	8.66	13.30
0.0742	1.155	9.22	15.20
0.0692	1.215	10.04	19.00
0.0666	1.250	10.81	22.80
0.0626	1.295	11.94	30.40

to the center of a neighbouring small ion [20, 21]. The factor w of the Debye–Hückel equation is dependent on the ionic strength, which is not known for our solutions. We therefore use the ionic strength in absence of the protein. According to Scatchard and co-workers [20, 21] the moles of ligand bound per mole of protein ($\bar{v}$) can be calculated from the change of pH by means of the equation:

$$\Delta \text{ pH} = -0.868\, w\, \bar{v}\, \bar{Z}$$

($\bar{Z} = -2$ for the divalent anion of bromosulphthalein) The results of this calculation are shown in Table III. If $\bar{v}$ is plotted against the log concentration of the free dye (difference between total and bound concentrations) it becomes obvious that the experimental values are close to a curve calculated for 14 sites capable of binding bromosulphthalein with an association constant of $K = 3.2 \cdot 10^3$ (Fig. 8).

The different curves for the three classes of binding sites measured by equilibrium dialysis [6] in which $n_1 = 1$, $k_1 = 1.7 \cdot 10^7$; $n_2 = 2$, $k_2 = 1.6 \cdot 10^6$ and $n_3 = 14$, $k_3 = 6 \cdot 10^3$ indicate that the isoelectric point of bovine serum albumin can be changed only during binding at the low affinity binding sites.

As already known from the literature [22] the "ΔpH method" performed with monovalent aromatic anions gives results in good agreement with those determined by dialysis.

DISCUSSION

All results with the various methods employed are in agreement with the proposed three high and several low affinity binding sites for bromosulphthalein at the

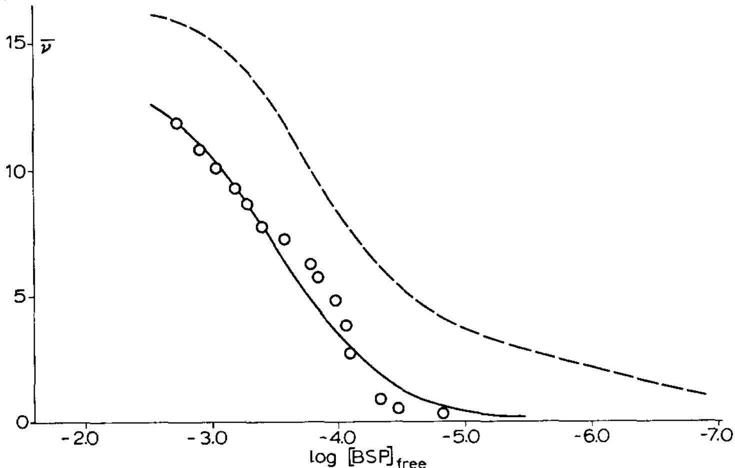

Fig. 8. Plot of $\bar{\nu}$ (number of moles bromosulphthalein bound to one molecule albumin) against log concentration of the unbound fraction of the dye. Broken line for $n_1 = 1$ binding site with the association constant $k_1 = 1.7 \cdot 10^7$, $n_2 = 2$ with $k_2 = 1.6 \cdot 10^6$ and $n_3 = 14$ with $k_3 = 6 \cdot 10^3$ [6]. Solid line for one set of groups with $n = 14$ and $k = 3.2 \cdot 10^3$.

albumin molecule [6, 23]. Binding of the dye is linked to an alteration of the tertiary structure of the albumin molecule as demonstrated by Scholtan and Gloxhuber [23] by measuring optical rotation dispersion. In addition our results do show that excessive binding of dye even changes the secondary structure of albumin.

During binding at the high affinity sites no conformational change of the albumins could be noted as seen by the identical kinetics of the H–^{2}H exchange and the unchanged fine structure of the peptide carbonyl bands. Binding of additional molecules of the dye modifies the mobility (as defined by Hvidt and Nielsen [12]) of the protein by breaking hydrogen bonds of the α-helical structure. As a consequence the exchange of the slow exchanging hydrogens becomes more rapid. Very high concentrations of the dye can even cause an exchange of the strongly hydrophobic region of the protein and can also alter the structure and position of the amide bands. A similar process can be observed with ovalbumin after binding with various dyes [24], which, as we could notice, already shows denaturation and precipitation by addition of a molar excess of bromosulphthalein molecules.

At the pH values of the measurements bromosulphthalein is present mostly in its lactone form. As indicated by the presence of the lactone carbonyl vibrations and the decrease of the visible absorption at 580 nm the dye is mainly bound in this form. The appearance of a weak band at 591 nm must be attributed to bound quinoic dye. Its amount, however, cannot exceed 2 or 3% of the total bromosulphthalein. As known from the literature [25] the electronic transitions of the phthaleins are susceptible to polar effects. In hydrophobic media or during binding to an anion-exchange resin the sulfonate group can undergo electrostatic bonding. This increases the electron withdrawing power of these groups causing a slight redshift of the absorption maximum. The altered visible spectra of the quinoic dye bound to albumin as well as the spectra recorded in a medium of decreased polarity or from the dye bound to DEAE-Sephadex as models, support that electrostatic forces take prece-

dence over hydrophobic interactions at the low affinity binding sites. In contrast the interactions at the specific sites are mainly hydrophobic.

These results are supported by the observed pH shift of 1.25 units upon addition of the anionic dye to albumin. The limitations of ΔpH method especially for tightly binding ligands have been pointed out [22], but together with the studies on the bathochromic shift it may be concluded that for the low affinity binding sites of albumin electrostatic interactions with ionic ligands are responsible.

Information about the new environment around the dye after binding was obtained from the decrease of the intensity and the simultaneous shift in the frequency of its carbonyl band. Both effects indicate an increase in bond strength which must be due to a decreased polarity of the carbonyl bond and can be observed also when the C=O group is transferred to an environment less polar than water. These changes, confirmed by laser-Raman spectra, occur at a low ratio of bromosulphthalein/human serum albumin and therefore must be characteristic for the first binding sites. Occupation of more sites does not increase the band at 1775 cm^{-1} but causes broadening and appearance of new bands at lower frequencies.

In conclusion the infrared spectroscopic data are in accordance with the results obtained by other methods and a further development of this technique for the investigation of biological systems promises more insight into their molecular structure.

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