Kinetics of the Reactions of Unconjugated and Conjugated Bilirubins with \( p \)-Diazobenzenesulfonic Acid

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We report the kinetics of the reactions of unconjugated bilirubin and conjugated bilirubin with \( p \)-diazobenzenesulfonic acid in aqueous media. Our studies confirm that each reaction proceeds in two steps and that the second step is catalyzed by sulfanilic acid. In the presence of an excess of \( p \)-diazobenzenesulfonic acid and in the absence of sulfanilic acid, the reaction for either unconjugated or conjugated bilirubin proceeds in two successive first-order steps, the second step being much the slower. This study emphasizes the first step in the reaction for each species and includes data on the effects of \( p \)-diazobenzenesulfonic acid, albumin, benzoate, and caffeine concentrations, \( pH \) in the range from 4 to 12, and temperature. Mechanisms proposed for reactions with and without caffeine are used to develop rate equations, and the kinetic data are used to evaluate rate constants, acid dissociation constants for the different bilirubin species, and formation constants for bilirubin–caffeine complex species that are proposed.

Additional Keyphrases: van den Bergh reaction • variation, source of • assay for "direct" and "indirect" bilirubin • reaction mechanisms • constants for rate, dissociation, and formation • rapid-scanning spectrometry

The coupling reaction between diazotized sulfanilic acid and bilirubin, sometimes called the van den Bergh reaction (1), is used extensively to determine bilirubin. Common procedures for assay of so-called direct and indirect bilirubin make use of the difference in kinetic behavior of the different forms of bilirubin in the absence and presence of methanol (2). Although numerous papers (3) have described modifications of the original van den Bergh method, detailed kinetic studies of the system are few. Aside from a legitimate scientific interest in the reaction system, there are at least two practical reasons why a detailed understanding of the kinetic behavior of unconjugated bilirubin (UCB) and conjugated bilirubin (CB) with the reagent \( p \)-diazobenzenesulfonic acid, under a variety of conditions, is in order. The first is that more complete kinetic data are needed from which to judge how reliable or unreliable the common "direct" and "indirect" procedures for CB and UCB are likely to be. The other reason is that more complete kinetic data may permit improved analytical procedures to be developed.

One of the earliest kinetic studies involved unconjugated bilirubin at low \( pH \) in a chloroform–ethanol–water mixture and concluded that production of azobilirubin takes place in two steps, as follows:

\[
B + D \rightarrow A_1 + HPC + H^+ \quad (1)
\]

and

\[
HPC + D \rightarrow A_2 + CH_2O \quad (2)
\]

where \( B \) is bilirubin, \( D \) is \( p \)-diazobenzenesulfonic acid, \( HPC \) is hydroxypyrrromethene carbinol, and \( A_1 \) and \( A_2 \) are azobilirubin isomers (4). A later study, involving sera in aqueous media, showed that with a large excess of the diazonium salt, the reaction occurred in four pseudo-first-order phases that were attributed to conjugated and unconjugated bilirubin in serum (5). It was concluded that the so-called direct and indirect procedures give only estimates of the concentrations of CB and UCB. Other kinetic data have been reported (6), but the most extensive study involved a detailed investigation of the behavior of both bilirubin species in water, acetone–water, and chloroform–acetone–water solvent systems (7). While these studies have provided useful information, they still leave many important questions related to the kinetic behavior of the reaction system unanswered.

In this work we have attempted a more complete study of the kinetic behavior of unconjugated and conjugated bilirubin reacting with the diazonium salt, \( p \)-diazobenzenesulfonate, in aqueous media. Variables studied included UCB and CB concentrations, \( pH \) in the range from 4 to 12, diazonium salt concentration, albumin concentration, sodium benzoate, caffeine, and temperature. We then used these data to propose reaction mechanisms for UCB and CB, and to develop kinetic equations that describe the system under a wide variety of conditions. Numerical values are reported for rate constants, bilirubin-acid-dissociation constants, and bilirubin–caffeine formation constants.

The reaction is observed to be first order in bilirubin and in \( p \)-diazobenzenesulfonic acid concentrations. Caffeine impedes the reaction, and benzoate added in the presence of caffeine tends to counteract the effect of caffeine and enhance the reaction rate. We conclude that caffeine forms an unreactive complex species with bilirubin and that benzoate competes with the bilirubin for caffeine and in the process frees up some bilirubin that would otherwise be complexed with caffeine. The \( pH \) profiles suggest three different reactive species, with the most highly protonated bilirubin species being the least reactive and the least highly protonated species being the most reactive.
Materials and Methods

General Considerations

Early in this study we discovered that the sulfanilic acid used to prepare the diazonium salt acts as a catalyst for the second step in the formation of azobilirubin (reaction 2). In the presence of an excess of sulfanilic acid, the rate constant for reaction 2 approaches that of reaction 1, and the system exhibits complex kinetic behavior and is difficult to treat quantitatively. However, it can be shown that as the concentration of sulfanilic acid is reduced, the rate constant for reaction 2 is small compared to that for the first, and pseudo-first-order conditions can be maintained by working with an excess of p-diazobenzensulfonate. These studies were carried out under those conditions that yielded pseudo-first-order behavior, with bilirubin being the rate-limiting reactant.

The reactions are moderately rapid, having half-lives in the range from 0.002 to 0.4 s depending upon the reaction conditions. For this reason, we used a stopped-flow mixing system to achieve the needed mixing times.

Instrumentation

The stopped-flow mixing system, described in detail earlier (8), is described only briefly here. A commercially available stopped-flow system (Amino-Morrow; American Instrument Company, Silver Spring, Md. 20910) with a 10-mm path length cell (9) was modified to permit computer control of the sample- and reagent-mixing step by use of the sampling system described earlier (10, 11), with a stabilized photometer (12). Briefly, sample and reagent are drawn simultaneously into each of two sample loops. When the filling step is completed, the valve positions are changed so that the wash-out reagents can force the sample and reagent out of their respective loops, through a mixing chamber, and into the observation cuvet. The net results of these operations are that sample and reagent are thoroughly mixed in 5 ms or less, and the sample and reagent loops are flushed with the wash-out reagent and ready for the next run. All of these operations are controlled with the hierarchical combination of a minicomputer and microprocessor described earlier (8). The computer collects and processes the data with a variety of programs, also described earlier (8, 12).

Equilibrium spectral and pH measurements were made with conventional instrumentation. For spectral studies of reaction processes we used a vidicon-based rapid scanning spectrometer (14) fitted with a stopped-flow mixing head (15). The ionic strength (μ = ½ Σ z C z 2 , where C is concentration and z is the charge) was maintained at 1.2 with NaClO 4 , and pH values were not corrected for ionic strength when converted to hydrogen ion concentration.

Reagents

All reagents were prepared in doubly distilled water (Megapure Still; Corning Glass Works, Corning, N.Y. 14830). AR-grade chemicals, if available, were used to prepare the following stock solutions.

Unconjugated bilirubin. Synthetic solutions of UCB were prepared by the method of Doumas et al. (16) from a commercial preparation of the compound (lot no. 5256, Harleco, Gibbstown, N.J. 08027) certified by the College of American Pathologists. Samples (usually 171 μmol/liter or 100 mg/liter) were prepared to contain 0.0 to 80 g of human serum albumin (HSA; Fraction V, lot no. A2386; Sigma Chemical Co., St. Louis, Mo. 63178) per liter. Samples were kept frozen in the dark until needed.

Conjugated bilirubin. Bilirubin diglucuronide was isolated by the method of Lucassen (7) from bile of patients with a temporary biliary fistula. The brown amorphous powder is reported to be 80 to 90% bilirubin diglucuronide, the major impurities being water and sodium hydroxide (7). When stored in the dark over concentrated sulfuric acid in a vacuum desiccator, the powder was stable for at least one year. Samples containing, per liter, about 171 μmol of CB and 0.0 to 40 g of HSA, in water, were prepared and kept frozen at 30 °C in the dark until needed. Frozen samples containing HSA showed only slight decomposition during a month, but samples without HSA were labile, decomposing in a matter of days, as evidenced by the green color characteristic of oxidation of bilirubin.

Sulfanilic acid. Prepare a 0.05 mol/liter sulfanilic acid solution by dissolving 9.56 g of the reagent-grade sodium salt (Matheson, Coleman & Bell, Norwood, Ohio 45212) in water, adding 15 ml of concentrated hydrochloric acid (Mallinckrodt Inc., St. Louis, Mo. 63147), and diluting to 1 liter with water.

Sodium nitrite. Prepare a 0.1 mol/liter sodium nitrite (Baker Chemical Co., Phillipsburg, N.J. 08865) solution by dissolving 0.69 g of the reagent-grade salt in water and diluting to 100 ml.

Sodium perchlorate. Prepare a 2.0 mol/liter solution of sodium perchlorate (G. Frederick Smith Chemical Co., Columbus, Ohio 43216) by dissolving 245 g of the anhydrous salt in water, diluting to 1 liter, and filtering.

Caffeine. Prepare this solution by dissolving 65 g of caffeine (Sigma Chemical Co.), 97.5 g of sodium benzoate (Sigma Chemical Co.), and 1.0 g of disodium ethylenediaminetetraacetate (EDTA; Fisher Scientific Co., Fairlawn, N.J. 07410) in water and diluting to 1 liter. Filter the slightly turbid solution through a fine filter paper.

Sodium benzoate. Dissolve 97.5 g of sodium benzoate (Sigma Chemical Co.) and 1.0 g of EDTA (Fisher Scientific Co.) in water and dilute to 1 liter.

Sodium acetate, 2.0 mol/liter. Dissolve 164 g of the anhydrous salt (Mallinckrodt, Inc.) in water and dilute to 1 liter.

Sodium phosphate, monobasic, 2.0 mol/liter. Dissolve 276 g of NaH 2 PO 4 H 2 O (Mallinckrodt, Inc.) in water and dilute to 1 liter.

Sodium phosphate, dibasic, 2.0 mol/liter. Dissolve 284 g of Na 2 HPO 4 (Mallinckrodt Inc.) in water and dilute to 1 liter.

Sodium carbonate, 2.0 mol/liter. Dissolve 212 g of Na 2 CO 3 (Mallinckrodt Inc.) in water and dilute to 1 liter.

Sodium bicarbonate, 2.0 mol/liter. Dissolve 168 g of NaHCO 3 (Mallinckrodt, Inc.) in water and dilute to 1 liter.

The following procedures for preparing working solutions apply for 100-ml volumes of each solution with a final ionic strength of 1.2.

Buffer-caffeine solutions. Add 57.7 ml of caffeine stock solution, 37.5 ml of sodium acetate stock, and various amounts of concentrated hydrochloric acid to give the desired pH and ionic strength and dilute to 100 ml final volume. Prepare phosphate and carbonate buffers containing caffeine by adding 57.7 ml of caffeine stock and appropriate amounts of the stock phosphate and carbonate buffer components to give the desired pH and ionic strength, and dilute to 100 ml.

Buffers-sodium benzoate solutions. For experiments to be run in the absence of caffeine, acetate, carbonate, and phosphate buffers were prepared as described above except that 57.7 ml of sodium benzoate was substituted for the caffeine stock. Appropriate amounts of all buffers were used to give a final ionic strength of 1.2.

β-Naphthol. Dissolve 2.06 g of sodium acetate (Mallinckrodt, Inc.) in 30 ml of water, add 180 mg of β-naphthol (Aldrich Chemical Co., Milwaukee, Wis. 53233) and dilute to 500 ml with 95% ethyl alcohol.

p-Diazobenzensulfonic acid. Add 10 ml of sulfanilic acid
stock to 10 ml of the sodium nitrite stock and permit the mixture to react in the dark for 15 min, then dilute to 100 ml. The resulting p-diazobenzenesulfonic acid solution is about 5.0 mmol/liter, with an excess of nitrite—added to ensure that all of the sulfanilic acid reacts so that it will not be present to catalyze the second step in the coupling reaction (equation 2 above).

Prepare working solutions of p-diazobenzenesulfonic acid of desired concentrations by dilution with 2.0 mol/liter sodium perchlorate to give an ionic strength of 1.2. When stored in a dark bottle at room temperature, this reagent decomposes at rates of about 5% after 8 h and 13% after 24 h, with the formation of a yellow product.

Standardize the working solutions by a colorimetric method based on a coupling reaction with β-naphthol (7). Before applying the procedure, we had to determine the molar absorptivity of the coupling product. A small quantity of pure p-diazobenzenesulfonic acid was isolated as the chloride salt (17) and purified as described earlier (7). A series of standards (75 to 275 μmol/liter) in 1.2 mol/liter sodium perchlorate was prepared by weighing the dry salt and processed by the procedure described below. The slope of the absorbance vs. concentration plot of the coupling product yielded a value of (2.44 ± 0.04) × 10^4 liter mol⁻¹ cm⁻¹ for the molar absorptivity at 486 nm.

In the analysis procedure, dilute and mix exactly 1.00 ml of p-diazobenzenesulfonic acid with 4.00 ml of water. Then add 2.00 ml of β-naphthol solution to 180 μl of the diluted reagent, mix, and permit to react for 5 min, then measure the absorbance in a 1.00-cm cell at 486 nm vs. a blank containing 1.2 mol/liter sodium perchlorate substituted for the p-diazobenzenesulfonic acid. Compute the concentration as C, μmol/liter = 2.48 × 10^4 × A_486, which takes into account the dilution factors and the molar absorptivity of the coupling product.

**Procedures**

**Kinetic studies.** Bilirubin and buffer–caffeine or buffer solutions were mixed in ratios of 1 to 4, the resulting solutions were mixed in the stopped-flow system with an equal volume of p-diazobenzenesulfonic acid, and the absorbance was monitored as a function of time. Some experiments were done with the rapid-scanning vidicon spectrometer, but most results reported are based upon absorbance measurements at 530 nm.

**Data collection and processing.** For each single-wavelength experiment, 250 absorbance values (A_t) were measured at equally spaced time intervals during the first three half-lives of the reaction. Equilibrium absorbance values (A_∞) were computed as the average of 30 values recorded after six half-lives. The total change in absorbance from the start to finish of the reaction (A_t − A_0) and apparent first-order rate constants were computed with a multilinear least-squares regression program (13). Rapid-scan experiments consisted of 50 spectra taken at equal time intervals (0 to 200 ms), with 100 points per spectrum over a 350-nm wavelength range, usually centered near 525 nm.

**Results and Discussion**

Unless stated otherwise, all uncertainties below are quoted in terms of one standard deviation unit (±1 SD) and all kinetic data correspond to 25.0 ± 0.1 °C, ionic strength of 1.2, and caffeine, sodium benzoate, and bilirubin concentrations in the final reaction mixture of 77.3 mmol/liter, 156 mmol/liter, and 17.1 μmol/liter, respectively. Additional statistical information given for the regression data are the standard error of estimate (SEE) and the correlation coefficient (r).

**Spectral properties and responses.** Figure 1a represents the spectra of unconjugated bilirubin, azobilirubin, and the intermediate, hydroxyprromethene carbinol, in water at pH 7 with caffeine and sodium benzoate. The bilirubin and azobilirubin spectra were obtained with a Cary 14 recording spectrophotometer by recording spectra of unreacted bilirubin and of the reaction mixture after sufficient time for the reaction to go to completion. The hydroxyprromethene carbinol spectrum was obtained from data recorded with the vidicon spectrometer.

Figure 1b represents several spectra recorded with the vidicon spectrometer from 4 ms after mixing (top curve at 450 nm and bottom curve at 530 nm) to 200 ms after mixing (bottom curve at 450 nm and top curve at 430 nm). The increase in absorbance at 530 nm corresponds to the increase in azobilirubin (equation 1). Careful examination of the curves in the region of 450 nm will show that as the reaction proceeds, there is a gradual shift in the absorption maximum toward shorter wavelengths. This results from the formation of the hydroxyprromethene carbinol. When the first reaction (equation 1) has gone to completion after some 200 ms, then the spectrum represents the mixture of azobilirubin and hydroxyprromethene carbinol, and the spectrum of the latter was easily obtained by difference, because the spectrum of azobilirubin had already been determined. The spectra in Figure 1a are very similar to those reported by Overbeek et al. (4). Here the spectrum of the intermediate was obtained without the laborious isolation procedures used by the earlier workers. This experiment demonstrates the power of the rapid-scanning spectrometer in kinetic studies of fast reactions that involve intermediates such as the hydroxyprro-
methene carbinol. Similar results are obtained for conjugated bilirubin.

The isosbestic point at 495 nm is significant, because it shows that amounts of azobilirubin produced are equivalent to the amounts of bilirubin reacted during the observation time. For example, if the second step in the reaction proceeded to any significant extent to produce more azobilirubin from the hydroxypropyromethene carbinol during the observation time, then the isosbestic point would not appear, but rather the absorbance would increase gradually at that wavelength.

We chose to monitor the formation of azobilirubin at 530 nm in the remaining studies because there is little or no interference from bilirubin or the intermediate at this wavelength.

Response curves. The ascending curve in Figure 2 represents the absorbance at 530 nm as a function of time for a typical set of reaction conditions and the descending plot represents ln (A∞ - A) vs. time. The plot of A vs. t shows that the reaction is approaching completion after 0.2 s, and that reliable data are being collected within a few milliseconds (~4 ms) after commencing the mixing process. The fact that the descending plot is linear confirms pseudo-first-order behavior. The horizontal plot represents deviations of individual points in the plot of ln(A∞ - A) vs. t from a least-squares fit of the line. The fact that these "residuals" are scattered randomly about the horizontal line confirms a high degree of linearity. Pseudo-first-order rate constants are determined as slopes of plots of ln(A∞ - A) vs. t such as that in Figure 2.

Effects of human serum albumin. We noted earlier that bilirubin solutions tend to be unstable in the absence of HSA, and accordingly it is desirable to quantify effects of HSA on the kinetics of the reaction. Figure 3 illustrates the dependence of the pseudo-first-order rate constant on human serum albumin, with UCB as reactant. The increase from 0 to 80 g/liter caused a reduction of about 9% in the rate constant. This decrease is caused by a rapid reaction between the p-diazobenzenesulfonic acid and the tyrosine groups on the albumin (7), the net effect being that the amount of diazo reagent is diminished. All subsequent experiments are performed with 3 g/liter albumin, corresponding to rate constants approximately 0.7% lower for unconjugated bilirubin than they would be without any albumin. Results for conjugated bilirubin are the same in the presence and absence of HSA.

p-Diazobenzenesulfonic acid dependence. Effects of the diazo reagent concentration on the first-order rate constant for UCB and CB are presented in the upper two lines in Figure 4 for no caffeine in the reaction solution and in the lower two lines for caffeine present. Important points to note from these data are that in all cases the first-order rate constant is proportional to the diazo reagent concentration for the range examined and the rate constants when caffeine is absent significantly exceed those when it is present. Evidently caffeine tends to impede the reaction of bilirubin that is in solution. In each case, the rate constant for UCB exceeds that for CB, but this is dependent upon pH, as will be noted later. The proportional relationship between the pseudo-first-order rate constant and p-diazobenzenesulfonic acid concentration is consistent with the general reaction scheme suggested in equation 1.

pH dependency. Dependencies upon pH for UCB and CB with and without caffeine are presented in Figures 5a and 5b. The ordinate is plotted on a log scale so that the wide range of constants can be presented effectively and the observed rate constant is normalized to the value of p-diazobenzenesulfonic acid concentration to account for the proportional relationship illustrated in Figure 4. In other words, dependence of k0 on diazo reagent concentration has been normalized to unit concentration.

For reactions in the presence of caffeine (Figure 5a), both UCB and CB exhibit a flat region in the range from pH 6 to 7.5. Below pH 6 the UCB rate constant falls off rapidly, so that
the reaction would require hours to go to completion below pH 5; the constant increases rapidly above pH 7.5 until it eventually levels off again above pH 11, where the reaction is very fast. These data suggest at least three reactive species in this pH range for UCB. The behavior of CB parallels that for UCB except that the rapid decrease in the rate constant below pH 6 was not observed experimentally.

Some additional points need to be made in connection with the data in the presence of caffeine. First, the rate constants for UCB below pH 5.5 are only estimates because absorbance changes were very small. Also, data were not collected below pH 5 because a precipitate (probably caffeine and benzoic acid) formed below this pH. Also, above pH 10.5 the uncertainty in the rate constants increased and the reported values are only estimates. Above pH 12 no reaction was observed because the diazonium salt is converted to an inactive hydroxide (18, 19).

The pH-dependency curves in the absence of caffeine are similar to those with caffeine, except that for CB the rate constant decreases between pH 4 and 6 as was the case for UCB in the presence of caffeine. These data also suggest there are three different reactive CB species in the pH range included in this study.

Some other points related to these data merit discussion. In the absence of caffeine, UCB precipitates below pH 7 and no kinetic data are reported for this range. Also, because sodium benzoate precipitates from the 156 mmol/liter solution below pH 5, kinetic studies for CB at lower pH values were performed with lower benzoate concentrations and extrapolated to 156 mmol/liter conditions with benzoate dependency data evaluated at higher pH (discussed later). Accordingly, the data below pH 5 for CB are only estimates. This procedure was used to extend the CB studies to pH 2; however, because there is little change between pH 4 and pH 2, the data below pH 4 are not included. Above pH 10, reliability of rate constants for both UCB and CB was degraded by the base-catalyzed decomposition of azobilirubin (7). This did not create a problem for the studies in the presence of caffeine, because caffeine inhibits the decomposition reaction.

Effects of pH on the total change in absorbance are presented (Figure 6) for solutions containing caffeine. The low values of ΔA for UCB at low pH probably result from the decreased solubility of the species. The wavelength for maximum absorption of the azobilirubin product remained constant at 530 nm over the pH range 4–12 for both UCB and CB.
Caffeine. Figure 7 shows dependencies of the observed rate constants for UCB and CB on caffeine. In both cases, the caffeine acts to inhibit the reaction velocity. The data could not be extended above 0.2 mol/liter because caffeine precipitates.

Sodium benzoate. Effects of benzoate on the rate constants are presented in Figure 8. Benzoate tends to increase the reaction velocity for both UCB and CB in the presence of caffeine.

Temperature. Figure 9 presents effects of temperature on the rate constants for UCB and CB. Activation energies for the HB specie reaction are 2.58 ± 0.13 kcal/mol for UCB and 4.85 ± 0.31 kcal/mol for CB, and the values of log A in the Arrhenius equation \( k = A e^{-Ea/RT} \) are 6.93 ± 0.09 and 8.04 ± 0.22 for UCB and CB, respectively.

Nitrous acid and EDTA. The reaction velocities for both UCB and CB are independent of nitrous acid and EDTA concentrations.

Discussion

The \( p \)-diazobenzenesulfonic acid dependency data in Figure 4 demonstrate a first-order dependency on diazo reagent. The pH dependency data in Figures 5a and 5b suggest that there are three reactive bilirubin species, which will be designated as HB (low pH), HI (intermediate pH), and B (high pH). The caffeine-dependency data in Figure 7 can be explained on the basis of formation of the non-reactive bilirubin–caffeine species. The benzoate data in Figure 8 tend to support this proposal, because benzoate is added to solubilize caffeine by the formation of an association complex (20). If benzoate complexes some caffeine, then bilirubin would be released and the rate would be expected to increase. These observations can be formulated into mechanisms for the reaction in the presence and absence of caffeine. Because the situation is simpler when no caffeine is added, this situation is discussed first.

Proposed Mechanism without Caffeine.

Identifying the three reactive bilirubin species as \( H_2B \), HB, and B and recalling the first-order dependence upon the diazo reagent (D), we propose the following reaction sequence.

\[
H_2B + D \xrightarrow{k_1} A_1 + HPC + H^+ \quad (3a)
\]

\[
HB + D \xrightarrow{k_2} A_1 + HPC + H^+ \quad (3b)
\]

\[
B + D \xrightarrow{k_3} A_1 + HPC + H^+ \quad (3c)
\]

The rate equation can be written in terms of the total concentration of bilirubin, \( C_B \), as follows,

\[
\frac{d[A_1]}{dt} = k_0 C_B \quad (4a)
\]

where the total bilirubin concentration is given by

\[
C_B = [H_2B] + [HB] + [B] \quad (4b)
\]

assuming \( H_2B-D \), HB-D, and B-D are in negligibly small steady-state concentration. Assuming the \( H_2B \), HB, and B species are involved in acid–base equilibria with equilibrium constants,

\[
K_1 = \frac{[HB]}{[H^+]^2} \quad \text{and} \quad K_2 = \frac{[B]}{[H^+]^3} \quad (4c)
\]

and assuming \( k_1 >> k_{-1}, k_{22} >> k_{-2}, \) and \( k_{33} >> k_{-3} \), it can be shown that (see Appendix)

\[
k_0 = k_1[H^+]^2 + k_2K_1[H^+] + k_3K_2 \quad (4d)
\]

which identifies the expected dependency of the observed rate constant diazo concentration, \( C_D \), upon acidity, rate constants for the individual steps, and the equilibrium constants. Experimental data can be used to evaluate the constants in this expression.

Parameter evaluations. It is necessary to make several simplifying approximations in order to evaluate the constants in equation 4d. For \( T < \text{pH} < 9 \), the terms involving \( [H^+]^2 \) and the product \( K_1K_2 \) can be expected to be small compared to the other terms, and equation 4d simplifies to

\[
k_0 \approx k_1[H^+] + k_2 \quad (5a)
\]

If this is a valid relationship, then a plot of \( k_0/C_D \) should be linear with slope equal to \( k_1K_2 \) and intercept equal to \( k_2 \). Plots for UCB and CB are given in Figure 10 and the slope and intercept data are included in the legend of the figure. The intercepts correspond to \( k_2 = (1.18 ± 0.03) \times 10^4 \text{liter/mol-s}^{-1} \) for UCB and \( k_2 = (3.10 ± 0.02) \times 10^4 \text{liter/mol-s}^{-1} \) for CB. The slopes correspond to \( k_1K_2 = (1.84 ± 0.01) \times 10^4 \text{liter}^{-1} \) for UCB and \( k_1K_2 = (1.48 ± 0.01) \times 10^4 \text{liter}^{-1} \) for CB.

At high pH (\( \text{pH} > 11.5 \)) the rate constant is independent of hydrogen ion concentrations (all of the bilirubin is converted to the B species) and the observed rate constant under these conditions is equivalent to \( k_3 \). Values of \( k_3 \) estimated
from the data in Figure 5b are \( k_0 = 2.75 \times 10^6 \) liter mol\(^{-1}\) s\(^{-1}\) for UCB and \( k_0 = 1.01 \times 10^6 \) liter mol\(^{-1}\) s\(^{-1}\) for CB. Combining these values with values for \( k_3 K_2 \) in the last paragraph leads to values of \( K_2 = (1.84 \times 10^{-4})/(2.75 \times 10^6) \), or \( 6.7 \times 10^{-11} \) mol/liter for UCB, and \( K_2 = (1.48 \times 10^{-4})/(1.01 \times 10^6) \), or \( 1.46 \times 10^{-10} \) mol/liter for CB.

The data for the \( p \)-diazobenzensulfonic acid dependency in Figure 4 were obtained at a pH where the pH profiles are flat, suggesting that most of the bilirubin is present as the HB species. Thus, the observed rate constants, when normalized to unit diazo reagent concentration, are representative of \( k_2 \). In other words, the equation for the straight line plots in Figure 4 is

\[
k_0 = k_2 C_D
\]

so that the slopes of the plots are equal to \( k_2 \) for UCB and CB. Values of \( k_0 \) obtained from these slopes are \( k_0 = (1.12 \pm 0.001) \times 10^6 \) liter mol\(^{-1}\) s\(^{-1}\) for UCB and \( (3.01 \pm 0.001) \times 10^4 \) liter mol\(^{-1}\) s\(^{-1}\) for CB. These values for \( k_2 \) agree well with those obtained above from the pH data and equation 5a.

Average values of \( k_2 \) from these two data sets and values for \( k_3 \) and \( pK_2 \) are included in Table 1.

Experimental difficulties involving insolubility of UCB and sodium benzoate prevented our collection of reliable kinetic data in regions of the pH profile where \( k_1 \) and \( K_1 \) are the limiting parameters (pH < 5) so it is not possible to obtain values for \( k_1 \) and \( K_1 \) as directly as was done for \( k_2, k_3, \) and \( K_2 \). Therefore, a nonlinear regression program was used to evaluate constants that would fit equation 4b to the pH profile data in Figure 5b. Values of \( k_3, k_2, \) and \( K_2 \) obtained by procedures described above were substituted as known quantities into equation 4b and values of \( k_1 \) and \( K_1 \) were computed with the regression program. Values obtained were \( k_1 = 2.32 \times 10^4 \) liter mol\(^{-1}\) s\(^{-1}\) for CB, \( K_1 = 3.16 \times 10^{-6} \) mol/liter for UCB, and \( K_1 = 1.7 \times 10^{-5} \) mol/liter for CB. Because the data at lower pH values are incomplete for UCB, it was not possible to obtain a reliable value of \( k_1 \) for UCB from these data. A value reported earlier (6) for slightly different conditions is included in Table 1 along with our values for \( k_2, k_3, K_1, \) and \( K_2 \) for UCB and \( k_1, k_3, K_3, K_4, \) and \( K_2 \) for CB. The solid curves in Figure 5b represent values computed by the regression equation and the constants in Table 1.

With these values for \( k_1, k_2, k_3, K_1, \) and \( K_2 \) in hand, attention can now be turned to the data in the presence of caffeine.

\[
\frac{d[A]}{dt} = k_0 C_B
\]

Proposed Mechanism with Caffeine.

The caffeine and benzoate dependency data in Figures 7 and 8 suggest an association between caffeine and the bilirubin species. The reaction sequence in equations 6a thru 6c is proposed to account for these observations where \( H_2B-Caf, HB-Caf, \) and \( B-Caf \) are assumed to be inactive forms of bilirubin as far as the diazo coupling reaction is concerned. It is also assumed that the uncomplexed \( H_2B, HB, \) and \( B \) species would undergo the same reactions represented in equations 3a–3c above with the same values of \( k_1, k_2, k_3, K_1, \) and \( K_2 \) as already determined (Table 1) for data collected without caffeine.

To derive a rate equation, we again start with the rate equation expressed in terms of the observed rate constant and the total bilirubin concentration.

\[
H_2B + Caf \xrightleftharpoons{} K_{c1} H_2B-Caf
\]

\[
K_1 \xrightleftharpoons{} K_{c1} HB + Caf \xrightleftharpoons{} HB-Caf
\]

\[
K_2 \xrightleftharpoons{} K_{c2} B + Caf \xrightleftharpoons{} B-Caf
\]

Using this equation for \( C_B, \) the acid–base constant expressions given earlier, and the following expressions for the bilirubin–caffeine equilibria,

\[
K_{c1} = [H_2B-Caf]/[H_2B][Caf]; K_{c2} = [HB-Caf]/[HB][Caf]; K_{c3} = [B-Caf]/[B][Caf]
\]

it can be shown that the observed rate constant \( k_0 \), is given by

\[
k_0 = \frac{k_1 [H^+]^2 + k_2 K_1 [H^+] + k_3 K_1 K_2}{C_D [H^+]^2 (1 + K_1 C_{Caf}) + [H^+] K_1 (1 + K_2 C_{Caf}) + K_1 K_2 (1 + K_3 C_{Caf})}
\]
CB, however, the diazo reaction is orders of magnitude faster at low pH values because the hydrogen-bonded structure is distorted, owing to a mutual steric hindrance of the bulky glucuronic acid groups, thereby forming a highly nonplanar distorted configuration. As pH increases in the range 4–8, the dissociation of the propionic residues and glucuronic residues results in further distortion of the molecules caused by the breakdown of hydrogen bonding and an electrostatic repulsion of the negatively charged propionic or glucuronic groups. This results in a dramatic increase in rate for UCB of four orders of magnitude caused by the molecule going from a rigid to a distorted configuration in this pH range. The increase in rate for CB in this pH range is only a slight one since the molecule is already in a distorted configuration when the glucuronic groups are protonated. Therefore, the distortion of the bilirubin molecule with increasing pH reduces steric hindrance of the reaction, thereby increasing the reaction rate.

As pH increases from 8–12 a second proton is dissociated from UCB and CB, resulting in a large increase in rate for both molecules. This rate increase is similar for both molecules, as seen by examining Figure 5a. This parallel behavior indicates that the same proton in the equivalent pyrrole ring system of each molecule is involved in the dissociation. This proton is a pyrrole ring N-H dissociation. The increase in rate for both molecules caused by the dissociation of this proton can be explained by a combination of two effects, namely a further distortion of the molecule due to breaking of the last hydrogen bond in the reactive portions of the molecule, and an electrostatic attraction between the resonance-stabilized negative charge now on the pyrrole ring system and the positively charged diazonium ion.

Other effects of increasing pH are seen in Table 1, where the values for the 1:1 bilirubin–caffeine association constants decrease as the bilirubin species change from H₂B to HB to B. There appear to be several effects operating here. The values for K₁₂ and K₁₃ are approximately equal for UCB and CB, indicating that conjugation is no longer important for the HB and B species ability to bind caffeine. The K₁₁ values are quite different, though, with the caffeine–CB value approximately three times larger than the caffeine–UCB value. Here, the conjugation is an important factor, and the additional proton on the glucuronic acid moiety in H₂B greatly affects the ability of the molecule to bind caffeine.

The overall effect of the decreasing caffeine–bilirubin association constants with pH can be explained by two effects. As the molecules go from H₂B to HB to B, the hydrogen-bonded structure breaks down and the molecules go from a rigid structure in UCB, or a semirigid structure in CB, to a more distorted structure. If the strength of the complex depends on the bilirubin molecule being rigid and exhibiting a high degree of planarity (20), then we would expect the association constants to decrease as the structure becomes more distorted. The second effect parallels the first and can be explained by hydrogen bonding. If the caffeine–bilirubin com-
plex depends upon hydrogen bonds between caffeine and bilirubin for its stability, then the association constants would be expected to decrease as either the protons involved in this bonding are dissociated, or, as already mentioned, the disto-
tion of the molecule changes the structure so hydrogen bonding between caffeine and bilirubin is no longer favored. This explanation appears adequate to explain why the first caffeine–bilirubin association constant for CB is larger than the constant for UCB. A close examination of glucuronic acid shows that it contains many groups other than the COOH group that can hydrogen bond. Therefore, the proton on the CB species H2B appears instrumental in permitting this hy-
drogen bonding to take place.

As a result of the decreasing caffeine–bilirubin association constants with increasing pH, more free bilirubin is present and the rate increases.

Nature of the reactive species. Until now, the nature of the reacting bilirubin species has been described only as H2B, HB, and B. In this section the probable identity of these species will be discussed, with use of Figure 12, which represents the structure for UCB. The corresponding structure for CB is formed by substituting the propionic residues with glucuronic acid (Figure 11). There are six possible hydrogen ions that can dissociate in this molecule. The propionic acid hydrogen ions denoted by a in Figure 12, the hydrogen ions on the outer pyrrole rings involved in the lactam–lactim tautomerism (24) denoted by b, and the hydrogen ions on the central pyrrole rings denoted by c.

Several of the dissociations can be explained by titration data. Recently Lee et al. (25) titrated UCB, using nonaqueous titrations in various organic solvents. The measured half-
neutralization potentials were correlated with known pK values to determine pK_a's in water of 4.3 and 5.3 for the ionization of the propionic acid hydrogens (a). These results confirm those obtained earlier by titrating the UCB suspended solid in water (26). In DMF, two additional protons were titratable, resulting in a major spectral change. During titration, the absorbance at \( \lambda_{max} \) 460 nm decreased and a dark-red color appeared, with \( \lambda_{max} \) 550 nm. The major spectral change accompanying titration of the third and fourth equivalents suggests that the hydrogens are coming from the chromophore structure. Gray et al. (27) also implicates the ionization of the N-H groups in the chromophores of bilirubin from spectro-
photometric titrations. These data, together with the data of Corwin and Brunings (28), suggest that the hydrogen ions are being abstracted from the pyrroles. The pK_a's for the titration of the third and fourth protons were not reported by Lee et al.

With these data, the following assignments can be made for the H2B, HB, and B species. For UCB, the H2B species has 1 a proton dissociated. Corresponding titration results for CB are not available. The same assignments hold, though, except that dissociation of the glucuronic acid a protons occurs at a lower pH because glucuronic acid is a stronger acid than propionic acid. The pK_a = 5.5 value for UCB determined kinetically is in good agreement with the value of 5.3 determined by the titration by Lee et al. (25) for the dissociation of the second a proton.

In assigning the N-H dissociation, we assumed that the b proton dissociated instead of the c proton. This is the most probable dissociation, because the dipyrromethene units in bilirubin each contain a carbonyl group which is capable of accommodating the anionic change that results from the NH group dissociating. Thus, the N-hydrogen atoms of the end pyrrole groups in the bis-lactam structure should be more easily ionized than the corresponding hydrogen ions in the central pyrrole centers.

Miscellaneous. One point that merits attention is the probable effects of impurities in the conjugated bilirubin preparation on rate constants determined for the CB reactions. Because CB is the rate-limiting species in the pseudofirst-order reactions, it is not necessary to know its exact concentration to obtain reliable rate constants. Also, because all plots of \( \ln(A_\infty - A) \) vs. t for CB were linear, we can be confident that there were no other species present that reacted to produce an absorbance at 530 nm with rate constants that differed significantly from that of conjugated bilirubin. There are numerous other procedures available for isolating CB (7, 29, 30, 31), but the method used in this work (7) appears to have been satisfactory.

One alternative approach to preparing \( p \)-diazobenzensulfonic acid free of sulfanilic acid is to prepare the fluoroborate salt (32, 33). While this method would have the advantage that the salt could be weighed directly, we judged that the procedure used here, in which sulfanilic acid is reacted with an excess of nitrous acid, offered the best chance of yielding a product completely free of sulfanilic acid.

Data obtained in this work have been used to develop a fast kinetic method for the simultaneous determination of unconjugated and conjugated bilirubin, and results are reported in the following paper.

Appendix

Derivation of equation 4d

Starting with equation 4c, it is easily shown that \([HB] = K_1[H2B]/[H^+] + K_2[H2B]/[H^+]^2\). When these quantities are substituted into equation 4b, that equation rearranges to give

\[
[H2B] = C_B/(1 + K_1/[H^+] + K_1K_2/[H^+]^2)
\]

(A-1)

By using equations 3a–3c it is shown that it is rate of formation of azobilirubin is given by

\[
dA_1/dt = k_{11}[H2B-D] + k_{22}[HB-D] + k_3 [B-D]
\]

(A-2)

Applying the steady-state approximation to equation 3a, it is shown that

\[
d[H2B-D]/dt = \frac{0}{k_{11} [H2B-D]} - k_{11} [H2B-D]
\]

(A-3)

which rearranges to give

\[
[H2B-D] = \frac{k_1 [H2B]}{k_{11} + k_{11}}
\]

(A-4)

which reduces to

\[
[H2B-D] \approx \frac{k_1}{k_{11}} [H2B][D]
\]

(A-5)

if it is assumed that \( k_{11} >> k_{11} \).

Similar procedures applied to equations 3b and 3c give

\[
[HB-D] = \frac{k_2}{k_{22}} [HB][D]
\]

(A-6)

and

\[
[B-D] = \frac{k_3}{k_{33}} [B][D]
\]

(A-7)

These equations can be substituted into equation A-2 to give a rate equation that is explicit in \([H2B], [HB], [B], \) and \([D]\). Expressions for HB and B given above in terms of \( K_1, K_2, \) and \([H^+]\) can be used to make the resulting expression explicit in \([H2B], \) and equation A-1 can then be used to make the expression explicit in the analytical concentration of bilirubin, \( C_B, \) that is used in equation 4a to relate the initial rate and observed rate constant to bilirubin concentration. These
substitutions accompanied by appropriate algebraic manipulations lead to equation 4d.

Equation 7 is developed by applying these same procedures to equations 6a–6c in combination with equations 3a–3c.

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