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Bilirubin in Aqueous Solution. Absorption Spectrum, Aqueous Solubility, and Dissociation Constants

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This report analyzes the change of both spectrum and solubility of bilirubin with pH in buffer solutions. Bilirubin (AH_2) is a dibasic acid which dissociates by two steps ($AH_2 \overset{K_1}{\rightleftharpoons} AH_1 \overset{K_2}{\rightleftharpoons} A$) with increasing pH; the wavelengths at the resulting three isosbestic points are 457 nm (AH_2 and AH_1), 485 nm (AH_1 and A), and 472 nm (A and AH_2). The solubility increases with increasing pH, where the total concentration ($[AH_2]+[AH_1]+[A]$) changes subject to a second-order curve of $1/a_{H^+}$. From the spectral change and the three coefficients of the solubility curve, K_1 and K_2 values were estimated to be $(6.7\pm2.6)\times10^{-7}$ and $(3.4\pm1.0)\times10^{-8}$, respectively. These dissociation constants are much smaller than those of aliphatic carboxylic groups, and the reason for it was discussed together with the values already reported and their reliability.

Bilirubin is one of the major components of gallstone[1,2] and is solubilized together with cholesterol in the mixed micelle of bile salts and lecithin in human bile.[3-6] Indeed, gallstone formation has been much investigated for twenty years from the view point of solubilization,[4-7] but the mechanism of the formation has not been made clear yet. Through these studies, the aqueous solubility of free bilirubin has been found to play a very important role in an elucidation of gallstone formation, and hence the two dissociation constants of bilirubin which are closely related to its solubility have been tried to be speculated or determined by several investigators.[8,9] Nevertheless, the correct values have not been determined yet because of the extremely small solubility of bilirubin even at neutral pH.

Bilirubin decomposes easily under oxygen, and its photooxidation has been extensively studied.[10-12] So, its purification is an important requirement to have reliable experimental results, and several purification methods have been proposed.[13-15] In this report, the authors have examined the solution properties of purified bilirubin in buffer solutions from the spectrum and solubility changes with pH. In the preceding paper,[16] the solubility change with pH was found to be very useful to determine acidity constants of acids which are sparingly soluble in aqueous solution. The solubility method is a modification of the isoextraction method for determination of dissociation constants.[17]

Accordingly, the main attention has been paid only to the determination of the two dissociation constants of bilirubin using two methods, a spectrophotometric and a solubility method. In this paper, therefore, the authors do not relate the constants to bilirubin metabolism or other biochemical events but make clear the physicochemical properties of aqueous bilirubin in solution.

The constants obtained independently by the two methods were in reasonable agreement. In any event, it is the first time that the two constants have been determined reliably. Therefore, they will become invaluable for future study on gallstone formation and on the pathological influence of bilirubin upon the human body.

Experimental

Materials. Chloroform of guaranteed reagent grade was further purified by alternate washing with dilute acid and alkali three times each followed by distilled water several times and then stored under distrilled water in the dark. Bilirubin from Sigma Chem. Corp. was purified as described previously[13] in the most respects. The different repect is the following; the bilirubin was dissolved first in the chloroform which had been beforehand passed through the column of anhydrous sodium sulfate heated at 300 °C for three hours, and then the chloroform solution of bilirubin was run down the column again shielded from light. Other reagents were of analytical reagent grade and used without further purification. Water used was distilled twice from alkaline permanganate.

Rate of Decomposition. Immediately after dissolution of bilirubin in 0.05 mol dm^{-3} NaOH solution, an aliquot of the solution was transfered into a photometric cell containing buffer solution whose ionic strength was 0.05, and then the change of the optical density of the solution with time was followed for more than two hours by spectrophotometer (Hitachi 100-50), where the bilirubin concentration is 2.5×10^{-6} mol dm^{-3} and the temperature is at 25 °C.

Spectrum. The absorption spectra of bilirubin in buffer solution (H_3PO_4-NaH_2PO_4-Na_2HPO_4 for pH≤8 and H_3BO_3-NaH_2BO_3 for pH>8) whose ionic strength(I) is 0.05 were obtained at 25 °C; immediately after the bilirubin had dissolved in alkaline solution (I=0.05), an aliquot of the solution was introduced into the buffer solutions, where the bilirubin concentration was 1.39×10^{-6} mol dm^{-3}. It took less than five minutes to finish recording the absorption spectra after the bilirubin dissolution. Almost no change in pH of the solutions were observed before and after the spectrum measurement.

Solubility. The apparatus and procedure are the same as those in the previous paper.[16] In preparing buffer solutions, ionic strength and temperature were made to resemble human bile (I=0.15 and 37 °C) and the reagents

used were the same as in the spectrum section. As bilirubin is not only chemically unstable but also photosensitive, the solubility measurement was carried out in a dark room and under the conditions of short dissolution time. First of all, the amount of bilirubin powder to be suspended in the buffer solution (3 ml) which is necessary to reach dissolution equilibrium within one hour was first examined and was found to be about 30 mg. Then, the solubility was determined spectrophotometrically at 37 °C against $1/a_{H^+}$ of each buffer solution under the above conditions.

Results and Discussion

Purity. Elemental analysis of purified bilirubin was 67.71 (67.79)% for C, 6.28 (6.21)% for H, and 9.49 (9.58)% for N, where the values in parentheses are calculated ones. The absorption spectra of original and purified bilirubin in chloroform are shown in Fig. 1. Impurities absorbing below 350 nm in chloroform solution were almost completely removed by the above purification process. The molar absorption coefficients in chloroform and in aqueous alkali solution are 5.67×10^4 mol^{-1} dm^3 cm^{-1} at 455 nm and 5.45×10^4 mol^{-1} dm^3 cm^{-1} at 438 nm,

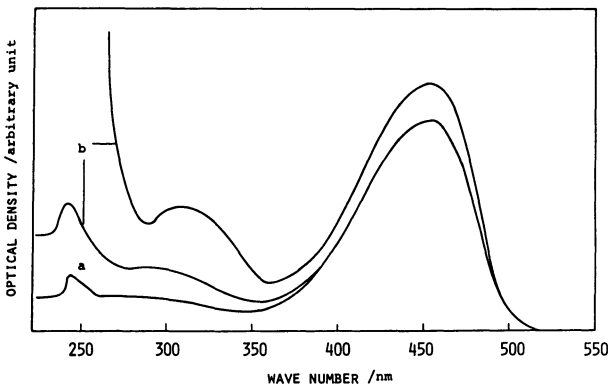

Fig. 1. Absorption spectra of purified(a) and unpurified bilirubin(b) in chloroform solution.

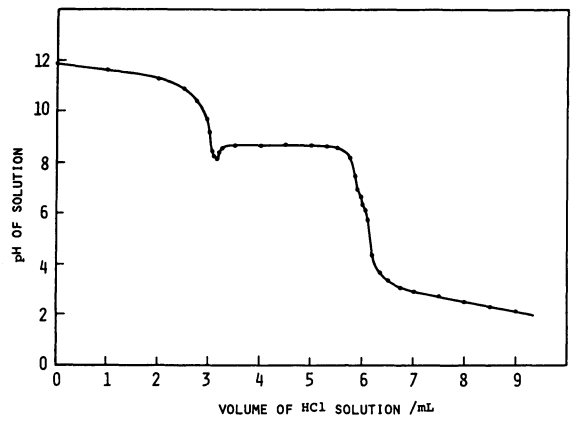

Fig. 2. Back-titration curve of bilirubin in alkali solution by HCl solution; 42.25 mg bilirubin dissolved in 10 ml of 3.018×10^{-2} mol dm^{-3} NaOH solution was titrated with 4.994×10^{-2} mol dm^{-3} HCl solution.

respectively. The former value can be compared with the literature value 6.07×10^4.[13] The titration curve is illustrated in Fig. 2. The result is appreciably different from that reported before.[9] The purity was found to go up to 99.8% with respect to two carboxyl groups from 90.4% of unpurified bilirubin. It is quite clear from this titration curve that bilirubin is dibasic acid in the pH region below 10 and that there exist two steps on pH decrease around the endpoint of titration due to different dissociation constants. The titration curve is quite similar to that of bile acids[18] which start micelle formation above their cmc,[19] but their physicochemical meaning is much different as will be clear later. The almost flat initial part of the curve represents the titration of excess NaOH. After neutralization of excess NaOH, the pH continues to decrease steeply with a small amount of HCl and then rises sharply again. With each successive addition of HCl, an almost equivalent amount of bilirubin is precipitated, where the pH of titration solution with bilirubin solid suspension hardly decreases keeping the value of 8.5 to the endpoint of the neutralization. The sharp rise of pH is accompanied by the precipitation of bilirubin. This indicates that supersaturation of bilirubin is broken at pH 8.15. The extent of the supersaturation can be estimated to be more than 10^{-4} mol dm^{-3} from the titration curve. Other acidic groups with $pK>10$ are out of the present discussion.[20]

Decomposition of Bilirubin in Buffer Solutions. Biomolecules are very easy to decompose, once they are taken out from their mother tissue. Thus, it is very important for the present investigation to know how easily bilirubin degrades in buffer solutions. As the decay kinetics of bilirubin is not the main purpose, very simple first-order kinetics is adopted, neglecting the effect of H^+ or OH^- ions;

$$OD = OD_0 \exp(-k^{app}t). \quad (1)$$

where an optical density(OD) is used in place of concentration, k^{app} is an apparent first-order rate constant, and t is time in unit of hour. The decay curves turned out to be approximately expressed by Eq. 1. The k^{app} values and the wavelength pursued are given in Table 1. From the results, more than 90% of birirubin can remain unchanged for one hour.

Spectrum Change with pH. It has become clear from the titration curve that bilirubin is a dibasic acid. Therefore, it dissociates stepwise from acidic to basic form with increasing pH;

$$\underset{\mathbf{1}}{AH_2} \overset{K_1}{\rightleftharpoons} \underset{\mathbf{2}}{AH_1^-} \overset{K_2}{\rightleftharpoons} \underset{\mathbf{3}}{A^{2-}}. \quad (2)$$

Figure 3 shows the absorption spectra in several buffer solutions. As is expected, the spectrum

Table 1. Apparent rate constant (k^{app}) of decomposition of bilirubin in buffer solutions of different pH at 25 °C

pH	λ^{obsd}/nm	$10^2\,k^{app}/h^{-1}$
12.68	438	18.0
8.84	438	4.5
8.15	438	2.7
7.96	440	7.3
7.37	450	9.0
7.07	450	11.5
6.38	453	10.1
6.09	440	5.1
4.41	440	7.8

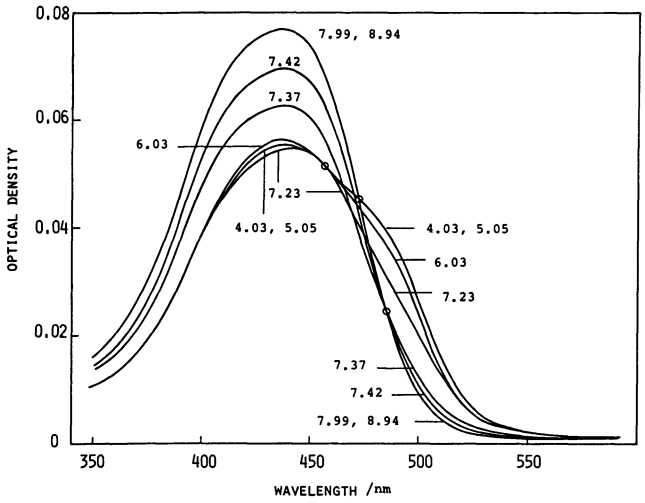

Fig. 3. Absorption spectra of bilirubin in buffer solutions with different pH and three isosbestic points(o): the number in the figure is pH of buffer solution and the bilirubin concentration is 1.39×10^{-6} mol dm^{-3}.

changes with pH of the solution. The concentration of each solution is 1.39×10^{-6} mol dm^{-3} which is higher than the solubility at pH 8, 1.0×10^{-6} mol dm^{-3} (Fig. 4). However, any precipitation has not been observed, because the concentration is so small that the supersaturation is easy to stand far up to 10^{-4} mol dm^{-3} as can be seen from the titration curve. The spectra of A^{2-} above pH 8 remain the same and has the absorption maximum at 438 nm with the molar absorption coefficient of 5.45×10^4 mol^{-1} dm^3 cm^{-1}. On the other hand, the spectra of AH_2 below pH 5 also remain unchanged, where the wavelength of the absorption maximum is 437 nm with the molar absorption coefficient of 3.92×10^4 mol^{-1} dm^3 cm^{-1}. The wavelength of three isosbestic points obtained from many spectra of different pH are 457 nm for AH_2 and AH_1^-, 485 nm for AH_1^- and A^{2-}, and 472 nm for A^{2-} and AH_2.

Solubility. The solubilities determined spectrophotometrically at 37 °C against $1/a_{H^+}$ of each buffer solution are shown in Fig. 4. Bilirubin which

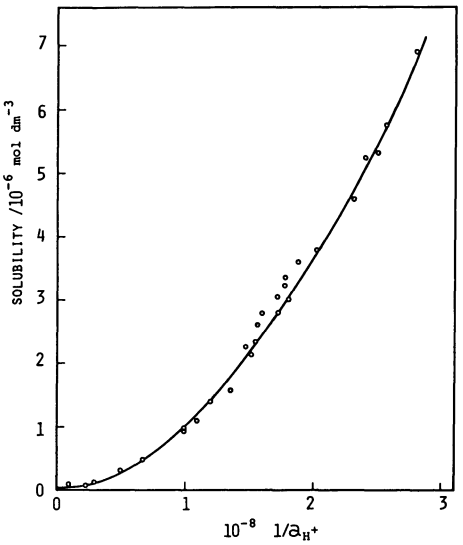

Fig. 4. Solubility of bilirubin in buffer solution as a function of $1/a_{H^+}$: ionic strength=0.15 and temperature=37 °C.

is a dibasic acid dissociates by two steps, and the total analytical concentration then becomes[16]

$$C_t = [AH_2] + [AH_1] + [A] = [AH_2]\left\{1+\frac{K_1}{\gamma_2}\frac{1}{a_{H^+}}+\frac{K_1K_2}{\gamma_3}\left(\frac{1}{a_{H^+}}\right)^2\right\}. \quad (3)$$

The concentration $[AH_2]$ should remain constant at constant temperature and ionic strength as far as AH_2 solid coexists in the solution, because AH_2 in the solution is in chemical equilibrium with solid AH_2. Since every parameter except $1/a_{H^+}$ of the right side of Eq. 3 is constant based on the above reasons, the total concentration C_t changes with the second-power curve of $1/a_{H^+}$. Three coefficients of the curve $(A+B(1/a_{H^+})+C(1/a_{H^+})^2)$ were estimated by applying the second-power curve to the solubility data where the method of least square was employed for the best fit between them, and they were found to be

$$A = 1.132\times10^{-9},\ B = 1.728\times10^{-15},$$

and

$$C = 8.176\times10^{-23}\ \text{mol dm}^{-3}.$$

Now it became clear that the total analytical concentration increases with increasing pH by being subject to the second-power curve of $1/a_{H^+}$ in this case, too. This means that dimerization or polymerization of bilirubin does not take place in these buffer solutions. It is incorrect that the logarithm of the solubility varies linearly with pH.[21]

Evaluation of Dissociation Constants

Spectrophotometric Method. Spectrum of bilirubin depends on the pH of its solution as seen

above. At any wavelength except that of the isosbestic point, the optical density can be expressed as

$$OD = \varepsilon_1 C_1 + \varepsilon_2 C_2 + \varepsilon_3 C_3 \quad (4)$$

where ε_i is molar absorption coefficient and C_i is molar concentration of species i. On the other hand, the total concentration is

$$C_t = C_1 + C_2 + C_3 \quad (5)$$

and they can be related with one another by the dissociation constants as

$$K_1 = \frac{C_2 \gamma_2 a_{H^+}}{C_1 \gamma_1} \quad (6)$$

and

$$K_2 = \frac{C_3 \gamma_3 a_{H^+}}{C_2 \gamma_2}, \quad (7)$$

where γ_i is the activity coefficient of i. By combining the above four equations, there results

$$\frac{\gamma_3 a_{H^+}^2}{K_1 K_2}\varepsilon_1 + \frac{\gamma_3 a_{H^+}}{\gamma_2 K_2}\varepsilon_2 + \varepsilon_3 = \frac{OD}{C_t}\left(\frac{\gamma_3 a_{H^+}^2}{K_1 K_2} + \frac{\gamma_3 a_{H^+}}{\gamma_2 K_2} + 1\right) \quad (8)$$

where γ_1 is assumed to be unity because of uncharged species of **1**. ε_1 and ε_3 are easily determined from the spectrum of low and high pH solutions of bilirubin, respectively, and their activity coefficients can be estimated at the ionic strength(I) of 0.05 and 25 °C from the equation suggested by Bates[22]

$$\log \gamma_i = -\frac{0.5|z_i|^2\sqrt{I}}{1+1.5\sqrt{I}} \quad (9)$$

where z_i is a valency of i. Now, it is theoretically possible to determine K_1, K_2, and ε_2 values from three different OD values of different pH solution at fixed wavelength and at known total concentration. Practically, however, the spectrum whose variation with pH is large can be used for the present purpose; only K_i which brings about larger spectrum change with small pH change can be determined with high accuracy. Two wavelengths, 490 and 438 nm, were used for the determination of K_1 and K_2 values, respectively. The molar absorption coefficients used are

$$\varepsilon_1 = 2.46 \times 10^4,$$
$$\varepsilon_3 = 1.24 \times 10^4 \text{ mol}^{-1}\text{ dm}^3\text{ cm}^{-1} \text{ at } 490 \text{ nm}$$

and

$$\varepsilon_1 = 3.88 \times 10^4,$$
$$\varepsilon_3 = 5.45 \times 10^4 \text{ mol}^{-1}\text{ dm}^3\text{ cm}^{-1} \text{ at } 438 \text{ nm}.$$

The K_i values thus obtained are

$$K_1 = (3.1\text{—}8.7) \times 10^{-7},$$
$$K_2 = (2.5\text{—}5.1) \times 10^{-8} \text{ at } 25\,^\circ\text{C}.$$

Solubility Method. As is clear from Eq. 3, the values of K_1 and K_2 can be obtained from the three coefficients of the second-power curve $(A+B(1/a_{H^+})+C(1/a_{H^+})^2)$ as

$$K_1 = \gamma_2 B/A, \quad (10)$$
$$K_2 = \gamma_3 C/\gamma_2 B, \quad (11)$$

From the three coefficients given above, K_1 and K_2 values become

$$K_1 = 9.7 \times 10^{-7} \text{ and } K_2 = 2.4 \times 10^{-8} \text{ at } 37\,^\circ\text{C}.$$

Although the A value cannot be precisely determined for a scarcely soluble acid like the present, B and C values are rather easy and more accurate to determine. Therefore, K_2 value is more reliable than K_1 value. In our preceding paper[16] the authors reported the method to determine K_1 value of dibasic acid by employing $[AH_2]$ as a parameter. However, this method can be used only when the two coefficients, B and C, are very accurate. In the present case, on the other hand, bilirubin is not so stable and these coefficients cannot be accurately determined, as is obvious from the plots in Fig. 4.[23] The feasible concentration range of AH_2 based on the range of K_1 values obtained above is shown in Fig. 5, supposing B value to be fairly accurate. Indeed there results some difference in value between the two dissociation constants depending on the methods employed, but the diminution of the difference is quite difficult in this case judging from the instability of bilirubin as is obvious in the section of its decomposition.

Now it can be said that K_1 value ranges from 3×10^{-7} to 1×10^{-6}, while K_2 value is between 2×10^{-8} and 5×10^{-8}, although the values obtained from the two methods are not coincident each other. The dissociation constants of aliphatic monocarboxylic acids as in bilirubin usually range from 10^{-5} to 10^{-3} and the second dissociation constants of corresponding dicarboxylic acids are between 10^{-8} and 10^{-5}.[24] The

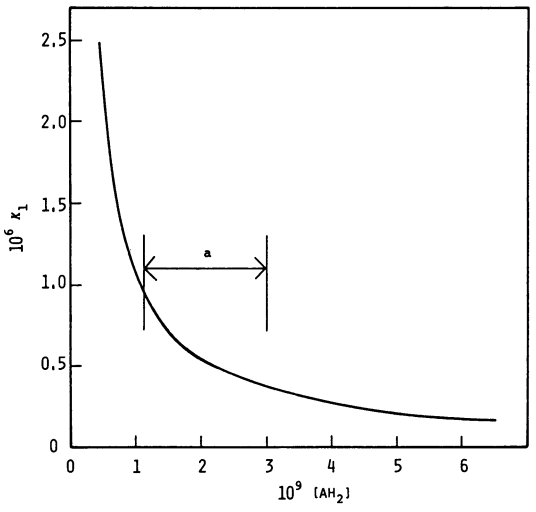

Fig. 5. Variation of K_1 value as a function of concentration of AH_2 and its feasible concentration range(a).

extreme small second dissociation constant of bilirubin can be ascribed partly to a free energy increase by electrostatic repulsion between two carboxylato groups[25] when the second dissociation takes place, as has been done in most cases. The distance between the two groups can range from 0 to 10 Å by CPK molecular model. This range can become the factor somehow to explain the difference between the obtained K_1 and K_2 values. The K_1 value of 10^{-6} at maximum also seems too small for the carboxyl group judging from dissociation constants of most aliphatic monocarboxylic acids. It is then highly possible that the smaller values of K_1 and K_2 are due to stabilization of the carboxyl groups by intramolecular hydrogen bond formation.

Finally, a few remarks on previous works by other investigators on the solubility, the absorption spectra, and the dissociation constants will be made. Overbeek *et al.* expected the dissociation constants for the two carboxyl groups, $K_1=4\times10^{-5}$ and $K_2=1\times10^{-5}$, and estimated the total solubility of 2×10^{-4} mol dm^{-3} at pH=8.[8] It is two hundred times higher than that of present case at the same pH, which indicates that their expected dissociation constants might be totally incorrect. On the other hand, the solubility determined by Brodersen, 9×10^{-8} mol dm^{-3} at pH=8,[21] is one tenth of the solubility determined by us because of extremely scanty solubility of solid bilirubin; 2.5 mg of solid bilirubin in 25 ml buffer solution was too small to reach the solubility equilibrium within one hour in our preliminary experiment. In addition, they attributed the spectrum change with time to bilirubin aggregation.[21] Even if it were true that the bilirubin aggregation took place during the process of spectral measurement in lower pH buffer solutions, the spectrum in Fig. 3 would be trustworthy with more than 98% confidence judging from k^{app} values in Table 1 obtained from higher bilirubin concentration than that for spectral measurement and from the time required for the measurement. Hansen *et al.* insisted that the dissociation constants(pK) of the two carboxyl groups should be well below 7 and obtained the calculated value of pK=4.4.[20] As mentioned above, the pH value with pH titration for the suspended neutralized precipitate bilirubin is around 8.5. If pK=4.4 is correct, $[A^{2-}]/[AH^-]$ must be larger than 10^4 at pH=8.5 and the total solubility becomes more than 10^{-1} mol dm^{-3}, $[AH_2]$ being assumed to be 10^{-9} mol dm^{-3}. Such a high solubility should not bring about the bilirubin precipitation during the back titration. On the other hand, Krasner and Yaffe concluded that both carboxyls have pK=7.55 at 26 °C from the titration curve.[9] The values are almost equal to the present K_2 value.

The strong dependence of the solubility of bilirubin on solution pH must have great importance for gallstone formation. The rapid decrease of solubility of bilirubin below pH 8 suggests that gallstone formation might be due to a lowering of pH in gall bladder. The solubility of bilirubin in buffer solutions has been extensively measured by several investigators. Nevertheless, the reported values have shown a wide variety depending on methods and conditions employed. However, the solubilities obtained here are rather accurate, and therefore we believe that they will become very useful for future study of the pathological function of bilirubin in the human body.

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