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MEASUREMENT OF THE ASSOCIATION OF CHOLEPHYLIC ORGANIC ANIONS WITH DIFFERENT BINDING PROTEINS

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Abstract—The binding of the colored cholephylic anions tetrabromosulfonphthalein (BSP), di-bromosulfonphthalein (DBSP), indocyanine green (ICG) and thymol blue (ThB) to a number of protein preparations including bovine serum albumin, human serum, rat hepatic cytosol and purified rat liver bilitranslocase has been studied by a direct spectrophotometric method. The experimentation provides extinction coefficients, dissociation constants and number of binding sites for the different complexes between dyes and the various proteins. Data obtained by this technique were in excellent agreement with those obtained on the same samples by ultrafiltration. The data presented indicate that the direct spectrophotometry applied to these dyes is simple, rapid and reproducible, making this the approach of choice during the purification of binding proteins when the binding capacity is the only useful criterion to follow the progress of the procedure.

A number of drugs as well as other diagnostic and physiologically relevant substances have in common the property of absorbing light in the visible region of the spectrum. Examples include rifamycins, indocyanine green, sulfobromophthalein as well as bile pigments. All of these substances undergo different metabolism at different body sites in which their free concentration may be determined by binding to proteins. It is relevant, therefore, to the pharmacologist, the biochemist or the clinician to evaluate the binding characteristics of colored small molecules to macro-molecules such as proteins. A number of techniques have been developed in the past to measure the concentration of free ligand in equilibrium with the bound one. The techniques include equilibrium- and dynamic-dialysis, gel-filtration, ultrafiltration and centrifugation at high speed. All of the approaches considered allow the measurement of the free compound in equilibrium with that bound to protein. None of the above-mentioned techniques for the study of the binding of a ligand to proteins is completely satisfactory. Unfortunately in many cases, the binding capacity of a protein for a specific compound remains the only identification criterion during, for instance, purification procedures. During purification of a binding protein, it is often very important that the measurement can be made in a sufficiently short time as to allow a rapid change in the isolation strategy. This requirement is not fulfilled by most techniques which may require hours to obtain the results. An additional possibility is open when dealing with colored substances. Dyes, as first shown by Klotz in 1946 [1], in most cases change their spectral properties upon binding to proteins. We decided therefore to investigate the possibility of this direct spectrophotometric approach to the problem of the binding of cholephylic organic anions to the proteins physiologically involved in their metabolism.

This study documents results obtained separately and largely independently by two groups of investigators, one in Italy and one in The Netherlands. When the authors realized that their data partially overlapped and were largely complementary, they decided to combine their results.

MATERIALS AND METHODS

Bovine serum albumin utilized in Groningen was purchased from Boseral Organon Technika (Amsterdam) and that in Trieste from Sigma Chemical Co. (St Louis, MO). DBSP was obtained from Serb (Paris, France), BSP and ThB from Merk (Darmstadt, West Germany), ICG from Hynson, Westcott and Dunning (Baltimore, U.S.A.). Dialysis tubing was Visking (Hoefelt, Den Haag, The Netherlands). Spectrophotometry was carried out in Groningen by means of a Zeiss spectrophotometer mod. M4Q III; in Trieste by a computer-assisted Carlo Erba Spectracomp 601 recording spectrophotometer. Female Wistar rats of the average weight of 234 g were sacrified by decapitation. Liver cytosol was prepared in 0.25 M sucrose containing 10 mM sodium phosphate buffer (pH 7.8) by homogenizing the organ previously perfused and rinsed with ice-cold saline solution, by means of a glass–Teflon Potter homogenizer and centrifugation at 105,000 *g* for 90 min. The supernatant was collected and protein determined by the method of Lowry [2]. Bilitranslocase was purified according to Lunazzi *et al.* [3]. Both

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bilitranslocase and bovine serum albumin were prepared or dissolved in 200 mM sodium phosphate buffer, 1 mM EDTA (pH 7.8).

THEORETICAL PRINCIPLE OF THE TECHNIQUE

The principle of the technique is based on the previous observation by Klotz [1] that the absorption spectrum of a colored substance may change upon binding to a macromolecule. Unbound chromophore has certain extinction coefficients at different wavelengths. When bound, the set of extinction coefficients changes. By choosing an appropriate wavelength, at which the absorbance of both bound and free ligand obey the Lambert–Beer's law, a differential extinction coefficient (Δ_ε) can be calculated. Such a value will be given by the difference of the two constants of free and bound forms of the dye respectively at the given wavelength ($\varepsilon_f - \varepsilon_b$). The extinction coefficient of the free chromophore may be measured separately from the absorbance of a given concentration of the dye at that wavelength. The extinction coefficient of the bound form, on the other hand, can be determined either in the presence of an excess protein or may be graphically extrapolated at infinite protein concentration (see under Results). Clearly in a solution containing both bound and unbound forms of the dye, the concentration of the free form (D_f) may be calculated from the following equation:

$$D_f = \frac{A - \varepsilon_b D_t}{\Delta_\varepsilon}$$

where A is the observed absorbance and D_t the concentration of the total dye present in the mixture. The equation is derived from Klotz [1] by multiplying the fraction of the free dye for the total dye concentration. Binding constants were computed by means of the CFT 3 multiparameter curve fitting program [6], adapted to our needs. The following equation for non-cooperative binding was used:

$$R = \sum_{i=1}^{\partial} \frac{n_i k_i [D]}{1 + k_i [D]}$$

where R are the moles of drug bound per mole of protein, n_i the number of sites with an intrinsic binding constant k_i and $[D]$ the molar concentration of the free dye.

The data were fitted for two classes of binding sites. No improvement could be achieved by fitting the data for a model with three categories of binding sites. The experimental points with molar ratios dye/bovine serum albumin lower than 0.5 yielded a poor fitting for BSP and ThB and were not taken into account for calculation of K_D and n. Such deviations are probably due to cooperative binding, as reported by others [7, 8]. The fitting of ICG showed a single class of binding sites.

EXPERIMENTAL PROCEDURE

The experimental application of the theory was carried out slightly differently in the two laboratories. In Groningen protein concentration was kept constant (0.05% bovine serum albumin) and the spectra compared in the presence of variable concentrations of the ligand. With BSP, DBSP and ThB, experiments were performed at pH 9.0 in the presence of 0.08 M Tris–HCl buffer, in 0.9% NaCl. ICG binding was followed under identical experimental conditions except for the buffer which was 0.08 M sodium phosphate (pH 7.4). Before the spectrophotometric reading, samples were kept at room temperature for 30 min. Routinely, the pH of the single solutions was checked. In contrast, the experimental approach in Trieste was based on the use of a constant dye concentration to which increasing amounts of binding proteins (bovine serum albumin, human serum, rat liver bilitranslocase, rat liver cytosol) were added. The protein was added in exactly equal amounts to the sample and the blank and the spectrum recorded immediately after. All the experiments were run at pH 7.8 in the presence of 200 mM sodium phosphate buffer, 1 mM EDTA. All spectra were automatically corrected for dilution.

Ultrafiltration was used as an alternative method to determine the free dye concentration. Assay mixtures identical to those used for the spectrophotometric determination were pipetted into Visking dialysis tubings. The dialysis bags were located into centrifuge tubes and spun down at 600 g for 60 min. The ultradialysate was assayed spectrophotometrically after alkalinisation with four volumes 0.1 N NaOH. All the experiments were carried out at room temperature.

RESULTS

Figure 1 shows the results of four experiments in which the four dyes under investigation were analyzed spectrophotometrically both in the presence and in the absence of bovine serum albumin. It is clear that, upon addition of the protein, a distinct change in the spectrum ensues which, in the case of sulfophthaleins (BSP, DBSP and ThB) corresponds to a marked decrease in the absorption maximum. On the contrary, with ICG the spectrum indicates an increase in the region at a wavelength higher than that of the maximal absorbance. This increase is accompanied by a decreased absorption at lower wavelengths, the maximum of the differential spectrum occurring at 810 nm.

Table 1 summarizes the results obtained when the four dyes bind to bovine serum albumin in solution. The table reports the values of the extinction coefficients of free and bound forms of the dyes, the appropriate wavelength, and the pH value of the experiments. In this regard, it should be emphasized that the pH of the solution is carefully maintained at a given value by an appropriate buffer system. It is necessary also to check that the addition of the protein is without any effect on this parameter. In addition, the number of high affinity binding sites obtained in this study and the corresponding dissociation constant of the complex of albumin with the different organic anions is listed. Both parameters are in good agreement with previous reported data [4, 5]. A direct comparison of the binding of three of the four dyes has been carried out in parallel with an ultra-filtration technique. The results of these experiments were nicely correlated with those obtained spectrophotometrically (BSP: $r = 0.95$;

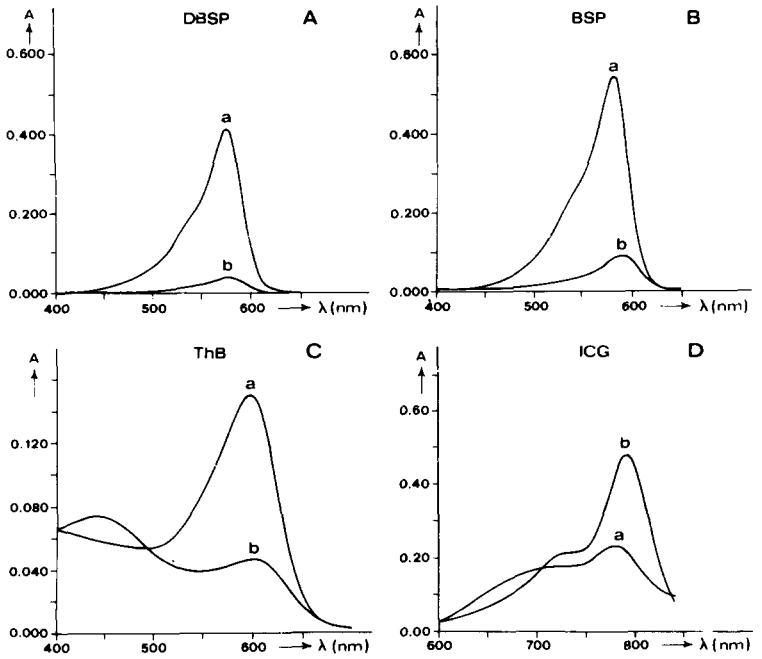

Fig. 1. Spectral effects of bovine serum albumin on different dyes. A: 9.6 μM DBSP at pH 9.0 before (curve a) and after (curve b) addition of 20 μM BSA; B: 10.1 μM BSP at pH 9.0 before (curve a) and after (curve b) 20 μM BSA; C: 10 μM ThB at pH 9.0 before (curve a) and after (curve b) addition of 20 μM BSA; D: 4.5 μM ICG at pH 7.4 before and after addition of 7.1 μM BSA.

DBSP: $r = 0.98$; ThB: $r = 0.96$, $P < 0.001$) (see Table 2).

Figure 2 shows the Scatchard plot [9] of the binding of the four dyes to bovine serum albumin. The measured values are represented by circles, the indicated curves are calculated on the basis of the binding constants and number of binding sites obtained from the computer fit.

The second type of approach, namely to maintain the dye concentration constant while progressively increasing the protein concentration, is exemplified in Fig. 3. The figure shows (upper curve) the visible spectrum of BSP in a strongly buffered system at pH 7.8. At this pH, only a fraction of BSP molecules is dissociated so that the extinction coefficient is 5.86 μmoles^{-1} × cm^2 as compared to 64 μmoles^{-1} × cm^2 at pH 13. With increasing amounts of bovine serum albumin (lower curves), the absorption spectrum is progressively depressed to almost a flat line at the highest albumin concentration. This indicates that, when bound to albumin at pH 7.8, BSP is virtually colorless. Obviously, the decrease in absorbance at 580 nm may be taken as a measure of the complex between albumin and BSP. The concentration of this complex may be quantified provided that one knows its extinction coefficient. This value may be derived from the experimental data by extrapolation to infinite protein concentration. This can be done, as shown in Fig. 4, by a double reciprocal plot of absorbance vs protein added. The intercept on the ordinate will represent the reciprocal of the absorbance when all the BSP is bound to albumin. This value is then subtracted from the original absorbance in the absence of protein and the difference divided by the BSP concentration in the system.

Figure 5 shows the Scatchard plot obtained with the data reported in Fig. 4. From this experiment, a K_D of 1.8 ± 0.2 μM (mean ± S.D.) has been calculated for the complex. The maximal binding

Table 1. Some parameters of bovine serum albumin binding to various dyes. ε_f and ε_b are defined as extinction coefficients of the free and bound forms of the dye respectively

	ε_f (μmoles^{-1} × cm^2)	ε_b (μmoles^{-1} × cm^2)	λ (nm)	pH	n (moles/mole)	K_D (μM)
DBSP	42.2	0.01	580	9.0	1.5	1.9
BSP	54.2	0.1	580	9.0	2.8	1.2
ThB	14.3	3.7	600	9.0	1.5	1.9
ICG	36.4	116.0	810	7.4	1.4	3.0

Table 2. Comparison of free concentrations (μmole/l) found by ultrafiltration (a) and by the spectrophotometric method (b)

DBSP		BSP		ThB	
a	b	a	b	a	b
0.26	0.30	0.41	0.31	1.1	1.9
1.9	1.7	0.62	0.64	3.8	3.4
4.6	5.0	1.1	1.1	7.0	6.0
8.5	9.6	2.0	2.2	8.7	8.3
14.5	15.4	7.5	7.8	12.4	11.6
18.3	20.9	9.5	11.2	17.5	15.4
35.1	36.3	20.2	22.1	47.4	45.6

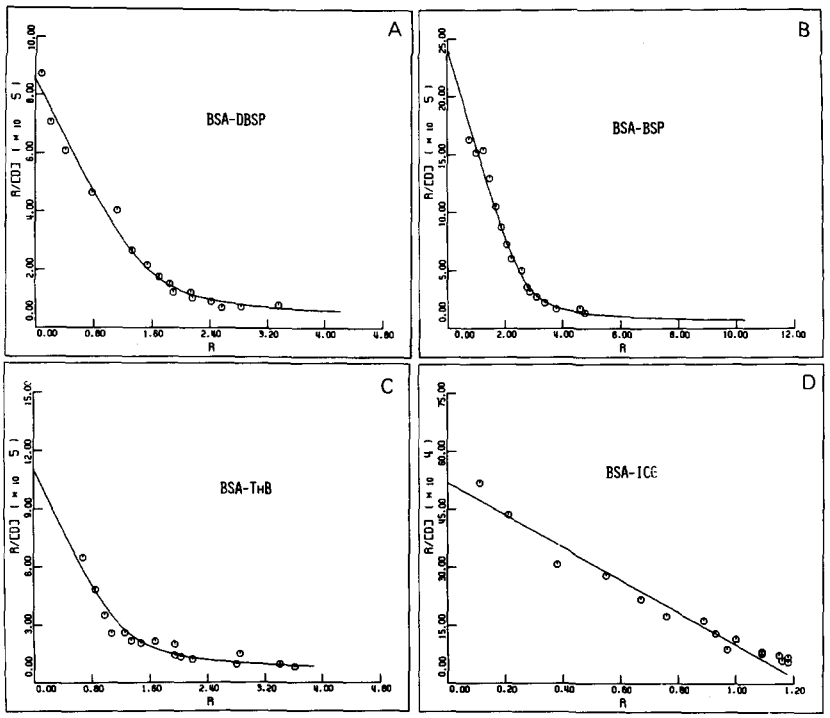

Fig. 2. Scatchard plot of the complex between bovine serum albumin and the different dyes. Experimental conditions as in the previous figure. For the calculation, see text. R = moles of dye bound per mole of protein: D = molar free dye concentration.

capacity is of the order of 75.8 ± 11.0 nmoles/mg protein, corresponding to approximately 5 moles BSP bound per mole albumin. These data are higher than those obtained at pH 9.0 (see Table 1). One of the possible interpretations of this discrepancy may be that at pH 7.8 additional binding sites on albumin molecules are measured.

This technique has been extended to a number of biological samples including human serum, rat liver cytosol and bilitranslocase preparations at different levels of purification [3]. Table 3 summarizes the results. It is interesting to note that the differential extinction coefficient of the complex is essentially the same with albumin and human serum. On the contrary, it is remarkably different with rat liver supernatant and, even more with bilitranslocase. The data indicate that the various complexes absorb differently at 580 nm.

DISCUSSION

The possibility to measure the binding of colored substances to macromolecules has been previously reported [1] and applied [11, 12]. Data presented here indicate that this approach may be applied to the study of BSP, DBSP, ThB and ICG to different

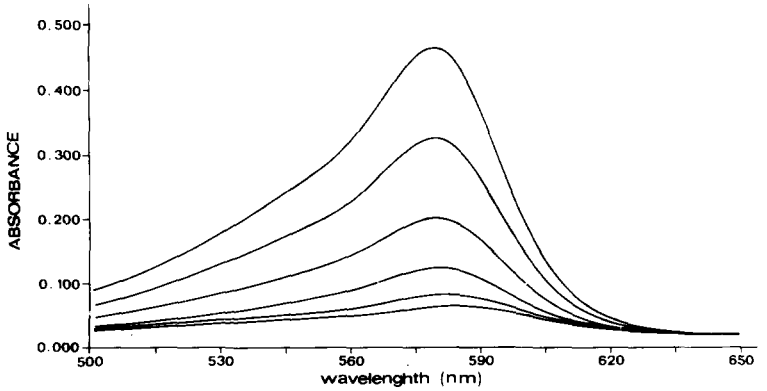

Fig. 3. Effect of albumin addition on the spectral properties of BSP at pH 7.8. Experimental conditions: upper curve 200 mM phosphate buffer, 1 mM EDTA, 75.2 μM BSP; lower curve after addition of aliquotes of 1 mg BSA.

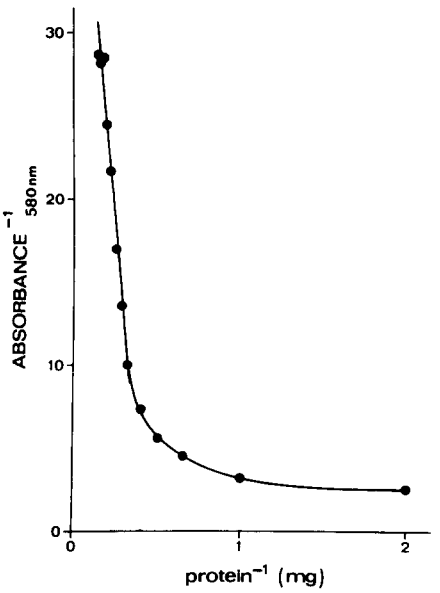

Fig. 4. Double reciprocal plot of change in absorbance vs BSA added. Experimental conditions as in the previous figures.

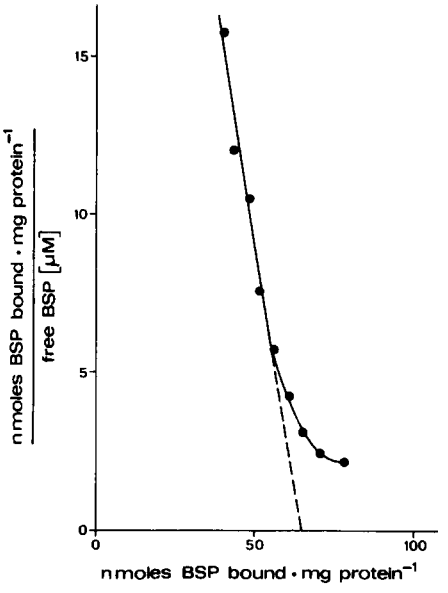

Fig. 5. Scatchard plot of BSP binding to BSA at pH 7.8. Experimental conditions as in the previous figures.

proteins and may be of general use whenever a colored compound changes its spectrum upon complexation with a macromolecule. One of the advantages over other techniques is the ability to differentiate rapidly whether or not a certain protein preparation binds a certain colored ligand. This is a particularly useful attribute in the screening of a series of biological samples such as for instance the fractions collected during chromatography. For a more accurate quantitative determination, however, it is necessary to calculate the differential extinction coefficient for each protein preparation, especially if more than one binding protein is likely to be present in the fraction. In the case of BSP and other dyes sensitive to pH changes, it is essential to determine that the spectral changes are not due to pH variations. This latter aspect can be overcome if a strong buffer system and appropriate blanks are included. Concentrated protein solutions with limited buffering capacity are to be preferred and, in addition, the pH of the protein solution should be as close as possible to the pH of the buffer. For DBSP, BSP and ThB a major shift in the spectrum (decrease in absorption) was seen upon addition of a binding protein. In contrast, when protein was added to ICG (pH 7.4) an increase in absorbance appeared. These spectral shifts can in principle be due to a change in dissociation of the dye or to a redistribution of charges within the dye molecule due to an interaction with the protein. Changes in absorption spectra have also been observed when protein disturbs the polymer–monomer equilibrium of a dye [1, 10]. With ICG, several aggregates are present in aqueous solution but the contribution of a shift in the polymer–monomer equilibrium to the change in absorbance is negligible under the conditions employed, however, in view of the low concentration used. When BSP and DBSP are bound to serum albumin, the absorption is reduced to zero, indicating that BSP and DBSP are bound to protein in such a way that they cannot be transformed into a quinoid form. This probably means that the phenolic OH groups play an important role in the binding. This effect can also be seen in the case of ThB. After binding to protein at pH 9.0, the maximum in the spectrum is shifted from 600 to 440 nm. ThB normally has a maximum at 440 nm when the pH is between 2.8 and 8.0. In this range the phenolic hydroxyl group is protonated. For these three dyes the greatest shift in spectrum occurred when the pH was near to the pK_a of the hydroxyl group. We found no significant shift in the spectrum when the pH

Table 3. Binding parameters of BSP by different protein preparations at pH 7.8. Data refer to mean ± S.D. In parentheses the number of experiments

	Number of experiments	K_D (μM)	n (nmoles/mg)	$(\varepsilon_f-\varepsilon_b)580$ nm (μmoles^{-1} × cm^2)
Bovine serum albumin	(6)	1.8 ± 0.2	75.8 ± 11.4	5.55 ± 0.05
Human serum	(6)	1.2 ± 0.3	40.5 ± 13.4	5.66 ± 0.01
Rat liver cytosol	(6)	2.2 ± 0.8	13.0 ± 7.7	5.59 ± 0.63
Bilitranslocase	(10)	1.4 ± 0.4	26.6 ± 4.7	2.69 ± 1.16

deviated more than 2 units from the pK_a of the hydroxyl group. Because the unbound ICG forms aggregates at relative high concentrations and it is unable to pass dialysis membranes, no ultrafiltration or dialysis studies for this dye can be performed. Consequently, the procedure described in this study, in addition to elaborative procedures such as probe-displacement methods or gel-filtration, is one of the very few alternatives for the determination of protein binding of ICG. The possibility exists that the technique can have wider application. The fact that colorless ligands can be indirectly shown to bind to a protein previously bound to a colored substance, suggests that the colored anion may be utilized as a probe for the binding of other colorless substances.

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