Principles of a competitive binding assay for the study of the interactions of poorly water-soluble ligands with their soluble binding partners

Application to bilirubin with the use of Sephadex G-10 as a competitive adsorbent

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1. A generally applicable method is described for obtaining experimental data on the interactions between a poorly water-soluble ligand and soluble binding factors, with the use of chemically inert solid adsorbent. The equilibrium distribution of the ligand between the liquid phase containing the soluble binders and the adsorbent must be measured and knowledge of the binding isotherm of the adsorbent is required. Procedures are given for the calculation of the binding parameters. 2. The method has been applied to quantify the interactions of bilirubin with serum and liver cytosol from the rat, Sephadex G-10 serving as the competing adsorbent. Reversible adsorption keeps the concentration of the free ligand low, thereby preventing formation of colloidal bilirubin. The sensitivity of the procedure accommodates the rather high binding affinities by which bilirubin generally interacts with its specific binding proteins. 3. The binding activities of serum and liver cytosol are of comparable magnitude. Binding of bilirubin by rat serum can be described by two independent binding sites, the affinities of which differ by two orders of magnitude. Only the site with the higher affinity appears to be of physiological importance. The major bilirubin-binding sites of rat liver cytosol seem to contribute equally to the overall binding activity of this preparation, provided that GSH is present.

INTRODUCTION

Quantitative data on the interactions of bilirubin, the major product of haem catabolism, with its physiological binding proteins are difficult to obtain. The rather high association constants make it necessary to measure small concentrations of unbound bilirubin in the presence of excess bound pigment. Furthermore, the solubility of bilirubin in water is very low, probably below 7 nM at pH 7.4 (Brodersen, 1979, 1980). When the solubility of bilirubin is exceeded, the pigment tends to form a colloidal suspension (Brodersen & Theilgaard, 1969; Brodersen *et al.*, 1972). Besides physical polymorphism, chemical and photochemical instability of bilirubin are other interfering factors (McDonagh, 1979). Finally, bilirubin freely dissolved in aqueous solution shows great tendency to adsorb on many interfaces, thus excluding the use of, e.g., equilibrium dialysis.

To study the interactions of bilirubin with binding proteins several spectral methods have been employed (for reviews see McDonagh, 1979; Brodersen, 1982; Meuwissen, 1984). All these methods suffer from insensitivity when free bilirubin concentrations below the dissociation constant of the complex must be determined. In a more sensitive procedure the enzymic breakdown of unbound bilirubin by horseradish peroxidase is monitored as a function of time (Jacobsen, 1969; Jacobsen & Wennberg, 1974; Brodersen, 1974). The method can be

applied to human albumin, but not to rat albumin, because in the latter case protein-bound bilirubin is oxidized (Frandsen & Brodersen, 1986). The value of the peroxidase method for assays on serum and plasma has been questioned (Brodersen *et al.*, 1979).

In the present paper we describe a new method for the quantitative determination of the association of bilirubin with binding protein, in which the above-mentioned problems are avoided.

EXPERIMENTAL

Animals

Male adult Wistar rats weighing between 250 and 300 g were used. They were kept on a standard diet and had free access to water. The animals were kept without food overnight before they were killed.

Materials

Sephadex G-10 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Before use, Sephadex G-10 was sieved on a 90 μ m-pore-size sieve (Endecotts Test Sieves, London, U.K.) to remove fines. Bilirubin (p.a.) was from E. Merck (Darmstadt, Germany). Bovine albumin (fraction V) obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), rat albumin (fraction V) from Miles Laboratories (Kankakee, IL,

Abbreviation used: in agreement with the Joint Commission of IUPAC and IUB, bilirubin IX α (4Z,15Z) is denoted as bilirubin.

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U.S.A.) and freeze-dried specific anti-(rat albumin) serum from Nordic Immunology (Tilburg, The Netherlands) were used as such. All other chemicals were of reagent grade.

General methods

Bilirubin was purified as described by Fog (1964) and stored in the dark *in vacuo* over P_2O_5 . All manipulations involving the light-sensitive pigment were carried out with protection from direct light. Bilirubin concentration, in the form of azo derivatives, was measured at 546 nm with a mercury-lamp photometer (Netheler und Hintz, Eppendorf, Germany). The azo pigments were prepared by using the *p*-iodoaniline method of Van Roy *et al.* (1971), as modified by Mertens *et al.* (1972). The sensitivity of the method was enhanced by increasing sample volume and diazo reagent volume from 0.5 to 1 ml. The volume of the organic extractant *n*-butyl acetate was increased from 2 to 2.6 ml, so that a 4 cm semi-micro cuvette could be filled adequately. Concentration of bilirubin was calculated from c (μM) = $A_{546}^{1\text{ cm}} \times 13.85$. Absorption spectra were obtained with an SP8-250 spectrophotometer (Pye-Unicam, Cambridge, U.K.).

Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Albumin concentration in rat serum was determined by radial immunodiffusion (Mancini *et al.*, 1965), with the use of rat serum albumin for calibration. In most experiments the buffer used was KH_2PO_4 / Na_2HPO_4 (1:1), pH 7.4, containing 1 mM- Na_2EDTA , and is referred to as standard phosphate buffer.

All procedures and experiments were performed at 4 °C, except when stated otherwise.

Preparation of biological samples

To prepare liver cytosol, a rat was anaesthetized with diethyl ether and killed by decapitation. The liver was freed from blood by perfusion *in situ* with ice-cold sucrose medium (0.25 M-sucrose containing 1 mM- Na_2EDTA), excised and homogenized at 0 °C in sucrose medium (1:1, w/v) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100 000 g_{av} for 60 min in an R-40 angle rotor in an L2-65B preparative ultracentrifuge (Beckman Instruments, Spinco Division, Palo Alto, CA, U.S.A.). The supernatant, referred to below as the cytosol, was collected by lateral puncture of the clamped centrifuge tube to avoid contamination by floating lipid, and used immediately. Blood was obtained by puncture of the abdominal aorta, and the prepared serum was either used immediately or stored at -20 °C.

Preparation of bilirubin-Sephadex complex

Purified bilirubin (20 mg) was dissolved in approx. 1.5 ml of 100 mM- $NaOH$ and diluted to 400 ml with glycine/ $NaOH$ buffer (1:1), pH 9.2. Without delay, 100 g of dry Sephadex G-10 was added under constant stirring and the suspension further mixed for 15 min. After decantation, the yellow-coloured Sephadex was stirred for 45 min in 300 ml of Tris/ HCl buffer (1:1), pH 8.0, followed by five similar washings with 300 ml of a solution containing equal volumes of standard phosphate buffer and distilled water. The yellow slurry was freeze-dried in Petri dishes covered with Parafilm provided with small perforations. Portions (5 g) of the final product were transferred to small brown glass vials

with a perforated cap and stored *in vacuo* over P_2O_5 in the dark. The dry yellow powder is referred to as bilirubin-Sephadex complex. For control purposes and for use in the binding assay, Sephadex G-10 was treated identically except for omission of bilirubin (bilirubin-free Sephadex).

Specific bilirubin content of bilirubin-Sephadex complex

Portions (300.0 mg) of bilirubin-Sephadex complex were weighed into at least six 12.5 ml glass test tubes (internal diameter 1.20–1.25 cm) with a ground-glass joint. After addition of 3 ml of a solution of bovine albumin (10 mg/ml) in standard phosphate buffer, the tubes were stoppered and placed in the horizontal position in a roller drum (Bellco Glass, Vineland, NJ, U.S.A.) rotating in the vertical plane at 20 rev./min. After overnight equilibration (approx. 16 h), the test tubes were centrifuged for 2 min at 450 g and the bilirubin concentration of the supernatants was determined. At an albumin concentration of 10 mg/ml virtually all the pigment becomes dissolved, permitting the amount of bilirubin originally adsorbed to the matrix to be calculated from:

$$y_0 = c_t \cdot v^* / m \quad (1)$$

where y_0 is the specific bilirubin content (mol/g) of the bilirubin-Sephadex G-10 complex as prepared, c_t the total bilirubin concentration (mol/l) in the liquid phase, m the mass (g) of the adsorbent and v^* the volume (l) of the liquid phase corrected for the water regain of Sephadex G-10. This correction, which amounts to about 10% for Sephadex G-10 [$v^* = v/1.1$, with v the volume (l) of the added liquid], is applied because protein-bound pigment does not penetrate the inner volume of the gel.

Detection of colloidal bilirubin

Colloidal bilirubin is characterized by a spectral shoulder around 500 nm (Brodersen & Theilgaard, 1969). The appearance of the shoulder and hence the presence of colloidal pigment can be monitored sensitively by first-derivative spectrophotometry, giving a biphasic negative peak with minima at 460 and 510 nm. A monophasic negative peak is observed (minimum at 460 nm) when bilirubin is genuinely dissolved or is present as a supersaturated solution.

Quality control of bilirubin-Sephadex complex

The hygroscopic bilirubin-Sephadex complex must be kept rigorously dry during storage, otherwise spurious results are obtained. When 100 mg of bilirubin-Sephadex complex that is not completely dry is extracted with 1 ml of chloroform, a yellow liquid phase ($A_{450}^{1\text{ cm}}$ approx. 0.8) is observed, this being due to the presence in the complex of an acid form of bilirubin, probably colloidal bilirubin (Brodersen & Theilgaard, 1969). The extraction test performed on the dry complex yields $A_{450}^{1\text{ cm}}$ less than 0.015. A specific bilirubin content of the complex of 0.3 $\mu\text{mol/g}$ is advocated. Larger contents easily lead to colloid formation.

Characterization of the adsorption of bilirubin by Sephadex G-10

The adsorption isotherm of bilirubin for Sephadex G-10 was established by incubating bilirubin-Sephadex complex of various specific bilirubin contents with standard phosphate buffer. Separate test tubes were

Fig. 1. Adsorption isotherm of Sephadex G-10 for bilirubin

Bilirubin–Sephadex G-10 complex in standard phosphate buffer was used. The free bilirubin concentration c_t (ordinate) is a linear function of the specific ligand content of the adsorbent y at distribution equilibrium (abscissa), which is calculated from eqn. (6). The Freundlich constant k_F is given by the inverse of the slope of the linear regression line. Each point is the average of at least three replicate incubations.

provided with different amounts of bilirubin–Sephadex (0, 10, ..., 100 % of the total amount) and adjusted to 300 mg with complementary amounts of bilirubin-free Sephadex, so that each test tube had the same amount of adsorbent, but a different specific bilirubin content. After the addition of 3 ml of standard phosphate buffer, the mixtures were incubated for 2 min by rotation on the roller drum, then centrifuged, and the bilirubin concentration in each supernatant was determined and an adsorption isotherm constructed (Fig. 1).

Protocol of the binding assay

Binding isotherms of soluble binders were obtained by allowing the exchange of bilirubin to occur at different amounts of ligand for a fixed concentration of binding factors and a constant amount of adsorbent, according to a procedure that is essentially the same as that used to establish the adsorption isotherm. Routinely, incubation was overnight (approx. 16 h).

Calculations

When a compound in solution is adsorbed reversibly by a solid, the equilibrium interaction can be expressed by the Freundlich adsorption isotherm (Glasstone, 1962):

$$y = k_F \cdot c_t^n \quad (2)$$

where the specific ligand content at equilibrium y is defined as the amount of ligand adsorbed per unit mass of adsorbent (mol/g), c_t is the concentration of unbound ligand (mol/l), k_F is the Freundlich constant (l/g) and n a dimensionless empirical parameter. In the concentration range used in this work, the adsorption of bilirubin on Sephadex G-10 is linear (Fig. 1) and n thus equals 1. From the mass balance for the liquid phase, $c_t = c_b + c_r$, follows:

$$y = k_F \cdot (c_t - c_b) \quad (3)$$

where c_t is the total and c_b the bound ligand concentration (mol/l). A second expression of y is derived from the mass balance for the system as a whole, at equilibrium:

$$y = (A - c_t \cdot v^*)/m \quad (4)$$

where A is the total amount (mol) of ligand, which is calculated from a third mass balance describing the system before exchange has taken place:

$$A = f \cdot y_0 \cdot m + c_0 \cdot v \quad (5)$$

where y_0 is the specific ligand content (mol/g) of the ligand–adsorbent complex as prepared and c_0 the concentration (mol/l) of ligand in the applied sample; f is the ratio of the mass of ligand–adsorbent complex to the total mass of adsorbent and thus varies between 0 and 1. Incorporation of the latter equation into eqn. (4) yields:

$$y = (f \cdot y_0 \cdot m + c_0 \cdot v - c_t \cdot v^*)/m \quad (6)$$

Equating eqns. (3) and (6) leads to:

$$c_b = [c_t(m \cdot k_F + v^*) - (f \cdot y_0 \cdot m + c_0 \cdot v)]/k_F \cdot m \quad (7)$$

Alternatively, the free concentration can be calculated as:

$$c_t = (f \cdot y_0 \cdot m + c_0 \cdot v - c_t \cdot v^*)/k_F \cdot m \quad (8)$$

Eqn. (7) or (8) is used to calculate either c_b or c_t as a function of the value of c_t obtained from the experiment. The other value is calculated from $c_t = c_b + c_r$.

The equation used for fitting is the generalized binding equation (Edsall & Wyman, 1958):

$$c_b = \sum_i Bc_i \cdot K_i \cdot c_t / (1 + K_i \cdot c_t) \quad (9)$$

where Bc_i is the binding capacity and K_i the association constant of the binding sites of class i . The fitting procedure involves non-linear least-squares fitting by using the Marquardt algorithm, essentially in accordance with Bevington (1969).

For simulation purposes and for calculation of the best approximations to the experimental data, the following equations were used; for a binding factor with a single binding site:

$$K_i \cdot (Bc - c_b) \cdot c_t - c_b = 0 \quad (10)$$

and for two independent classes of binding site ($i = 1, 2$):

$$K_1 \cdot K_2 \cdot [(Bc_1 + Bc_2) - c_b] \cdot c_t^2 + [K_1 \cdot (Bc_1 - c_b) + K_2 \cdot (Bc_2 - c_b)] \cdot c_t - c_b = 0 \quad (11)$$

These equations were solved for c_t by using the Newton–Raphson algorithm (Stark, 1970) after introduction a suitable range of values of c_b . A Scatchard-type graph was obtained by plotting (c_b/c_t) as a function of c_b .

Data analysis, non-linear least-squares fitting and simulations were performed with a Hewlett–Packard HP1000F RTE-IVB minicomputer system with graphic capabilities.

RESULTS

Qualitative observations

Solutions that contain binding activity, such as serum, liver cytosol and albumin, did solubilize Sephadex-adsorbed bilirubin. In contrast, solutions of non-binding proteins such as lysozyme and β -lactoglobulin had no

Fig. 2. Principle of the competitive binding assay: simulation of the 'experimental' binding curve

The ordinate of the 'experimental' curve (—) represents the total ligand concentration c_t in the liquid phase at distribution equilibrium with the solid adsorbent phase, plotted as a function of the total amount A of ligand in the binding system (abscissa). Both quantities are in fractional units. The upper limiting line (----) is reached when sufficient binding activity is present in the liquid phase to keep all the ligand soluble. The dot-dashed line (---) represents an upper limit of the free concentration that can only be reached when soluble binding factors are absent and thus represents the binding isotherm of the adsorbent (not to scale). At equilibrium, the adsorbed (y) and soluble portions of the ligand (c_t) are related by simple equations to the Freundlich constant (k_F), the free ligand concentration (c_f) and bound ligand concentration (c_b).

such effect. Apparent solubilization of bilirubin by distilled water could be traced to the formation of colloidal bilirubin, as shown by first-derivative spectrophotometry. In standard phosphate buffer, in the absence of binding protein, supersaturated bilirubin solutions were observed after short incubation (1–5 min), longer incubation periods yielding colloidal suspensions. Solid bilirubin was not dissolved by binding protein.

Principle of the binding assay

From the equilibrium distribution of a ligand between a solid adsorbent and a binder in solution, binding parameters of the soluble binding factor can be calculated provided that the adsorption isotherm of the adsorbent for the ligand has been established. Having obtained the unbound equilibrium concentration c_f from the Freundlich isotherm and the bound ligand concentration c_b , in the assay, from $c_b = c_t - c_f$, the (c_t , c_b) data pairs are used to calculate the binding parameters of the binding protein in the soluble phase. In the particular case of bilirubin it was found advantageous and even necessary to adsorb the pigment on Sephadex G-10 before incubation with binding protein, since addition of a concentrated bilirubinate solution to an aqueous system involves the risk of colloid formation.

Simulation of 'experimental' binding curves

With the use of eqn. (10), simulations were performed to gain a qualitative understanding of the results to be

Fig. 3. Simulation of 'experimental' binding curves at fixed binding affinity

The binding affinity parameter was kept constant ($K = 10^8 \text{ M}^{-1}$) and the binding capacity Bc was varied. Bc : curve 1, $5 \mu\text{M}$; curve 2, $10 \mu\text{M}$; curve 3, $20 \mu\text{M}$; curve 4, $50 \mu\text{M}$; curve 5, $100 \mu\text{M}$. Maximum specific ligand content y_0 , $0.350 \mu\text{mol/g}$, was set to 1; k_F is 0.66 l/g .

expected with the experimental protocol described above. The general form of the binding curve is shown in Fig. 2, where the total ligand concentration in the liquid phase c_t is plotted as a function of the total ligand content A . This type of graph is referred to as the 'experimental' curve. When the binding capability of the liquid phase largely exceeds that of the adsorbent, virtually all the ligand is confined to the liquid phase (Fig. 2, upper limiting line). The difference between the 'experimental' curve and the upper limiting line is a measure of the amount of ligand that is associated with the solid phase.

In Fig. 3, a family of curves is shown that were obtained with the concentration of the soluble binding factors variable and the association constant fixed. At low values of the binding capacity, the experimental curve clears the upper limiting line at relatively low ligand content and tends to a plateau value with increasing amount of ligand (Fig. 3, curve 1). This is so because, for the greater part of the curve, there is an excess of available ligand with respect to the concentration of soluble binding sites. With the binding capacity increasing, the region of near-saturation is progressively displaced to the right and eventually disappears from the experimental window. Concurrently, the experimental curve approaches the upper limiting line more closely (Fig. 3, curve 5). If one is primarily interested in the binding capacity one thus should select the amount of soluble binding sites well within the ligand content of the assay, so that near-saturation of the binding sites can be attained. On the other hand, if one is primarily interested in the binding affinity, it is advantageous to perform the assay at higher values of the binding capacity, so that the ligand concentration c_t present in the aqueous phase changes appreciably over the range covered by the amount of ligand A . Here a balance must be struck, since too high a binder concentration will result in an 'experimental' curve too

Fig. 4. Simulation of 'experimental' binding curves at fixed binding capacity

The binding capacity parameter was kept constant ($Bc = 10 \mu\text{M}$) and the affinity constant K was varied. K : curve 1, 10^5 M^{-1} ; curve 2, 10^6 M^{-1} ; curve 3, 10^7 M^{-1} ; curve 4, 10^8 M^{-1} ; curve 5, 10^9 M^{-1} . Maximum specific ligand content y_0 , $0.350 \mu\text{mol/g}$, was set to 1; k_F is 0.66 l/g .

close to the upper limiting line. The specific ligand content y of the adsorbent at equilibrium then tends to zero, the data thus becoming less accurate.

In Fig. 4, the binding capacity was fixed and the affinity parameter K varied in consecutive steps of one order of magnitude. The conditions were chosen such that the maximum amount of available ligand exceeded the binding capacity. At low K values, the soluble binding sites are too weak to compete successfully with the adsorbent and only a low concentration of ligand is achieved in the soluble phase (Fig. 4, curve 1). The curve almost coincides with the binding isotherm of the adsorbent and carries little information with regard to the soluble binders. At the other extreme, their affinity is so high that virtually all the ligand is confined to the aqueous phase when the amount of ligand available is below the binding capacity of the soluble phase (Fig. 4, curve 5, left-hand part). When the amount of soluble binding sites is less than the amount of ligand available, the total ligand concentration c_t increases with the amount of ligand up to a value that corresponds to near-saturation of the binding sites. Beyond this point only the free concentration further increases on top of the saturating concentration of ligand (Fig. 4, curve 5, right-hand part). At these high affinity values the 'experimental' curve resembles a titration curve, delineating the binding capacity of the liquid phase, but lacks information about the binding affinity. Thus the information content of the 'experimental' binding curves becomes limited both at low and high affinity values, relative to the binding strength of the adsorbent. Between these limits there is a region where the sensitivity of the binding assay is at maximum. Fig. 5 provides an estimate of the relative sensitivity of the method for different values of the binding capacity Bc . With Sephadex G-10 as the adsorbent, the method has a useful range of several orders of magnitude in terms of the association parameter K (10^5 M^{-1} to 10^9 M^{-1}).

Fig. 5. Sensitivity of the competitive binding assay

Curves such as are depicted in Fig. 3 were generated for $\log K$ values between 4.5 and 9.5, changed sequentially by a factor $\frac{1}{3}$. At the maximum curvature of the individual curves, the differences Δc_t between two consecutive curves, relative to the binding capacity Bc , were plotted as a function of $\log K$. The data sets so obtained were approximated by polynomial multiple regression (Bevington, 1969). Simulations were for binding capacities Bc of 1, 15 and $30 \mu\text{M}$, which span the range of practical applicability of the present method.

Characterization of the adsorption of bilirubin by Sephadex G-10

Adsorption is linear with the specific bilirubin content at equilibrium at least up to $0.3 \mu\text{mol/g}$ and is represented by the adsorption isotherm $y = k_F \cdot c_t$ with the Freundlich constant k_F equal to 0.632 ± 0.0217 (S.E.M.) l/g in standard phosphate buffer (Fig. 1). Linear adsorption isotherms were obtained at several temperatures between 5°C and 37°C . Within this range, the dependence of the Freundlich constant k_F on temperature t ($^\circ\text{C}$) is given by $k_F = k_{F(5^\circ\text{C})} + (\Delta k_F / \Delta t) \cdot t$ with $k_{F(5^\circ\text{C})} = 0.626 \pm 0.0185$ (S.E.M.) and $(\Delta k_F / \Delta t) = -8.0 \times 10^{-3} \pm 0.84 \times 10^{-3}$ (S.E.M.).

Equilibration times

With binding protein present, the incubation time was routinely overnight, which is largely sufficient for bilirubin and its binding proteins to reach equilibrium distribution (Fig. 6). With liver cytosol, equilibrium was attained in about 2 h, whereas serum required at least 7–8 h. The extents of binding did not change during overnight incubation, indicating that, in these conditions, bilirubin and its binding proteins remain stable. The data for serum are best approximated by a sum of two exponentials (Fig. 6b). In contrast, solubilization of bilirubin by cytosol can be described by a single exponential (Fig. 6a).

Fig. 6. Rates of desorption of bilirubin from bilirubin-Sephadex G-10 complex

(a) Time course of solubilization of bilirubin from bilirubin-Sephadex G-10 complex by preparations containing specific bilirubin-binding proteins. Separate test tubes were used for each time point and the assays were performed in duplicate. ●, Rat serum, diluted 1:80 (v/v) with standard phosphate buffer, pH 7.4; ▲, cytosol from the same rat, diluted 1:4 (v/v) with distilled water, pH 6.8. (b) Deconvolution of the fitted curve for serum (A) into two exponential curves (B) and (C).

Examples of application

To illustrate the procedures, a selection of representative experiments is given. Binding data for rat serum are presented in Table 1. Fig. 7 shows 'experimental' binding curves for serum and liver cytosol obtained from the same rat. The effect of GSH on the binding of bilirubin to rat liver cytosol is shown in Fig. 8. We have experimental evidence that the method can also work with the conjugated bilirubins and with haem, with Sephadex G-10 as the adsorbent (results not shown).

DISCUSSION

Aromatic compounds such as porphyrins, haem and bilirubin adsorb onto cross-linked dextrans such as Sephadex (Gelotte, 1960; Determann & Walter, 1968; Porath, 1968; Meuwissen & Heirwegh, 1970). The degree of adsorption of bilirubin decreases with decreasing cross-linking of Sephadex (results not shown), supporting the view that the cross-linking alkyl ether bridges are involved in the binding (Determann & Walter, 1968). The present binding assay is based on this adsorptive property. The assumptions that Sephadex G-10 is inert

Table 1. Bilirubin-binding parameters of rat serum

Rat serum was diluted 1:80 (v/v) with standard phosphate buffer. The binding isotherm was obtained and analysed as outlined in the Experimental section. The number of binding sites n_i was obtained by dividing the value of the binding capacity by the concentration of albumin, for which an M_r of 64000 was assumed (Sober, 1970). In the fitting procedure the binding capacities were constrained (i.e. $Bc_1 = Bc_2$), after it was found that, in the unconstrained condition, individual values of Bc_i of comparable magnitude were obtained. Standard errors were obtained from non-linear least-squares analysis.

n_1	$10^{-6} \times K_1$ (M^{-1})	n_2	$10^{-6} \times K_2$ (M^{-1})
1.10 ± 0.031	72.2 ± 2.64	1.10 ± 0.031	0.74 ± 0.157

Fig. 7. Bilirubin binding by serum and by liver cytosol from the rat

Serum and liver cytosol, obtained from the same rat, diluted with standard phosphate buffer, pH 7.4 (serum, 1:40 v/v), and with phosphate buffer, pH 7.0 (1:0.008) (cytosol, 1:1 v/v), were incubated for 16 h with bilirubin-Sephadex G-10 complex and analysed, as outlined in the Experimental section. The total bilirubin concentration c_t in the liquid phase at distribution equilibrium is plotted as a function of the total amount of ligand A present in the assay system ('experimental' plot). $y_0 = 0.200 \mu\text{mol/g}$. ○, Serum; ●, cytosol; —, lines of best fit, obtained by non-linear least-squares fitting; ----, limiting curve.

towards binding protein and that the presence of protein does not influence the adsorption isotherm of Sephadex G-10 for bilirubin appear reasonable.

Colloid formation presents a major problem in all experimental work with aqueous solutions of bilirubin

Fig. 8. Bilirubin-binding isotherms of rat liver cytosol

Liver cytosol was diluted 1:4 (v/v) with phosphate buffer, pH 7.0 ($I=0.01$), and divided into two portions. To one portion GSH was added to a final concentration of 2 mM. Binding data were then obtained as described in the Experimental section. The results are presented as a Scatchard-type graph. The data obtained with added GSH ($\circ$) could be fitted by eqn. (9) by using a single set of binding sites ($i=1$). The following binding parameters were obtained: $K^{app} = 2.30 \times 10^8 \text{ M}^{-1}$; $Bc = 3.06 \mu\text{M}$. The data set in the absence of GSH ($\square$) could not be fitted by assuming independent binding sites (eqn. 9). [From Meuwissen & Heirwegh (1978), with permission.]

near neutral pH (Brodersen & Theilgaard, 1969; Lee & Gartner, 1976). Since, even in the presence of sufficient binding protein, the solubility of the pigment can be exceeded locally in the system, the pigment is introduced into the assay system in adsorbed form, resulting, at equilibrium, in a low free concentration in the liquid phase. Although, depending on the amount of bilirubin-Sephadex, the free concentration can exceed the solubility of bilirubin in the absence of binding protein, the pigment appeared to remain in a state of supersaturation (Brodersen & Theilgaard, 1969), at least for the short time periods required to attain equilibrium. The presence of sufficient binding activity in the solution further lowers the free bilirubin concentration. Thus the occurrence of colloidal bilirubin in a protein solution after incubation with bilirubin-Sephadex complex indicates either that the protein is a non-specific binder or that its concentration is too low. The complex is particularly prone to colloid formation when it has become wet during storage. Results obtained by incubating bilirubin-Sephadex with distilled water indicate that colloidal bilirubin acts as a competitive auto-binder, so that the

size of the aggregates and thus the concentration of bilirubin in the liquid phase keeps increasing.

In the Sephadex-adsorbed state, bilirubin is easily exchangeable, probably in the di-anionic form, in view of the preparation procedure of the complex and the observation that Sephadex-bound pigment cannot be extracted with chloroform. This interpretation is strengthened by the observation that solid bilirubin in the acid form is not dissolved by albumin.

For a given k_F , the sensitivity of the method varies with the binding affinity and the binding capacity (Fig. 5). The competitive method described in this paper is sufficiently sensitive to determine the relatively large association constants (K values approx. 10^8 M^{-1}), which are characteristic for the binding of bilirubin *in vivo* and *in vitro*.

The binding capability of the sample must be adjusted to that of the adsorbent. A concentration of binding sites between 5 and $20 \mu\text{M}$ is adequate for the bilirubin-binding proteins in serum and liver cytosol of the rat. In the particular case of bilirubin and Sephadex G-10, it is therefore necessary to use binding protein at concentrations much smaller than the concentrations *in vivo*. This is advantageous when sample material is in short supply. Dilution of biological preparations also diminishes the effects of possible endogenous competitive inhibitors (Cashore *et al.*, 1983). To detect such inhibitors undiluted material should preferably be used, but this would require a bilirubin-adsorbent complex much richer in bilirubin than presently available. However, with enriched bilirubin-Sephadex G-10 preparations, the detection of apparent binding capacities up to 30 mol of bilirubin/mol of albumin was traced to formation of colloidal bilirubin (Meuwissen & Heirwegh, 1970). The only alternative to increase the assayable binder concentration is to use a stronger adsorbent. Prospective adsorbents should be inert towards binding protein. Cholestyramine (Schmid *et al.*, 1965) and CaCO_3 (Rutkowski, 1967, 1980) have been used as adsorbents for bilirubin, but their adsorption isotherms were not characterized at the time. More recently, bilirubin adsorption by cholestyramine and several other adsorbents has been investigated (Henning *et al.*, 1986). Unfortunately, most of these adsorbents are ionized and therefore expected to interact with protein.

The results given in Table 1 and Figs. 7 and 8 illustrate applications of the method to biological preparations containing bilirubin-binding protein. Fig. 7 illustrates the nature of the 'experimental' curves obtained with serum and cytosol from the rat. The steep portion of the 'experimental' curve of cytosol, compared with that of serum, is somewhat nearer to the limiting line, showing that the binding strength of cytosol is at least as high as that of serum. This conclusion is strengthened by faster desorption of bilirubin from bilirubin-Sephadex complex by cytosol compared with serum (Fig. 6). The results obtained with rat serum (Table 1) are compatible with a binding model comprising two independent binding sites having binding affinities that differ by two orders of magnitude. The binding capacity Bc correlates with the albumin concentration in a molar ratio close to 2:1. The rate of transfer of adsorbed bilirubin to rat serum can be expressed by the sum of two exponentials of equal amplitude (Fig. 6), suggesting that each exponential curve corresponds to a separate class of sites, in accordance with the results of the equilibrium measure-

ments (Table 1). Calculation with these binding parameters show that, in rat serum, the second site binds no more than 1% of the endogenous pigment, which is about 1 μ M (Muraca *et al.*, 1987). Binding to the second, weaker, site thus probably is of little physiological importance.

Rat liver cytosol has at its disposal several specific bilirubin-binding sites, including the ligandins (Litwack *et al.*, 1971; Meuwissen & Zeegers, 1981; Meuwissen, 1984), which are glutathione *S*-transferases (Habig *et al.*, 1974). The binding isotherm of rat liver cytosol (Fig. 8), assayed in the presence of GSH, appears to be linear, supporting the conclusion that the binding sites of cytosol have similar binding capacities and affinities, in agreement with the results of sedimentation analysis and gel filtration studies (Meuwissen *et al.*, 1977*a,b*; Meuwissen, 1984). When GSH is not present in sufficient concentration, the isotherm cannot be described by a simple binding equilibrium (Fig. 8). Considering the fact that the concentration of GSH inside the liver cell is high (8–10 mM according to Tateishi *et al.*, 1977), the curve in the presence of GSH probably best reflects the physiological binding capability of cytosol for bilirubin. Keeping some thiol groups of the binding proteins reduced and/or their GSH-binding sites occupied seem to be required to preserve the bilirubin-binding potential of rat liver cytosol. It is gratifying that the major conclusions about the binding parameters obtained by an equilibrium technique are entirely corroborated by kinetic desorption analysis.

The procedures described in this paper are to be considered a general approach, with bilirubin and Sephadex G-10 being used as an illustrative example. The binding of any ligand for which a suitable reference adsorbent can be found could be studied according to the principles described here.

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