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Affinity of Human Serum Albumin for Bilirubin Varies with Albumin Concentration and Buffer Composition

RESULTS OF A NOVEL ULTRAFILTRATION METHOD*

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Albumin binding is a crucial determinant of bilirubin clearance in health and bilirubin toxicity in certain disease states. However, prior attempts to measure the affinity of albumin for bilirubin have yielded highly variable results, reflecting both differing conditions and the confounding influence of impurities. We therefore have devised a method based on serial ultrafiltration that successively removes impurities in [^{14}C]bilirubin until a stable binding affinity is achieved, and then we used it to assess the effect of albumin concentration and buffer composition on binding. The apparent binding affinity of human serum albumin for [^{14}C]bilirubin was strongly dependent on assay conditions, falling from (5.09 ± 0.24) × 10^7 liters/mol at lower albumin concentrations (15 μM) to (0.54 ± 0.05) × 10^7 liters/mol at higher albumin concentrations (300 μM). To determine whether radioactive impurities were responsible for this change, we estimated impurities in the stock bilirubin using a novel modeling approach and found them to be 0.11–0.13%. Formation of new impurities during the study and their affinity for albumin were also estimated. After correction for impurities, the binding affinity remained heavily dependent on the albumin concentration (range (5.37 ± 0.26) × 10^7 liters/mol to (0.65 ± 0.03) × 10^7 liters/mol). Affinities decreased by about half in the presence of chloride (50 mM). Thus, the affinity of human albumin for bilirubin is not constant, but varies with both albumin concentration and buffer composition. Binding may be considerably less avid at physiological albumin concentrations than previously believed.

Bilirubin is a potentially toxic product of heme catabolism that is normally cleared from plasma by the liver, conjugated with glucuronic acid and excreted into bile (1). Newborn infants with low levels of bilirubin glucuronosyltransferase and people with a severe genetic deficiency of this enzyme are at risk for developing bilirubin toxicity, which occurs when bilirubin levels in cells become sufficiently elevated to interfere with normal cellular functions (2). Deposition in brain tissues produces the most severe toxicity, known as kernicterus (1), whereas deposition in skin and mucous membranes results in the yellow jaundice characteristic of liver disease.

Plasma albumin limits the toxicity of bilirubin by reducing the unbound bilirubin concentration and thereby competing with tissues for bilirubin binding (3). Extremely avid binding to albumin may be detrimental, however, because it limits the rate of hepatic removal of bilirubin from the plasma (4). Thus, the affinity of albumin for bilirubin may reflect a compromise between the need to prevent excessive binding to tissues and the need for efficient hepatic elimination.

Attempts to measure the equilibrium binding constant (K_F)[1] of albumin for bilirubin have been hampered by the difficulty in measuring the extremely low concentrations of unbound bilirubin typically present in plasma, reportedly less than 0.005% of total bilirubin at physiological albumin concentrations (3). For this reason, investigators have usually measured the binding affinity at much lower albumin concentrations where the unbound fraction of bilirubin is correspondingly larger. The value of K_F thus determined is then used to predict the unbound bilirubin concentration for physiologic albumin and bilirubin concentrations. This approach assumes that K_F is a constant that is independent of the albumin concentration.

This assumption has never been adequately tested and has recently been questioned (5, 6). Moreover, many properties of albumin are known to depend on its concentration (7–10). In particular, the binding affinity of albumin for a variety of metabolites and drugs is reduced at higher albumin concentra-

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[1] The abbreviations used are: K_F, actual formation (association) constant for binding of bilirubin to HSA; B_F, actual unbound bilirubin concentration; B_T, actual total bilirubin concentration; $B_{f,}$ apparent unbound bilirubin concentration; $B_{t,}$ apparent total bilirubin concentration; HSA, human serum albumin concentration; I, concentration of unbound impurities in solution (derived variable); I_0, initial impurity concentration in the stock bilirubin (fitted variable); k, rate of formation of new impurity per cycle (fitted variable); $K'_{f,}$ apparent formation (association) constant for binding of bilirubin to HSA; K_I, formation (association) constant of I for HSA (fitted variable); R, ratio of total to unbound impurity in solution.

tion (8, 9, 11–16). Thus, the assumption that the K_F for bilirubin is unaffected by albumin concentration may not be valid.

Prior studies of bilirubin binding to human serum albumin have produced estimates for K_F that vary by more than 100-fold (3). One possible explanation is the use of different experimental conditions. However, an even greater confounding factor may have been contamination by impurities. Studies that use radiolabeled bilirubin must contend with the presence of radiolabeled impurities, both those present initially and those generated during the study (*e.g.* by photodegradation, Ref. 17). Impurities that bind weakly to albumin but cannot be distinguished from genuine bilirubin may reduce the apparent binding affinity dramatically. For example, if the unbound concentration of bilirubin is 0.01%, then the presence of a nonbinding impurity at a level of 0.1% would reduce the apparent binding affinity ~10-fold. Current methods do not allow preparation of bilirubin with a purity of >99.99%. Thus, any binding study using labeled bilirubin must compensate in some way for impurities.

In the current study, we present a method that uses serial ultrafiltration to progressively and selectively remove weakly bound impurities until the K_F value approaches a constant. Our results indicate that the binding affinity of human serum albumin for bilirubin is not a constant, as previously assumed (3), but is modulated by both albumin concentration and buffer composition. Mathematical modeling of these data allowed us to estimate not only the true binding affinity, but also the amount of initial impurity, the rate of impurity formation during sample processing, and the affinity of the impurities for albumin.

EXPERIMENTAL PROCEDURES

To minimize photodegradation of bilirubin (18–20), samples were maintained in complete darkness throughout all procedures except when being transferred into and out of the centrifuge and during aliquot removal and remixing of the retentates. Illumination was provided by red lamps with no detectable emission below 600 nm; this has been shown to produce no detectable photodegradation of bilirubin in deoxygenated albumin solution at pH 7.4 (19). Photoisomerization is also unlikely because of the lack of emission in the range of wavelengths at which bilirubin absorbs light.

Preparation of Stock [^{14}C]Bilirubin—Radiolabeled bilirubin conjugates were purified from bile after intravenous infusion of δ-[5-^{14}C]aminolevulinic acid (PerkinElmer Life Sciences, Boston, MA) to bile-fistula rats using a protocol approved by the Animal Committee of Northwestern University Medical School and the Chicago VA Lakeside Medical Center. Purified ^{14}C-unconjugated bilirubin was then obtained by a modification of the method of Ostrow *et al*. (21). Briefly, proteins were removed from pooled bile using reverse-phase C18 cartridges (Mega Bond Elut, Analytichem, Harbor City, CA). Following elution, the conjugated bilirubin was precipitated with lead ion, and contaminating lipids were removed by ethanol extraction followed by two water washes. Unconjugated bilirubin was produced by alkaline hydrolysis of the resulting pigments. After extraction into chloroform, the labeled bilirubin was further purified by two cycles of alkaline extraction followed by recrystallization to constant specific activity according to McDonagh and Assisi (22). The resulting [^{14}C]bilirubin had a specific radioactivity of 5×10^4 dpm/μg or 0.022 μCi/μg, as assessed by scintillation counting and diazo assay (23), and met criteria for purity described previously (21). The [^{14}C]bilirubin solution was distributed among glass vials, evaporated to dryness, sealed under argon, and finally stored in the dark at −20 °C until use (18). Assays at 4-week intervals confirmed the stability of the stored [^{14}C]bilirubin (specific activity within ±2% of baseline).

Preparation of Solutions—Sucrose buffer solutions contained 10–300 μM human serum albumin (fatty acid free, catalog A-3782, lot 93H9343, Sigma) plus 0.25 M sucrose and 20 mM HEPES, adjusted with Tris to pH 7.4. For chloride buffer solutions, 50 mM KCl was substituted isosmotically for 100 mM sucrose. In two experiments at albumin concentrations of 30–35 μM, 50 mM NaCl, or potassium gluconate were used instead of KCl. On the day of the study, [^{14}C]bilirubin from one vial was dissolved in deoxygenated chloroform, and the last measurable traces of polar impurities were removed by serial extraction with 20 volumes of 0.1 M phosphate buffer, pH 7.0, until a constant solvent partition ratio was obtained (five cycles). The chloroform was evaporated under a stream of argon and the [^{14}C]bilirubin dissolved in 30 μl of Me_2SO and diluted into 3.0 ml of deoxygenated albumin solution. The target bilirubin/HSA molar ratio was 0.25 for most experiments but was reduced to 0.10 at the highest albumin concentrations to conserve [^{14}C]bilirubin. Radioassay and diazo assay (23) were performed in duplicate on 0.5 ml of the solution, and the remaining 2.5 ml was used immediately for ultrafiltration studies.

Measurement of Bilirubin Albumin Binding by Serial Ultrafiltration—Ultrafiltration was performed at 25 °C using Centricon 10 ultrafiltration devices (Amicon, Danvers, MA). Experiments for each day were performed in duplicate, and each experiment using chloride buffer was paired with an identical experiment using sucrose buffer (four tubes total). Centricons, presoaked overnight in appropriate buffer, were flushed dry with argon and loaded with 2.5 ml of buffered [^{14}C]bilirubin-HSA solution. Five sequential centrifugation cycles were then performed at 42 ± 6 min intervals (mean ± S.D., n = 112). An initial centrifugation (10 min at 2500 × g) allowed the Centricon membrane to become equilibrated with unbound bilirubin so that the ultrafiltrate composition would accurately reflect the unbound bilirubin concentration in the albumin solution. Subsequent centrifugations (cycles 2 through 5) were for 20 min at 4500 × g. After each centrifugation, 30 μl of stirred retentate, and the entire filtrate were taken for analysis of protein concentration and radioactivity. The residual retentate was then diluted back to its original volume with the same buffer, mixed gently for 2 min, and 30 μl taken for analysis. In selected experiments to assess gradient formation, 10-μl fluid samples were taken from the extreme top and bottom of the retentate fluid column immediately following the final centrifugation using a 25 μl fine-tip Hamilton syringe.

Retentate samples (10 μl) were assayed for total albumin concentration by the bicinchonic acid method (24) with correction for the small reactivity of the HEPES buffer using a protein-free blank. Control studies indicated that bilirubin did not interfere with this assay at the concentrations used. Radioactivity in the remaining 20 μl of retentate, and the entire ultrafiltrate was determined by scintillation counting. Apparent total [B_t] and unbound [B_f] bilirubin concentrations were calculated from the specific activity of [^{14}C]bilirubin and the dpm/ml of the retentate (sampled before and after each centrifugation) and filtrate, respectively. Apparent bound bilirubin concentrations were calculated as B_t-B_f using the mean dpm/ml and HSA concentrations in the retentate fluid before and after the centrifugation. Control studies were performed in the absence of HSA, using 30 and 45 nM [^{14}C]bilirubin, comparable with B_f values observed in the presence of HSA.

Calculation of the Apparent Equilibrium Binding Constant—The apparent equilibrium binding constant, K'_f, was calculated using the familiar mass action equation, Equation 1, in which the numerator contains the apparent concentration of albumin-bilirubin complexes, while the denominator is the product of the apparent unbound bilirubin concentration [B_f] and the concentration of unoccupied albumin binding sites [HSA-B_t +B_f]. [B_t] is the measured total bilirubin concentration.

$$K'_f = \frac{B_t - B_f}{B_f(HSA - B_t + B_f)} \quad \text{(Eq. 1)}$$

As used here, K'_f is the first stepwise binding constant of albumin as defined by Spector *et al.* (25). Although albumin has multiple bilirubin binding sites (3), this study considers only the first sequential site to be occupied because the molar ratio of B_t to HSA was always $\ll$ 1.0.

Equation for Predicting K'_f from the Impurity Concentration—Equation 1 may be modified to express K'_f in terms of actual concentrations by replacing B_f and B_t with their equivalents, B_F + I and B_T + I, as shown in Equation 2,

$$K'_f = \frac{(B_T + I) - (B_F + I)}{(B_F + I)\,(HSA - (B_T + I) + (B_F + I))} \quad \text{(Eq. 2)}$$

where B_F and B_T are the true concentrations of unbound and total bilirubin and I is the concentration of unbound impurities.

Measurement of Impurity Concentration by Curve Fitting—Our modeling strategy was to determine I by finding the values that minimize the differences between K'_f predicted by Equation 2 and K'_f measured using Equation 1. Thus, the impurity concentration at each centrifugation cycle for each pair of Centricon tubes (containing sucrose and chloride buffer, respectively) was determined by iteratively varying the values of I to minimize the sum of the squares between the observed values of K'_f in Equation 1 and the values predicted by Equation 2 for each cycle (after replacing B_F with B_f -I and B_T with B_t -I). K_F was then

calculated for each Centricon pair using the standard mass action equation.

Note that Equation 2 alone does not allow us to distinguish impurity from unbound bilirubin, as both are efficiently filtered through the membrane. We can calculate I if we know B_F, or can calculate B_F if we know I, but not both. To determine both values, we take advantage of the fact that removal of impurity by successive filtrations is much more efficient than removal of bilirubin, reflecting the more avid binding of bilirubin to albumin. As the amount of impurity in the solution decreases with each successive cycle, the value of K'_f approaches K_F. Because I and B_F behave differently, they can be separated by our curve-fitting routine.

The amount of impurity remaining for any given cycle, n, is also affected by sampling and formation of new impurity during that cycle. The impurity concentration for a given centrifugation cycle, I_n, is the sum of initial impurity I_0 plus any impurity that has formed during sample processing minus all impurity that has been removed by filtration or sampling. This may be expressed as Equation 3,

$$I_n = I_0(1 - F)^{n-1} + \sum_{i=2}^{n} kB_T(1 - F)^{i-2}, \quad \text{(Eq. 3)}$$

where k is the rate of impurity formation per centrifugation cycle, and F is the fraction of the total impurity that is removed by filtration per cycle. Note that both I_0 and newly generated impurity are decreased for each subsequent centrifugation cycle by the fraction, F, of impurity removed each cycle. If the impurity does not bind to albumin, F is simply the fraction of the solution removed by filtration and sampling (0.48). If the impurity does bind to albumin, then F is 0.48 multiplied by the fraction of the impurity that is unbound as shown in Equation 4,

$$F = \frac{0.48}{1 + HSA\,K_I}, \quad \text{(Eq. 4)}$$

where K_I is the binding affinity of the impurity for albumin. If both I_0 and k are zero, Equation 3 simplifies to zero, and we have the traditional case in which no impurity is present.

Our approach uses the change in B_f with successive centrifugation cycles to determine the impurity concentration, I. If impurities are present, B_f will typically decrease with each cycle after the first because of progressive, selective removal of poorly bound impurities by filtration, eventually approaching a steady value. For convenience, B_f values were converted to their equivalent K'_f values using Equation 1 prior to fitting. For each Centricon tube, the sum of the squares of the differences between observed and model-predicted K'_f values for cycles 2 through 5 was iteratively minimized using a derivative-free (simplex) method. The value of I for each cycle was calculated from Equation 3 and used to determine the true binding affinity, K_F as described below. A Microsoft Excel program to perform the curve fitting is available from the first author.

Calculation of the True Binding Constant from Experimental Data—The true binding affinity K_F for each centrifugation is found by subtracting the impurity concentration for that cycle I_n from B_f and B_t everywhere they appear in Equation 1. Modifying Equation 1 and defining R as the ratio of total to unbound impurity, we get Equation 5.

$$K_F = \frac{(B_t - RI_n) - (B_f - I_n)}{(B_f - I_n)(HSA - (B_t - RI_n) + (B_f - I_n))} = \frac{B_T - B_F}{B_F(HSA - B_T + B_F)} \quad \text{(Eq. 5)}$$

Model Discrimination—We sought the simplest impurity model that could adequately account for the data. All models considered the amount of initial impurity, I_0; the rate of new impurity formation per cycle, k; and the affinity of binding of impurity to albumin, K_I, although one or more of these values could be set to zero prior to fitting. Where k was not zero, we further considered whether k was acting on the total concentration of bilirubin, the unbound concentration of bilirubin, or was a constant independent of either concentration. The best model was selected using three criteria. First, curve fitting of the data must converge on physiologically reasonable (*e.g.* non-negative) values for the fitted parameters for each pair of Centricon tubes. Second, all parameters must be adequately identified by the data (S.E. of the fitted values $\ll$100% of the mean). Models that fulfilled the above criteria were further compared according to goodness of fit (determined by the lowest sum of the squares for a given number of unknown parameters). Models with fewer unknown parameters were favored over more complex models if they could adequately account for the data.

Statistical Analysis—Differences in values between individual parameters were determined using the appropriate paired or unpaired two-tailed t test with $p < 0.05$ being judged as significant. Unless otherwise specified, parameter values are listed as mean ± S.E.

RESULTS

Validation of Ultrafiltration Method—In eight control studies without albumin, 8–18% of the [^{14}C]bilirubin adsorbed to the walls and frit of the Centricon device during the first centrifugation. With subsequent cycles, no additional adsorption was detected and the concentration of [^{14}C]bilirubin in the filtrate averaged 98 ± 2% of that in the retentate. Thus, no correction for binding to the Centricon was needed for cycles 2 through 5. Subsequent analysis used data from these cycles only.

Binding of bilirubin to the Centricon tubes during the first centrifugation was also seen in the presence of albumin, although it was much less pronounced. Radioactivity in the filtrate was 52 ± 30% greater (mean ± S.D., n = 112) after the second cycle than after the first. Assay of ultrafiltrates showed no detectable protein even after 10-fold concentration by lyophilization, indicating that the Centricon membranes were essentially impermeable to albumin. Our assay would have been sensitive to leakage of as little as 0.005 μg/ml albumin in the filtrate. This corresponds to leakage of less than 0.00005% of the albumin from a 1% solution, a value that is at least 1000 times lower than the corresponding fraction of radioactivity filtered (mean 0.22%, range 0.06–1.42%). Total albumin concentration in the solution was not affected by filtration followed by redilution to the original volume. However, total HSA and [^{14}C]bilirubin concentrations in the retained solutions declined by about 10% overall during cycles 2 through 5 because of removal of samples for assay followed by redilution to the original volume. Because we measured the values of [HSA] and [B_t] for each cycle, these small changes had no effect on results.

In control experiments at albumin concentrations of 13–17 and 80–90 μM, K'_f values did not differ between B_t/HSA molar ratios of 0.44–0.48 and 0.23–0.26 (data not shown). However, molar ratios >0.55 resulted in significantly larger values of K'_f, possibly reflecting formation of non-filtered bilirubin aggregates when B_F exceeds its solubility in water. To avoid problems related to saturation of the unbound bilirubin concentration, all studies were performed using molar ratios of 0.1–0.25.

Gradient Formation—At the conclusion of each centrifugation cycle, a steep gradient of increased yellow color was visible at the bottom of the retentate fluid. To determine the magnitude of these gradients, 25 μl samples were taken from the extreme top and bottom of the retentate in 4 control Centricons after the 5th spin cycle, being careful not to disturb the gradient. All tubes contained 40–50 μM HSA, whereas two contained sucrose buffer and two contained chloride buffer. Measured concentrations of HSA were 4.0 ± 0.4-fold higher at the bottom than at the top of the retentate column, whereas the comparable value for [^{14}C]bilirubin was 2.8 ± 0.3 (mean ± S.E., $p <$ 0.05 HSA *versus* [^{14}C]bilirubin). The potential impact of these gradients on our conclusions is considered later.

Results of Traditional Analysis—Most prior binding studies have analyzed their data assuming that no impurity is present. To allow comparison with these earlier studies, we first analyzed our data using cycles 2–5 as replicates without any correction for impurities. This analysis suggests a dramatic decrease in the apparent binding affinity of HSA for bilirubin at higher albumin concentrations (Fig. 1). The concentration-dependent change in K'_f was 9.5-fold in sucrose buffer (from $(5.09 \pm 0.24) \times 10^7$ to $(5.36 \pm 0.51) \times 10^6$ liters/mol) and 8.7-fold in chloride buffer (from $(2.19 \pm 0.14) \times 10^7$ to $(2.52 \pm 0.28) \times 10^6$ liters/mol). Moreover, K'_f was significantly greater

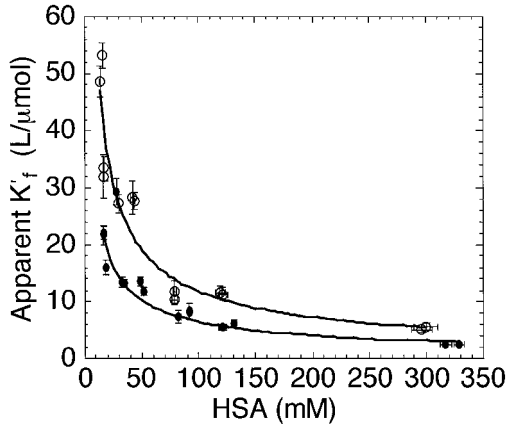

FIG. 1. **Apparent binding affinity of HSA for bilirubin as a function of albumin concentration assuming no impurity is present.** Each point represents a single Centricon tube, with data from centrifugations 2–5 treated as replicates. A 49–57% reduction in apparent binding affinity is present at all HSA concentrations in the presence of chloride (50 mM). This traditional analysis does not exclude the possibility that the apparent reduction is because of poorly bound radiolabeled impurities that constitute an increasingly large fraction of the filtered radioactivity at higher albumin concentrations. Albumin concentrations shown are the mean of values measured in the mixed retentate before and after filtration. Actual albumin concentrations immediately above the ultrafiltration membrane were larger by a factor of 4 or more because of gradient formation (see text). ○, sucrose; ●, chloride. *Error bars* are ± S.E.

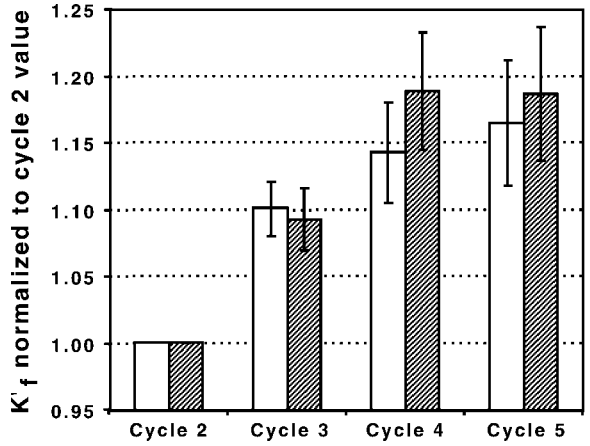

FIG. 2. **Increase in apparent binding affinity with number of centrifugation cycles.** Mean K'_f values (expressed as a fraction of their individual values for cycle 2) increased from the 2nd to the 5th cycle by 17 ± 5% in sucrose buffer (*open bars*) and 19 ± 5% in chloride buffer (*shaded bars*). The incremental increase in K'_f became less marked with each succeeding cycle, suggesting that impurity was lost by ultrafiltration more rapidly than it was being formed until a balance between these two processes was approached. *Error bars* are ± S.E.

in sucrose buffer than in chloride buffer (mean ratio 2.03 ± 0.54, $p < 0.0002$).

Analysis of Impurities—The decrease in K'_f with rising albumin concentration could also be explained by a poorly bound impurity that constitutes a relatively minor fraction of the unbound radioactivity at lower albumin concentrations, but most of the unbound radioactivity at higher albumin concentrations. If so, the true binding affinity K_F might actually be constant. This alternative interpretation must be excluded before variation in binding affinity with albumin concentration can be accepted. We therefore estimated the impurity concentration from the change in B_f with successive centrifugation cycles.

Change in Apparent Binding Affinity with Successive Cycles—The radioactivity in the filtrate (B_f) gradually decreased from the 2nd to the 5th centrifugation cycles by a mean of 18%, presumably reflecting progressive removal of impurities by filtration. Because the HSA concentration remained nearly constant, this decline resulted in a corresponding increase in the apparent binding affinity of albumin for bilirubin, K'_f (Fig. 2). The increase in K'_f became less marked with each succeeding cycle, suggesting that impurity was being lost by ultrafiltration more rapidly than it was being formed until a balance between these two processes was approached. The total increase in K'_f over cycles 2–5 averaged 17 ± 5% in the absence of KCl and 19 ± 5% with 50 mM KCl present ($n = 14$, mean ± S.E., Fig. 2). In one study, K'_f declined substantially over all cycles, presumably because of very high rates of impurity formation and/or development of leaks in the filter membrane. These data were excluded from subsequent analysis by replacing them with data from a replicate study done under identical conditions.

Estimation of Impurities by Modeling—The amount of impurity needed to cause the observed change in K'_f for each pair of Centricon tubes was estimated by modeling the K'_f data for each cycle as described in "Experimental Procedures." All models considered included the initial impurity level, I_0, as a fitted variable (allowed range, 0–100% of filtered radioactivity). All models also included possible binding of impurity to albumin by incorporating the binding affinity of impurity for albumin, K_I, as a fitted parameter (allowed range, 0-K_F).

Models that were considered differed only by whether impurity was generated during each cycle and if so, whether the rate constant for impurity formation, k, was proportional to the unbound bilirubin concentration (model A), the total bilirubin concentration (model B), or a constant independent of the bilirubin concentration (zero-order, model C). Thus, the unknown parameters in the fitting process were I_0, K_I, and k.

Both models A and B satisfactorily accounted for the data with reasonable values of the unknown parameters (Table I). All parameter values were adequately identified by the data. Thus, the uncertainty (S.E.) in I_0 was only 16% of the mean for both models whereas the uncertainty in K_I was 18% for model A and 49% for model B. The corresponding uncertainties for k were 9 and 15% for models A and B in sucrose buffer, and 6 and 17% in chloride buffer. The goodness of fit was comparable for models A and B (ratio of the r^2 coefficients of variation was 1.0). Thus, both models A and B appear to be reasonable interpretations of the data. In contrast, fits using model C did not converge for most experiments despite trying a wide range of starting parameter values. Model C was thus eliminated from further consideration.

From this we conclude that impurity is generated during the centrifugation process with a rate that is proportional to the bilirubin concentration. However, our data are not sufficient to determine whether impurity formation is proportional to the unbound or to the total bilirubin concentration. Fortunately, our conclusions are identical whether model A or model B is used to analyze the data. Specific results are presented for each model below.

Initial Impurity—The fitted value of I_0 was identical for both models A and B (0.11–0.14% of total radioactivity, Table I). This is expected because the level of preformed impurity should be independent of whether subsequent impurity is generated from unbound or total bilirubin. Thus, I_0 cannot be used to discriminate between models A and B.

Rate of Impurity Formation—Model A: The overall rate of impurity formation according to model A was 29.6 ± 0.2% of the unbound bilirubin concentration per cycle and was smaller in chloride (21.5 ± 2.2%) than sucrose (37.7 ± 3.4%) buffer ($p <$ 0.005, Table I). *Model B:* The mean rate of impurity formation according to model B was 0.0302 ± 0.0048% of the total bilirubin

TABLE I

Estimates of impurity levels and formation rates using different model assumptions

Results are mean ± S.E. (n = 14). For each Centricon pair (containing identical concentrations of bilirubin and albumin in either sucrose or chloride buffer), K'_f values for cycles 2 through 5 were fitted to models A, B, and C, and the resulting data averaged to give the values shown. I_0 is the amount of unbound radiolabeled impurity present in the bilirubin solution prior to the first centrifugation, expressed as a percent of the total bilirubin concentration. K_I is the apparent binding constant of the albumin for impurity, while k is the fraction of the unbound (model A) or total (model B) bilirubin converted to impurity per centrifugation cycle. Model C assumes zero-order degradation of bilirubin, in which the rate of impurity formation is a constant independent of the unbound or total bilirubin concentrations. Because >99.8% of bilirubin was bound at all albumin concentrations studied, bound and total bilirubin concentrations are considered identical for modeling purposes.

Model	Reaction order	Source of impurity	I_0	K_I	k with sucrose	k with chloride
			% of Bt	*liter/mole*	*cycle^{-1}*	*cycle^{-1}*
A	first	unbound	0.134 ± 0.023[a]	3550 ± 627[a]	$3.77 \pm 0.34 \times 10^{-1}$	$2.15 \pm 0.22 \times 10^{-1}$[b]
B	first	total	0.115 ± 0.018[a]	482 ± 236	$2.53 \pm 0.39 \times 10^{-4}$	$3.50 \pm 0.61 \times 10^{-4}$[b]
C	zero	—	fits did not converge			

[a] $p < 0.00005$ *versus* zero.
[b] $p < 0.005$ *versus* sucrose.

per cycle and was slightly larger in chloride (0.035 ± 0.006%) than sucrose (0.025 ± 0.004%) buffer ($p < 0.005$, Table I).

Binding Affinity of Impurity for Albumin—The biggest difference between models A and B was the binding affinity of the impurity for albumin (K_I), which was 3,550 ± 627 liters/mol for model A, and only 482 ± 236 liters/mol for model B. The latter value is low enough that less than 10% of the impurity should be bound even at the highest albumin concentrations used and is not significantly different from zero ($p = 0.06$). For model A, however, the fraction of bound impurity over the range of HSA concentrations studied is 5–53% percent.

Indifference of K_F Values to Model Selection—Although we were unable to differentiate between model A and model B, resulting values for K_F turned out to be virtually identical for both models (mean ratio 1.03 ± 0.03 for chloride buffer and 0.97 ± 0.07 for sucrose buffer, n = 14 for each). This indifference to model selection reflects the fact that I_0, which is treated the same in both models, was the most important source of impurity. Values for model A were arbitrarily selected for Fig. 3.

Effect of Buffer Composition—At all albumin concentrations studied, binding was considerably less avid in the presence of 50 mM KCl than in its absence. This conclusion holds whether or not the data have been corrected for impurity. Thus, the uncorrected affinity (K'_f) was on average 2.03 ± 0.54-fold greater in the absence of chloride (Fig. 1, $p < 0.0001$), whereas the corrected affinity (K_F) was 2.63 ± 0.10-fold greater in the absence of chloride (Fig. 3, $p < 0.0001$). The relative reduction in K_F with rising albumin concentration was, however, unaffected by buffer composition (Fig. 3, *inset*). Ion substitution studies (Fig. 4) showed that 50 mM NaCl decreased K'_f to the same extent as 50 mM KCl, whereas 50 mM potassium gluconate did not. Thus, the reduction in affinity appears to be due to chloride ion and is not due to sodium ion or overall ionic strength.

Effect of Albumin Concentration—The value of K_F decreased more than 6-fold as the HSA concentration increased from 18 to 320 μM (Fig. 3). Thus, K_F decreased from (5.37 ± 0.26) to (0.65 ± 0.03) × 10^7 liters/mol in sucrose buffer and from (1.70 ± 0.06) to (0.28 ± 0.01) × 10^7 liters/mol in chloride buffer. These changes were highly significant ($p < 0.0001$). Most of the decline occurred at albumin concentrations below 100 μM (0.67 g/dl), concentrations that are below the range encountered in health and disease (typically 750–200 μM, Ref. 26). However, if we multiply the albumin concentrations by a factor of 4–10 to adjust for albumin gradients generated during centrifugation, the shift in K_F occurs within the physiologic range.

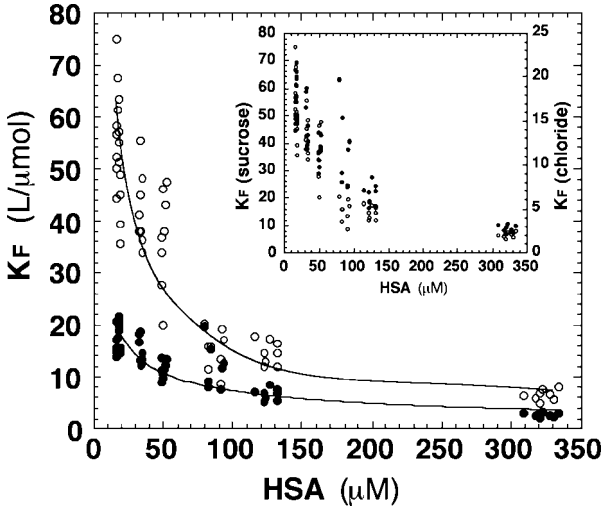

FIG. 3. **Binding affinity of HSA for bilirubin as a function of albumin concentration after correction for impurities.** The average correction is small (compare with Fig. 1), reflecting the fact that most impurities are removed by filtration. The relative decline in affinity is similar with and without chloride (see *inset*). Albumin concentrations listed are the mean of values measured in the mixed retentate before and after filtration. Actual albumin concentrations immediately above the ultrafiltration membrane were larger by a factor of 4 or more because of gradient formation (see text). ○, sucrose; ●, chloride.

DISCUSSION

Most prior studies of the binding of bilirubin to albumin have assumed that poorly bound impurities constitute a sufficiently small fraction of the unbound bilirubin concentration that they may be ignored. Whereas this may be true at very low albumin concentrations, selective binding of bilirubin but not impurities at higher albumin concentrations magnifies the impact of any impurities present. For this reason, and to decrease consumption of expensive HSA and bilirubin, most investigators have avoided measuring K_F at physiologic albumin concentrations.

In this study, we started with a highly purified preparation of [^{14}C]bilirubin and then estimated the initial impurity level, which averaged 0.13 ± 0.02% of total bilirubin. We also estimated the rate of new impurity formation, which averaged 0.030 ± 0.005% of total bilirubin (or 26.6 ± 3.8% of unbound bilirubin) per centrifugation cycle. By knowing these values, we were able to estimate the true binding affinity K_F over a wide range of albumin concentrations up to about two-thirds of physiologic values. The required correction was relatively small, reflecting relatively low levels of impurities in our crystallized and serially pre-extracted bilirubin preparation that were further reduced by serial ultrafiltration. Higher albumin concentrations were not studied because ultrafiltration rates became too slow under the conditions of our assay.

Our results indicate that the binding affinity of albumin for bilirubin falls with rising albumin concentration by a factor of 6 or more. An earlier study from our laboratory using [^{3}H]bilirubin reached a similar conclusion, but yielded much lower

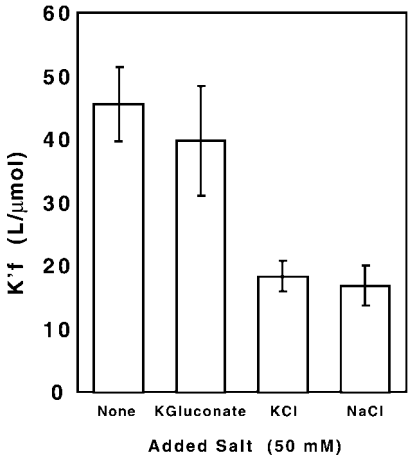

FIG. 4. **Effect of buffer composition on binding affinity of albumin for bilirubin.** K'_f was measured at pH 7.4, 23–25 °C, at HSA of 15–17 μM and B_t/HSA ratios averaging 0.25, using four different buffer compositions. Each value is the mean ± S.E. of eight K'_f values, derived from four ultrafiltration cycles in each of two Centricon-10 devices. The *left bar* shows the mean K'_f in standard sucrose buffer (20 mM HEPES-Tris buffer containing 250 mM sucrose). The other *bars* show the effects of isosmotically substituting 50 mM of the indicated salt for 100 mM sucrose. Buffers that lacked chloride had significantly greater K'_f values than those with chloride ($p < 0.00001$), whereas changes in cation or ionic strength had no apparent effect.

values for K'_f (5). This discrepancy probably reflects higher levels of unbound impurities in our earlier study because of exchange of tritium with the solvent. Because impurities were not measured, it is not possible to determine K_F.

Ahlfors (6) recently reported a 2.4-fold reduction in the affinity of human serum for bilirubin when measured at a 1:2.5 serum dilution when compared with at a 1:44 dilution. This study, which uses a sophisticated peroxidase assay, confirmed his earlier observations using a simpler method (27). Interestingly, the steep increase in B_f with rising albumin concentrations reported in his earlier paper closely mirrors the change in K'_f seen in our present work, but was attributed to factors related to the assay method and to inhibitors of binding, rather than an intrinsic change in binding affinity (27). The greater reduction of K_F found in our study may reflect use of purified albumin instead of whole serum, differences in buffer composition, and correction for poorly bound impurities. In particular, serum contains fatty acids that are known to modulate the binding affinity of albumin for bilirubin (28). Direct comparison of these studies is further limited by the fact that the buffer compositions at the two dilutions studied by Ahlfors (27) were not comparable due to dilution of serum electrolytes, and newborn serum contains significant amounts of α-fetoprotein (29), which also binds avidly to bilirubin (30).

The concentration dependence of K_F is not without precedent. The binding affinity of albumin has been reported to vary with albumin concentration for numerous other metabolites and drugs, including cortisol (8, 11), sulfobromophthalein (12), thiopental (13), phenytoin (14), tryptophan (14, 15), sulfadiazine (9), salicylate (9), phenylbutazone (9), and a benzoic acid derivative (16). The mechanism of this effect is unknown, but may reflect formation of reversible aggregates of albumin at higher concentrations. The osmotic activity of albumin molecules is lower at physiologic concentrations than in dilute solution (7, 8). The mean radius of dissolved albumin molecules increases by 8% when the concentration is raised from 0.4% to 4% (9). Zini et al. (10) found evidence for reversible aggregates using electrophoresis, and suggested that only one-half of the albumin was monomeric at physiologic concentrations. Aggregation could greatly alter the binding affinity of albumin. After binding to bilirubin, albumin undergoes a conformational relaxation that increases the binding energy and affinity (31). Anything that inhibits this change would reduce K_F. For example, binding of fatty acids to separate binding sites on albumin reduces its affinity for bilirubin, presumably because albumin cannot optimize its conformation for both ligands simultaneously (28). A similar loss of conformational freedom might result from protein-protein interactions at higher albumin concentrations.

Preliminary mathematical modeling of our data suggest that the decrease in K'_F with albumin concentration may be explained by the tendency of albumin to self-aggregate at higher concentrations (7, 8, 10, 32–35), which appears to cause a reduction of binding affinity for various other ligands (8, 9, 16, 36, 37). Studies to test this hypothesis are underway but are beyond the scope of the present work.

Effect of Buffer Composition—Binding was considerably less avid in the presence of KCl than in the presence of sucrose. Ion substitution studies showed that this effect was because of chloride anion and not due to the cation, or, as previously suggested (3), to a change in ionic strength. Limited literature data suggest that chloride can bind the region 2 binding site of albumin, which is not responsible for bilirubin binding (38). However, chloride is known to modulate the conformation of albumin (39) and could thus allosterically modulate the binding affinity for bilirubin, as has previously been shown for warfarin (40). Alternatively, chloride, which is present in plasma at concentrations six orders of magnitude larger than unbound bilirubin, might competitively displace the bilirubin anion from its primary binding site on lysine 242 of albumin (41). Studies of a range of chloride concentrations and a variety of other anions are needed to determine the specificity and mechanism of the chloride effect on binding.

Effects of Model Selection—Our primary conclusions do not depend on the model used to analyze the data. Indeed, the increase in K_F caused by correction for impurities was small (compare Fig. 1 with Fig. 3), and the effect of concentration on albumin binding was similar for both models A and B. Similarly, the effect of chloride on binding was similar both before and after correcting for impurities. Thus, our conclusions appear to be robust and model-independent.

The increase in K_F compared with K'_f was highly significant, indicating that correction of the binding constant for impurities was necessary to get an accurate binding constant ($p < 0.0002$ by Student's paired t test). The required correction was relatively small because we used multiple steps to remove polar impurities from the [^{14}C]bilirubin prior to use. This left relatively little impurity to correct for by modeling. Modeling was nevertheless essential, however, because without it, there would have been no way to know how closely K'_f approximated K_F.

We included k and K_I in our models for two reasons. First, bilirubin is known to undergo photooxidation (18–20) and photoisomerization (42), and some impurities must therefore have formed despite all precautions. Second, the binding affinity of the impurities for albumin is not necessarily zero. Photoisomers of bilirubin bind to albumin, although with lower affinity than native bilirubin (17, 43). Likewise, bilirubin photooxidation products include molecules such as biliverdin that may bind to albumin (17, 20, 44). If binding occurs, the fraction of the impurities removed with each centrifugation cycle will be less than the fraction of the total volume filtered. Whereas we were able to identify the values of k and K_I, we were unable to determine whether the newly formed impurities were derived mainly from unbound or total bilirubin. Fortunately, similar values for K_F were returned for each Centricon pair regardless of which model was used to analyze the data.

Serial ultrafiltration offers two major advantages for measuring K_F. First, a much higher fraction of the poorly bound impurity is filtered each cycle than of authentic bilirubin. This leads to removal of nearly all preformed impurity after a sufficient number of filtration cycles, and is one of the reasons why K'_f and K_F are so similar in our study. We have previously used a similar sequential strategy to measure the binding affinity of [^{14}C]palmitate, another avidly bound ligand of albumin (45). Second, the impurity level may be estimated from the change in the fraction of filtered radioactivity with each cycle, allowing correction of the affinity for the effects of impurities.

On the other hand, ultrafiltration has been criticized as a method for measuring unbound bilirubin because leakage of even a small amount of albumin (with its bound bilirubin) across the membrane can invalidate the results (46). Fortunately, ultrafiltration membranes have improved dramatically since this concern was raised. Using a sensitive protein assay on concentrated samples of the filtrate, we found that leakage was at least 100 times below the level needed to produce a 10% error in our results.

Limitations of the Current Approach—A limitation of this study is that ultrafiltration generates concentration gradients within the retentate because of the selective passage of solvent and unbound bilirubin, but not albumin, from the thin layer of solution located just above filter membrane. Direct measurements indicated that the albumin concentration just above the membrane was ~4-fold greater than in the bulk solution. Gradients may have been still larger if significant dissipation occurred prior to and during sampling.

At the relatively low centrifugal forces used here, sedimentation of albumin and bilirubin is negligible, and movement of both solutes toward the membrane is mainly by solvent drag. The gradient becomes gradually steeper during the centrifugation, but the concentrations of albumin and bilirubin in the bulk solution above the gradient remains unchanged. The albumin concentrations reported above are the means of the pre- and post-centrifugation values in the mixed retentate, and do not take into consideration gradient formation.

Despite this uncertainty, the major conclusions of this paper appear secure for several reasons. First, the albumin concentrations above the membrane, whereas not identical to the measured values in Figs. 1 and 3, should be proportional to them. Thus, the main effect of gradients is to shift the steep portion of the curves in Figs. 1 and 3 rightward, toward higher albumin concentrations. Ahlfors binding data in serum (6, 27), obtained with the peroxidase method, suggests that this shift is not marked and that the steep decline in K_F occurs below physiological albumin concentrations. Second, B_F should not be affected by gradient formation at a constant ratio of bilirubin to albumin unless K_F is, in fact, concentration-dependent. The law of mass action states that for HSA concentrations sufficient to bind nearly all of the bilirubin, B_F at each level of the retentate is entirely determined by K_F and the ratio of bilirubin to albumin (4). Because B_F changes with albumin concentration, despite the fact that this ratio is constant, it follows that K_F must vary with the albumin concentration.

Thus, the major uncertainty is not whether binding of bilirubin is concentration dependent, but the precise range of albumin concentrations over which the change occurs. If it coincides with the physiologic range, an increase in the binding affinity of albumin for bilirubin might help compensate for the lower concentration of albumin associated with certain disease states.

Interpretation of our data is also limited by the fact that none of the buffers tested contained physiological ion concentrations. These buffers were chosen to allow comparison with earlier vesicle transport studies using the same preparations of [^{14}C]bilirubin and albumin (47, 48) and not to simulate neonatal plasma. Thus, neither our values of K_F, nor those obtained by other methods (3) may represent the true affinity of bilirubin for HSA in the plasma of jaundiced neonates, and further studies are needed to determine the K_F values in neonatal plasma.

Physiological and Clinical Implications—Our value for K_F using 60 μM HSA in sucrose buffer (Fig. 3) is less than half the most widely accepted literature value of $\sim 6 \times 10^7$ liters/mol, which was measured under comparable conditions (~60 μM albumin and low chloride concentrations) using the peroxidase method (3, 41, 49). Although application of the peroxidase method to newborn serum is associated with a number of limitations (6, 50–53), a substantially improved version of this method was recently reported by Ahlfors that gives K_F values for a 1:2.5 dilution of plasma (about 120 μM HSA, 40 mM chloride) of 1.7×10^7 *versus* 0.7×10^7 liters/mol for our study under comparable conditions (Fig. 3). The difference may reflect the presence of fatty acids in serum, which increase the affinity of albumin for bilirubin ~3-fold (28).

Accurate knowledge of K_F at physiologic albumin concentrations is important for understanding the mechanisms of hepatic bilirubin clearance and bilirubin encephalopathy. Efficient hepatic clearance of avidly bound molecules can be difficult to reconcile with the very small concentrations of the unbound form thought to be present outside the plasma membrane (54). If unbound concentrations are larger at physiological albumin concentrations than currently believed, this problem disappears.

Conversely, the current results also create some problems. Many bilirubin transport studies have assumed that K_F remains constant when other variables change. Failure of this assumption may require reinterpretation of these studies. For example, the enhanced uptake of bilirubin that is observed when KCl and valinomycin are added to vesicle suspensions (5, 48) may result not just from enhanced electrical potential across the membrane, but also from reduced albumin binding because of chloride. Similarly, addition of chloride, but not other anions, has been shown to stimulate uptake of bilirubin and other organic anions from albumin solutions by perfused liver and cultured hepatocytes (55). Although this effect may reflect a chloride requirement of the membrane transporter as suggested by the authors, it could also reflect higher unbound bilirubin concentrations in the medium in the presence of chloride. In conclusion, the dependence of K_F on conditions underscores, as previously emphasized (5), the need to measure unbound concentrations using the same albumin and ligand preparations and the same solution compositions as are used for transport measurements.

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Affinity of Human Serum Albumin for Bilirubin Varies with Albumin Concentration and Buffer Composition: RESULTS OF A NOVEL ULTRAFILTRATION METHOD

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