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

RESULTS OF A NOVEL ULTRAFILTRATION METHOD*

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Albunin 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 [14C]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 [14C]bilirubin was strongly dependent on assay conditions, falling from (0.39 ± 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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† The abbreviations used are: K_F, actual formation (association) constant for binding of bilirubin to HSA; B_T, actual unbound bilirubin concentration; B_a, apparent unbound bilirubin concentration; B_p, apparent total bilirubin concentration; B_T, apparent total bilirubin concentration; HSA, human serum albumin concentration; I, concentration of unbound impurities in solution (derived variable); k_i, 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_F, formation (association) constant of f 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 radio-labeled 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 [14C]Bilirubin—**Radiolabeled bilirubin conjugates were purified from bile after intravenous infusion of 29954 of 30 mCi of 14C-labeled bilirubin dissolved in 30 μl of MeSO 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 [14C]bilirubin. Radio-assay 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). Centricrons, presoaked overnight in appropriate buffer, were flushed dry with argon and loaded with 2.5 ml of buffered [14C]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 5000 × 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 [14C]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 [14C]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-$B_f$ + $B_t$]. $[B_f]$ is the total measured bilirubin concentration.

$$K_F = \frac{B_t - B_f}{[\text{HSA}] - [B_f] + [B_t]} \quad (\text{Eq. 1})$$

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 $B_f$ to HSA was always ≪ 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,

$$K_F = \frac{(B_t - I) - (B_f - I)}{(B_t + I) ([\text{HSA}] - (B_f + I) + (B_t + I))} \quad (\text{Eq. 2})$$

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 if we know \(B_B\), or can calculate \(B_B\) if we know \(I_0\). And that is the case for each pair of Centricon tubes. Second, all parameters must be adequately identified by the data (S.E. of the fitted values <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.

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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 Centrifugation Cycles**—The radioactivity in the filtrate \(B_f\) gradually decreased from the 2nd to the 5th centrifugation cycles by a mean of 18% decreasing 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.

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 \(I_0\), \(K_F\), 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 \(K_F\) 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.
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 ($K_F$), 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 ($K'$) 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 $K'$ to the same extent as 50 mM KCl, whereas 50 mM potassium glucanate 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) × 107 liters/mol in sucrose buffer and from (1.70 ± 0.06) to (0.28 ± 0.01) × 107 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.

**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

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**TABLE I Estimates of impurity levels and formation rates using different model assumptions**

| Model | Reaction order | Source of impurity | $I_0$ | $K_F$ with sucrose | $K_F$ with chloride |
|-------|----------------|------------------|-------|---------------------|---------------------|
| A     | first          | unbound          | 0.134 ± 0.023 $^a$ | 3550 ± 627 $^a$ | 3.77 ± 0.34 × 10^{-1} |
| B     | first          | total            | 0.115 ± 0.018 $^a$ | 482 ± 236       | 2.53 ± 0.39 × 10^{-4} |
| C     | zero           |                  | —             | 3.8% of unbound $f$ | 3.50 ± 0.61 × 10^{-4} |

$^a$ p < 0.00005 versus zero.

$^b$ p < 0.005 versus sucrose.
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 a 1:1.44 dilution. This study, which uses a sophisticated peroxidase assay, confirmed his earlier observations using a simpler method (27). Interestingly, the steep increase in $B_t$ 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 $\alpha$-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 $K_F$ 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 $K_F$, 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 \( K_P \) are so similar in our study. We have previously used a similar sequential strategy to measure the binding affinity of \(^{14}\text{C}\)bilirubin and albumin (47, 48) and not to simulate neonatal plasma. Thus, neither our values of \( K_P \), 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 \( \mu \text{M} \) 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 (\( \sim 60 \mu \text{M} \) 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 \( \mu \text{M} \), 40 \( \text{mM} \) chloride) of \( 1.7 \times 10^7 \) versus \( 0.7 \times 10^7 \) 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 \( \sim 3\)-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 physiologic 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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