

Factors Affecting the Binding of Bilirubin to Serum Albumins: Validation and Application of the Peroxidase Method

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ABSTRACT: The unbound “free” bilirubin concentration (B_f), not the total bilirubin concentration, is the critical determinant of cellular uptake and toxicity of bilirubin. We compared B_f measured by a modified peroxidase method with published data obtained with ultrafiltration and examined conditions that affect the affinity (K_F) of human (HSA) and bovine (BSA) serum albumin for bilirubin. The peroxidase and ultrafiltration methods yielded similar K_F values that decreased with increasing HSA concentration and the presence of 50 mM chloride. When related to ionic strength, inhibition of BSA-bilirubin binding by chloride, bromide, and sulfate were similar, whereas phosphate buffer had a smaller effect. K_F was lower at 37°C than at 25°C for HSA but not for BSA. K_F for BSA was similar at pH 7.4 and 8.0. BSA and FCS had similar binding properties. The close agreement of B_f and K_F values determined by the peroxidase method with published results obtained by ultrafiltration validates both methods and supports the use of the peroxidase method as a practical technique for measuring B_f under steady state conditions in minimally diluted serum or culture medium. (*Pediatr Res* 60: 724–728, 2006)

The unbound (“free”) concentration of unconjugated bilirubin (B_f) in plasma, although less than 0.1% of total bilirubin concentration, is the principal determinant of tissue uptake and toxicity of bilirubin, and plays a critical role in the pathogenesis of bilirubin encephalopathy in jaundiced newborns (1,2) and in patients with Crigler Najjar disease (3). Notwithstanding its biologic significance, B_f has rarely been measured in either clinical evaluation of jaundiced newborns or *in vitro* studies of bilirubin effects and toxicity. This avoidance is due in part to the perceived complexity of B_f assays and scepticism regarding their clinical value or accuracy (4–6).

Bilirubin-albumin binding has been studied using a variety of techniques, including fluorescent quenching, bilirubin fluorescence, circular dichroism, Sephadex gel filtration, optical rotary dispersion, dialysis, ultrafiltration, spectrophotometry, and enzymatic oxidation of bilirubin (peroxidase method). Reported association constants for HSA range from 6.7×10^6

M^{-1} to $>10^8 M^{-1}$ (7–10). The binding constant of HSA has been estimated to be 2–13 times greater than that of BSA (11–13). Considerable variation in serum binding of bilirubin has been reported in newborn infants (2). These differences may be due to direct binding competitors (*e.g.* sulfonamides), allosteric effects, electrolyte environment, or simply the assay technique, *e.g.* serum sample dilution (14).

The dependency of bilirubin binding affinity on HSA concentration as well as chloride concentration was demonstrated by Weisiger *et al.* (15) using a complicated procedure in which binding affinity of ^{14}C -bilirubin was calculated after the sequential removal of labeled impurities by serial ultrafiltration. A more practical technique for measuring B_f in clinical or laboratory settings was developed by Jacobsen and Wennberg (16) based on the observation that albumin-binding protects bilirubin from oxidation by HRP. Ahlfors (14,17) has modified the peroxidase method, emphasizing the need to measure B_f with two or more HRP concentrations and under the same conditions and albumin concentrations existing in plasma or incubation medium.

In this investigation, we replicated and extended the experimental conditions used in ultrafiltration studies (15) and examined the effects of albumin and electrolyte concentrations on bilirubin binding to HSA and BSA, using the modified peroxidase method (17). We then evaluated various experimental conditions that must be controlled when measuring B_f in tissue culture medium.

MATERIALS AND METHODS

Materials. PBS, DMEMHG, penicillin, and streptomycin were purchased from Euroclone, Milan, Italy. FCS, obtained from Invitrogen (Carlsbad, CA), contained 24 g/L albumin. Chloroform, HPLC grade was obtained from Carlo Erba, Milan, Italy. Bilirubin, DMSO (HPLC grade), HRP (EC.1.11.1.7/P 8125, Type I), hydrogen peroxide (H_2O_2 , 30% wt/vol), fatty acid-free HSA, and fatty acid-free BSA fraction V, were purchased from Sigma Chemical Co.-Aldrich, Milan, Italy.

Abbreviations: **B**, total unconjugated bilirubin concentration; **B/A**, molar ratio of bilirubin to albumin; **DMEMHG**, Dulbecco’s modified Eagle’s medium—high glucose; **HRP**, horseradish peroxidase; **HSA**, human serum albumin; **K_F** , calculated equilibrium binding constant of bilirubin to albumin; **k_p** , first-order rate constant for the oxidation of bilirubin by HRP

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Preparation of solution and reagents. Bilirubin was purified using the method of McDonagh and Assisi (18), divided into 50 μg aliquots, and stored at -20°C until used. Aliquots were dissolved in either 200 μL 0.01 N NaOH, followed by dilution with 2.64 mL H_2O (final concentration 30 μM) or 17 μL DMSO, yielding 5 mM (3 $\mu\text{g}/\text{mL}$).

Immediately after preparation, the bilirubin stock solution was added to PBS or DMEMHG containing 30 or 60 μM HSA or BSA, or 10% FCS (33 μM BSA). Final DMSO concentrations never exceeded 0.5% (vol/vol). When NaOH stock was added, the pH was adjusted to 7.4 with 0.01 N HCl. Total bilirubin concentrations (B) in albumin solutions were confirmed by the absorbance (A) at 468 nm (BSA) or 460 nm (HSA) assuming $\epsilon^{1\text{cm}} = 49,000$ A/mol for BSA and 48,000 A/mol for HSA (16).

HRP stock solution (1 mg/mL) was prepared by dissolving 2 mg HRP (EC.1.1.1.7/P 8125, Type I) in 2 mL PBS, pH 7.4, and stored at -20°C (19).

1.0% Hydrogen peroxide was prepared freshly each day by diluting 1 mL 30% H_2O_2 with 29 mL PBS.

Peroxidase method—basic principles and calculations. Unbound bilirubin is oxidized to mostly colorless compounds by peroxide (H_2O_2) in the presence of HRP with first-order kinetics; albumin-bound bilirubin is not oxidized. The rate constant for oxidation of bilirubin by HRP (kp) is determined by measuring the decrease in A_{440} following addition of HRP and H_2O_2 to dilute solutions of bilirubin (1–3 μM):

$$kp = V_o / ([B_f] \times [\text{HRP}]) \quad (1)$$

V_o , the initial oxidation velocity determined using a recording spectrophotometer, can be expressed either as the initial decrease in total bilirubin concentration (B) or simply $\Delta A_{440}/\text{min}$ (i.e. $A_o - A_t$)/t:

$$kp = \frac{\Delta A}{A_o [\text{HRP}] \cdot \Delta t} \quad (\text{mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}) \quad (1a)$$

Alternatively, kp may be calculated from an integrated form of eq. (1), which accounts for nonlinear oxidation as B decreases:

$$kp = - \frac{\ln\left(\frac{A_t}{A_o}\right)}{[\text{HRP}] \cdot t} \quad (2)$$

The steady state B_f in bilirubin-albumin solutions is determined by measuring the decrease in A_{460} (HSA) or A_{468} (BSA) over 30–60 s in the presence of HRP and H_2O_2 and calculated from the rate constant (kp), the concentration of HRP, and the initial change in bilirubin concentration (or ΔA) over time.

$$\beta_{f\text{SteadyState}} = \frac{\Delta A}{\epsilon^* \cdot kp \cdot [\text{HRP}] \cdot \Delta t} \quad (3)$$

where ϵ^* is the molar absorbance coefficient of bilirubin-albumin (BSA or HSA), or with the integrated form:

$$\beta_{f\text{SteadyState}} = \frac{A_o \cdot \ln\left(\frac{A_t}{A_o}\right)}{\epsilon^* \cdot kp \cdot [\text{HRP}] \cdot t} \quad (4)$$

The instantaneous decrease in A_{460} (HSA) or A_{468} (BSA) reflects the equilibrium B_f , but thereafter the rate of oxidation represents the steady state concentration of B_f reflecting bilirubin-albumin association/dissociation and enzyme oxidation (14,17). To determine the equilibrium B_f , the assay is performed with at least two concentrations of enzyme. As the bilirubin/albumin molar ratio (B/A) increases, albumin-bilirubin dissociation becomes rate limiting, and a significant underestimation of B_f will occur if several concentrations of HRP are not used. Because $1/[\text{steady-state } B_f]$ is proportional to $1/([B_f] + [\text{HRP}])$, the equilibrium B_f is determined from the inverse of the y intercept of a plot of $1/[\text{steady-state } B_f]$ versus $[\text{HRP}]$ (17).

The binding constant was calculated assuming a single albumin binding site because measurements were performed at low B/A ratios (0.2–0.3), where binding to other sites should be minimal (15). Because of these assumptions, we adopted the term K_F (formation constant), as used by Weisiger *et al.* (15), rather than the customary association constant, K_a , to describe binding. Thus:

$$K_F = (B - B_f) / (B_f ([\text{albumin}] - B + B_f)) \quad (5)$$

HRP standardization (kp). Stock solutions of bilirubin in NaOH or DMSO were protected from light and used within 1 h. Appropriate volumes were added to 2.8 mL of medium without albumin, to reach final bilirubin

concentrations of 1, 2, and 3 μM , and the concentration of bilirubin determined spectrophotometrically (Beckman DU 640 spectrophotometer) assuming $\epsilon^{1\text{cm}} = 47,500$ per mol at 440 nm (16). Then 10 μL of 1.0% H_2O_2 and 10 μL of diluted HRP (1.65×10^{-2} $\mu\text{g}/\text{mL}$) were added and the cuvette inverted twice without shaking the supersaturated solution. $\Delta A_{440\text{nm}}$ was recorded for 30–60 s and kp calculated as described above. Using the same (albumin-free) medium used for B_f determination, 5 replicates were run at each of 2–3 HRP concentrations, and the 10–15 measurements averaged to determine kp.

Measurement of B_f . B_f was measured using undiluted samples to avoid alteration in K_F . Following measurement of A_o at the appropriate wavelength, 5 μL of 1.0% H_2O_2 and 5–20 μL HRP (final concentration 0.6–2.5 $\mu\text{g}/\text{mL}$) were added to 3 mL bilirubin-albumin solution. The cuvette was inverted twice and ΔA measured for 30 s. The HRP concentration was adjusted to produce a ΔA of 10–20% over 1 min. Triplicate determinations were performed at each of 2 to 3 peroxidase concentrations (0.6, 1.2, and/or 2.5 $\mu\text{g}/\text{mL}$) and the steady state B_f was calculated as described above. The equilibrium B_f was then calculated from formula 5.

Comparison of peroxidase and ultrafiltration methods using HSA. B_f was measured using media of the same composition as those used in ultrafiltration studies (15) (250 mM sucrose + 50 mM KCl or 350 mM sucrose without chloride, HEPES 20 mM, pH 7.4, and variable HSA concentration). The B/A ratio was maintained at 0.2. Our studies were performed at 37°C rather than 25°C .

Bilirubin binding in solutions containing HSA, BSA, or FCS. *In vitro* studies of bilirubin toxicity often use bilirubin bound to HSA or BSA, or media containing FCS. The binding of bilirubin to HSA, BSA, and FCS was examined using albumin concentrations of 30 μM , and varying B, in PBS and DMEMHG, pH 7.4, at 37°C in the dark.

Bilirubin stability in solutions without or with albumin. We evaluated the effect of bilirubin solvent (0.01 N NaOH or DMSO) on aqueous stability and kp determination in DMEMHG and PBS, pH 7.4, 37°C . Final bilirubin concentrations of 1, 2, and 3 μM were confirmed by measuring absorbance at 440 nm, and stability was determined by monitoring ΔA_{440} . K_p was measured as noted above. The stability of bilirubin-BSA solutions (B/A ratio = 0.3) was evaluated by monitoring A_{468} over 24 h.

Other factors affecting K_p and bilirubin binding to BSA. Unless otherwise noted, the remaining studies were performed in DMEMHG medium, pH 7.4, at 37°C using 30 μM BSA.

The effects of inorganic anions on binding were studied at a B/A ratio of 0.2. Sodium salts of Cl^- , Br^- , or SO_4^{2-} were added to 250 mM sucrose, containing 20 mM HEPES; HEPES was omitted from studies of phosphate.

The effects of impurities in commercial bilirubin on kp and B_f determinations were determined by comparison with purified bilirubin. The effect of light (1000 lux) on B_f was evaluated using 60 μM BSA and 12 μM bilirubin in DMEMHG. The solution flask was placed 10 cm below a POLILUX 4000 58WT8 fluorescent lamp in a biologic safety cabin (Model-VBH72 MP/99; Steril, Milan, Italy). After 15, 30, and 60 min light exposure at 25°C , B_f was measured at 37°C .

The effects of temperature were studied at 25°C and 37°C . K_p was determined at bilirubin concentrations of 1.8 and 3.5 μM . B_f was measured using 60 μM BSA or HSA and B/A ratios of 0.23 and 0.17, respectively.

The effects of pH on kp and on binding by HSA and BSA were evaluated at pH 7.4 and 8.0. The pH was adjusted by addition of 0.01N HCl or NaOH. Effects on B_f were assessed at a B/A ratio of 0.3.

The effect of commonly used tissue culture additives (streptomycin, penicillin, glutamine) on binding were studied in DMEMHG using BSA, B/A 0.2 at 37°C .

Statistical analysis. Results are expressed as mean \pm SD of three to five assays per condition. Statistical analysis was performed using ANOVA combined with Student's two-tailed *t* test. Differences among the conditions were considered significant at $p < 0.05$.

RESULTS

Comparison of the peroxidase and ultrafiltration methods with HSA (Fig. 1). HEPES buffer, used for ultrafiltration, inhibited HRP activity, resulting in a kp an order of magnitude lower than in PBS. This required a higher concentration of HRP for measuring B_f in the presence of albumin, but the calculated B_f in a control serum (60 μM BSA, 12 μM B) was the same with either HEPES or PBS buffer (18.9 ± 1.7 nM versus 19.2 ± 0.3 nM, respectively).

Data obtained with the peroxidase method confirmed the findings of Weisiger *et al.* (15). The affinity of HSA for

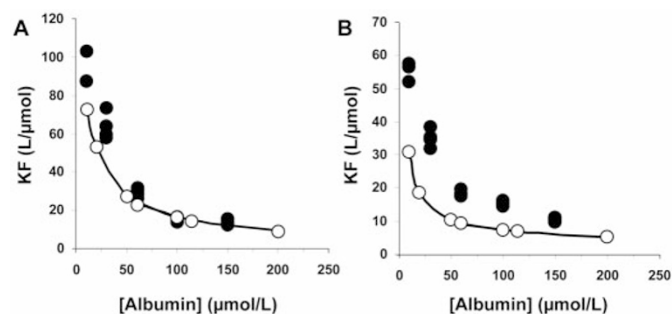


Figure 1. Effect of HSA concentration on the binding affinity (K_F) of bilirubin, 37°C, in the absence (A) or presence (B) of 50 mM KCl. Data are compared with reported K_F values determined in identical media by ultrafiltration (15). The bilirubin/albumin molar ratio was maintained at 0.2. Peroxidase method (●), ultrafiltration method (○).

bilirubin decreased with increasing albumin concentration and with the addition of chloride (Fig. 1). In the absence of chloride, at HSA concentrations of 60 μ M or above, the peroxidase and ultrafiltration methods yielded nearly identical results. At lower HSA concentrations, especially in the presence of chloride, the peroxidase method yielded a somewhat higher K_F than had ultrafiltration, but the patterns were similar.

Comparison of K_F for HSA and BSA using the peroxidase method. The effects of albumin and electrolyte concentrations on bilirubin binding to BSA had unique features (Table 1). In the absence of chloride, K_{F-HSA} was about two times higher than K_{F-BSA} at each albumin concentration (1.6 \times at 150 μ M to 2.7 \times at 10 μ M). However, in the presence of 50 mM Cl^- , the ratio K_{F-HSA}/K_{F-BSA} increased from 3 \times at 150 μ M to about 10 \times at 10 μ M albumin. With BSA, the dilution effect was mitigated by 50 mM Cl^- . In the presence of chloride anion, K_{F-HSA} increased 7 \times when albumin was diluted from 150 μ M to 10 μ M, whereas K_{F-BSA} increased less than 2 \times and remained nearly constant with BSA concentrations of 60 μ M or higher. However, the effect of chloride on BSA binding was substantial. With HSA, chloride decreased K_F about 40% at all HSA concentrations, whereas K_{F-BSA} decreased 3-fold with 150 μ M BSA and more than 6-fold with albumin concentrations of 30 μ M or lower.

B_f measurements in culture media containing BSA, HSA, or 10% FCS. *In vitro* studies of bilirubin toxicity often use bilirubin bound to HSA or BSA, and many cell cultures

require media containing FCS. We, therefore, compared the binding of bilirubin to HSA, BSA, and FCS in DMEMHG.

Addition of H_2O_2 alone to DMEMHG with 10% bilirubin-FCS revealed intrinsic oxidase activity that followed saturation kinetics, with a V_{max} of 0.07 $\Delta A_{468}/min$ at about 20 μ M bilirubin (data not shown). This background oxidation rate, which was not diminished by pretreating the medium with a heme-adsorbing resin, was subtracted from the oxidation rate obtained with added HRP to obtain the corrected oxidation rate:

$$\Delta A/min_{total} - \Delta A/min_{H_2O_2} = \Delta A/min_{corrected}$$

Background oxidation was not observed when either HSA or BSA was added to DMEMHG in the absence of FCS.

Incubation of bilirubin/BSA in DMEMHG in the dark at 37°C resulted in a 13% decay of bilirubin (ΔA_{468}) in 24 h but only 7% over 6 h. By contrast, there was no decrease in A_{468} when bilirubin/BSA was dissolved in PBS.

Titration of bilirubin using a constant albumin concentration of 30 μ M in DMEMHG (Fig. 2) revealed a greater binding affinity with HSA than with either BSA or FCS. B_f was similar with BSA and FCS up to a B/A ratio of 0.5 but rose steeply at higher B/A ratios with FCS. Addition of glutamine (2 mM), streptomycin (100 mg/L), and 1% (vol/vol) penicillin (100,000 U/L) to culture medium (B/A 0.2) did not alter B_f (37 ± 5 nM versus 39 ± 7 nM in controls).

Effect of inorganic anions on binding affinity of BSA for bilirubin. The addition of 118 mM NaCl increased B_f from 10 to 179 nM and, as shown in Figure 3A, decreased K_F from 28.7 to 1.3 μM^{-1} . Bromide behaved similarly (data not shown). Divalent sulfate anion decreased K_F more than chloride, with inhibition of bilirubin binding by 97% or more at sulfate concentrations of 50 mM or higher. In contrast, phosphate (50 mM, pH 7.4) produced only a 30% reduction in K_F .

For each anion, $\log K_F$ was negatively correlated with the square root of ionic strength (Fig. 3B). Chloride, bromide (not shown), and sulfate produced similar decreases in binding with increasing ionic strength; the effect of phosphate was much less at all ionic strengths.

Effect of bilirubin solvent and medium on bilirubin stability and K_p determination. DMSO is frequently used as a bilirubin solvent for *in vitro* toxicity studies, while NaOH is usually

Table 1. Effect of albumin concentration and chloride anion on bilirubin binding by HSA and BSA

[Albumin] (μ M)	K_F ($\times 10^6 M^{-1}$)			
	HSA		BSA	
	No Cl^-	+50 mM Cl^-	No Cl^-	+50 mM Cl^-
10	107 \pm 12	61.3 \pm 9.5	39.4 \pm 5.3	6.2 \pm 0.6
30	55.7 \pm 6.0	31.5 \pm 2.4	28.7 \pm 7.6	4.4 \pm 0.9
60	26 \pm 1.6	17.0 \pm 0.8	13.9 \pm 1.6	3.7 \pm 0.4
100	15.9 \pm 1.1	13.8 \pm 0.6	9.6 \pm 1.8	3.1 \pm 0.3
150	16.0 \pm 1.3	9.6 \pm 0.6	10.0 \pm 0.8	3.3 \pm 0.2

K_F was calculated from B_f values determined by the peroxidase method, using a constant B/A ratio of 0.2. The buffer (20 mM HEPES, pH 7.4) contained either 350 mM sucrose or 250 mM sucrose + 50 mM KCl. Data are expressed as mean \pm SD of three experiments.

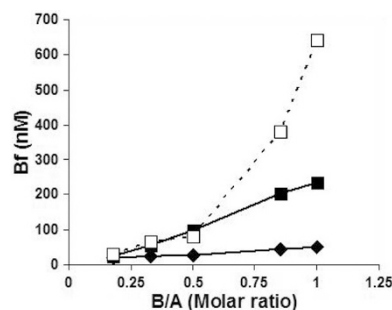


Figure 2. Effect of albumin species on the concentration of unbound bilirubin (B_f) at various bilirubin/albumin (B/A) molar ratios, comparing FCS (□), BSA (■), and HSA (◆), each 30 μ M. Medium was DMEMHG. B_f was measured at 37°C, pH = 7.4, using HRP concentrations of 0.6, 1.2, and 2.5 μ g/mL.

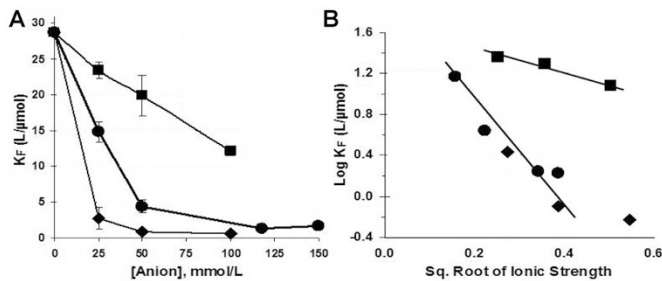


Figure 3. Effect of sodium salts of different anions on binding of bilirubin to BSA. The experiments were performed at 37°C in 250 mM sucrose, 20 mM HEPES, pH 7.4, containing 30 μM BSA at a constant bilirubin/albumin molar ratio of 0.2. Data represent the mean ± SD of three experiments. For 50 and 100 mM phosphate vs no phosphate, $p < 0.02$ and $p < 0.002$, respectively. For each concentration of chloride or sulfate vs no anion, $p < 0.001$. Phosphate (■), chloride (●), sulfate (◆).

used for peroxidase standardization. Bilirubin dissolved in DMSO precipitated when added to DMEMHG, so DMSO could not be used to determine k_p in this medium. In PBS, spontaneous absorbance decay was greater for bilirubin dissolved in NaOH ($\Delta A/\text{min} = 0.0028$) than in DMSO ($\Delta A/\text{min} = 0.0014$).

K_p values in PBS were not significantly different for bilirubin dissolved with NaOH ($463 \pm 53 \text{ mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$) or DMSO ($491 \pm 30 \text{ mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$) and were unaffected by bilirubin concentration (1, 2, or 3 μM). K_p did not differ significantly between DMEMHG ($445 \pm 41 \text{ mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$) and PBS ($463 \pm 53 \text{ mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$) when bilirubin was dissolved in NaOH.

Effect of bilirubin purity and light exposure on binding to BSA in DMEMHG. Purified and commercial bilirubin yielded similar k_p values (439 ± 24 versus $450 \pm 44 \text{ mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$, respectively) and B_f values (21.1 ± 1.8 versus $21.2 \pm 0.9 \text{ nM}$, respectively). After 15, 30, and 60 min exposure of bilirubin-BSA to intense light, B_f remained unchanged ($25.0 \pm 1.1 \text{ nM}$ at time 0 versus $25.4 \pm 0.6 \text{ nM}$ after 1 h) despite a 14% drop in total bilirubin.

Effect of temperature on K_p and B_f . In DMEMHG, the k_p at 25°C, $282 \pm 34 \text{ mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$, was much lower than at 37°C, $445 \pm 41 \text{ mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$. In the small sample size studied ($n = 5$), we did not observe a temperature effect on binding to BSA (B_f measured at 25°C was $41.3 \pm 4.8 \text{ nM}$ versus $34.5 \pm 5.6 \text{ nM}$ at 37°C, $p = 0.07$). Bilirubin binding to HSA decreased slightly at the higher temperature ($B_f = 22.8 \pm 1.6 \text{ nM}$ at 25°C versus $31.3 \pm 6.1 \text{ nM}$ at 37°C, $p = 0.03$), consistent with observations of Jacobsen (20).

Effect of pH on K_p and B_f in DMEMHG. Tissue culture is frequently buffered by a CO_2 -bicarbonate system, resulting in an increased pH when removed from the CO_2 atmosphere. The k_p of 463 ± 31 at pH 7.4 fell to $402 \pm 29 \text{ mL} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$ at 8.0 ($p < 0.03$). Nonetheless, B_f values for bilirubin-BSA did not differ between pH 7.4 and 8.0 (51 ± 9.0 and $48 \pm 8.0 \text{ nM}$, respectively). Similar results have been reported using HSA (21,22).

DISCUSSION

Our data confirm the effects of albumin and chloride concentration on bilirubin binding to HSA (15). There was good

agreement with the ultrafiltration data (15), especially at HSA concentrations of 60 μM or higher in the absence of chloride. At low HSA concentrations, the peroxidase method yielded somewhat higher K_F values than observed with ultrafiltration (15). This difference may be due in part to an albumin gradient created by ultracentrifugation, engendering higher HSA concentrations at the interface of the retentate with the filter. This would increase filtered bilirubin, yielding a higher apparent B_f and lower K_F .

The range of K_F values for HSA observed under various conditions in our study ($6.9 \times 10^6 \text{ M}^{-1}$ to $1.1 \times 10^8 \text{ M}^{-1}$, Table 1) reflects the range of association constants reported in the literature using a variety of experimental techniques. Interestingly, the higher published values ($K_F > 10^8 \text{ M}^{-1}$) were performed with very dilute albumin solutions (7,10), whereas the lower K_F of $6.7 \times 10^6 \text{ M}^{-1}$, reported by Lamola *et al.* (8), used 440 μM HSA. The effect of albumin concentration on binding was far greater with HSA than with BSA (Table 1).

Our studies extend to BSA the previously reported inhibitory effect of 50 mM chloride anion on HSA binding (15) and also measured the effect of higher (physiologic) chloride concentrations. Chloride, bromide, and sulfate have the same effect on binding when analyzed as a function of ionic strength. Dehydration has been implicated in potentiating kernicterus, but variations in anion concentration within the range observed clinically should have little impact on bilirubin binding (Fig. 3A). The effect of phosphate is much smaller though still related to ionic strength, confirming the findings of Jacobsen (20). In contrast, Weisiger *et al.* (15) found that gluconate anion had no effect on bilirubin binding. Although the inhibition of organic anion binding by chloride roughly parallels the extent of binding by chloride (23,24), chloride binds to many more sites on albumin than does bilirubin (24). Thus, the relative roles of ionic strength, allosteric effects, and specific binding competition with anions remain uncertain.

A potential error in measuring B_f with the peroxidase method is that the HRP activity (k_p) must be standardized with supersaturated bilirubin solutions. Aggregation of bilirubin might impair its oxidation, yielding a falsely low k_p and overestimate of B_f . However, we found no differences in k_p values measured at bilirubin concentrations of 1, 2, and 3 μM, suggesting that aggregation did not affect oxidation rates during the brief duration of the assay.

Water-soluble photoisomers, which form rapidly when bilirubin is exposed to light, cannot be distinguished from native ZZ-bilirubin IX α by either light absorbance or diazo reaction, but are thought to bind less tightly to albumin (25). Although photoisomer concentration was not determined, we found no effect on B_f of short-term, intense exposure of bilirubin-BSA to light. This result is explicable only if the photoproducts are bound to the bilirubin site on BSA, contribute to absorbance at 440 nm, and/or are substrates oxidized by HRP. Itoh *et al.* (26) previously demonstrated that the initial rate of the peroxidase reaction with HSA is not accelerated by either (ZE)-photobilirubin or (EZ)-cyclobilirubin when present at clinically relevant concentrations.

Most reported *in vitro* studies examining bilirubin effects or toxicity use HSA or BSA as a bilirubin stabilizer or donor, but rarely measure B_f and often use initial bilirubin concentrations that greatly exceed physiologic or pathologic significance (27). Even fewer studies measured the more meaningful equilibrium concentration of bilirubin during or after incubation (22,28). Ostrow *et al.* (27) recently calculated the B_f from molar bilirubin/albumin ratios (B/A) reported in several studies by applying ultrafiltration-determined binding constants for HSA in similar media (15). This study confirms, however, that calculated B_f 's are unreliable since binding affinity varies with albumin species, albumin concentration, and the buffer medium used.

Tissue culture media typically contain 10% FCS, providing a BSA concentration of about 33 μ M. Thus, bilirubin can be added directly to the medium rather than replacing medium with albumin solution. However, when using FCS, we found it important to correct for background oxidase activity. We also confirmed that commonly used culture medium additives, glutamine, streptomycin, and penicillin, do not alter binding.

In conclusion, our goal was to validate a practical method for measuring B_f and to adapt the method for studies using tissue culture media. The peroxidase method yielded K_F values concordant with ultrafiltration data (15) obtained with nearly identical experimental conditions, and consistent with published values for K_F . Compared with HSA, BSA has a lower binding affinity, greater sensitivity to chloride and less sensitivity to temperature. Our findings emphasize the need to measure HRP activity (kp) and B_f under the same conditions and in the same media used experimentally. We recommend that B_f be measured under steady state or equilibrium conditions in all studies in which albumin is used as a reservoir for bilirubin. This would facilitate interpretation of dose-response relationships and improve comparisons of *in vitro* studies conducted in different laboratories.

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