7. Kleinman, L. J., Petering, H. G., and Sutherland, J. M.: Blood carbonic anhydrase activity and zinc concentration in infants with respiratory distress syndrome. *N. Eng. J. Med.*, 277: 1157 (1967).
8. Kleinman, L. J., Sell, J. E., and Petering, H. G.: Carbonic anhydrase isoenzymes in infants with respiratory distress syndrome. *Amer. J. Dis. Child.* 124: 696 (1972).
9. Kotas, R. V., and Avery, M. E.: Accelerated appearance of pulmonary surfactant in the fetal rabbit. *J. Appl. Physiol.*, 30: 358 (1971).
10. Logan, R. W., Crooks, S. M., Hutchison, J. H., and Kerr, M. M.: Blood carbonic anhydrase activity in the newborn. *Arch. Dis. Childhood*, 48: 256 (1973).
11. Motoyama, E. K., Orzalesi, M. M., Kikkawa, Y., Kaibara, M., Wu, B., Zigos, C. J., and Cook, C. D.: Effect of cortisol on the maturation of fetal rabbit lungs. *Pediatrics*, 38: 547 (1971).
12. Murphy, B. E. P.: Evidence of cortisol deficiency at birth in infants with the respiratory distress syndrome. *J. Clin. Endocrinol.*, 38: 158 (1974).
13. Narumi, S., and Miyamoto, E.: Activation and phosphorylation of carbonic anhydrase by adenosine 3',5'-monophosphate-dependent protein kinases. *Biochem. Biophys. Acta*, 350: 215 (1974).
14. Roughton, F. J. W., and Booth, V. H.: The effect of substrate concentration, pH, and other factors upon the activity of carbon anhydrase. *Biochem. J.*, 40: 319 (1946).
15. Russell, B. J., Nugent, L., and Chernick, V.: Effects of steroids on the enzymatic pathways of lecithin production in fetal rabbits. *Biol. Neonate*, 24: 306 (1974).
16. Sell, J. E., and Petering, H. G.: Carbonic anhydrases from human neonatal erythrocytes. *J. Lab. Clin. Med.*, 84: 369 (1974).
17. Stadie, W. C.: A method for the determination of methemoglobin in blood. *J. Biol. Chem.*, 41: 237 (1920).
18. Stevenson, S. S.: Carbonic anhydrase in newborn infants. *J. Clin. Invest.*, 22: 403 (1943).
19. Suzuki, S., Ogawa, E., and Shibata, K.: Experimental studies on the carbonic anhydrase activity. IX. The mode of action of adrenocortical steroids, especially aldosterone on the carbonic anhydrase in mice and rats. *Endocrinol. Jap.*, 14: 107 (1967).
20. Wang, N. S., Kotas, R. V., Avery, M. E., and Thurlbeck, W. M.: Accelerated appearance of osmiophilic bodies in fetal lungs following steroid injection. *J. Appl. Physiol.*, 30: 362 (1971).
21. We are grateful to Mrs. P. Trudel for typing the manuscript.
22. This research was supported by the Medical Research Council of Canada and the Children's Hospital of Winnipeg Research Foundation, Inc.
23. Requests for reprints should be addressed to: V. Chernick, M.D., Children's Centre, 685 Bannatyne Ave., Winnipeg, Manitoba R3E 0W1 (Canada).
24. Accepted for publication March 17, 1976.

Copyright © 1976 International Pediatric Research Foundation, Inc.

Printed in U.S.A.

Pediat. Res. 10: 782-788 (1976)

Bilirubin promethazine
induction Rh erythroblastosis
liver

Spectrophotometric Characteristics of Bilirubin

KWANG-SUN LEE AND LAWRENCE M. GARTNER⁽⁴³⁾

Department of Pediatrics, Rose F. Kennedy Center and Liver Research Center, Albert Einstein College of Medicine, Bronx, New York

Extract

Spectrophotometric characteristics of bilirubin at low concentrations (0.005-2.500 mg/100 ml) have been studied under various physical conditions in order to gain a better understanding of the state of bilirubin when preparing "solutions" for laboratory use. Standing, minimal shaking, or stirring of the bilirubin preparations at pH 7.4 progressively reduced and altered the maximal spectral absorption of bilirubin (440 nm) in aqueous buffered media. The shift to 415-420 nm is attributed to oxidation of the pigment whereas shoulder formation is attributed to the formation of large size particles (flocclulants). In the presence of antioxidants (L-ascorbic acid and nitrogen gas) and EDTA the maximal absorption peak remained at 440 nm but decreased in magnitude concomitant with development of a progressively increasing shoulder at 480-560 nm. In the absence of antioxidants and EDTA maximal absorption shifted to 415-420 nm and the magnitude of 480-560 nm shoulder formation was less. At the higher concentrations of bilirubin and with reduction in pH of the buffer in the absence of antioxidants, the shift to lower wave lengths was reduced and 480-560 nm shoulder formation was increased. In the absence of antioxidants and EDTA at the lower concentrations of bilirubin and in more alkaline media, the reduction at 440 nm and the shift of maximal absorption to the shorter wave lengths was enhanced. At pH 12, stirring of antioxidant-EDTA-containing solutions of bilirubin resulted in neither a shift of maximal absorption to the shorter wave lengths nor the formation of 480-560 nm shoulder. The formation of 480-560 nm shoulder was accompanied by the visual appearance of turbidity. The formation of flocclulants when a "solution" is agitated indicates

that significant portions of the pigment were in fact, not in solution and must have existed previously as a finely dispersed colloidal sol or supersaturated solution which progressed to a colloidal sol.

Spectral curves of bilirubin, therefore, may represent a composite resulting from four physical states of bilirubin: (1) bilirubin truly in solution with the spectral peak at 440 nm; (2) bilirubin in the fine colloidal dispersion with spectral characteristics similar to those of bilirubin in solution; (3) bilirubin flocclulant giving 480-560 nm shoulder; and (4) oxidation products of bilirubin with the spectral peaks lower than 440 nm.

Increasing the pH of the aqueous media containing bilirubin (0.05 mg/100 ml) from 7.4 to 12.0 increased the molar extinction coefficient of bilirubin, $E_{440}^{1M, 1cm}$, progressively to a maximum at pH 12 of 6.35×10^4 . Very dilute bilirubin preparations (0.005-0.050 mg/100 ml) in aqueous media, pH 7.4, exhibited spectral evidence of rapid oxidation (more so at higher pH), but spectral shoulder formation was still observed after mechanical agitation. Thus, the solubility of bilirubin in 0.1 M phosphate buffer at pH 7.4 appears to be less than 0.005 mg/100 ml.

Speculation

Unbound bilirubin *in vivo*, at concentrations exceeding albumin binding capacity and its aqueous solubility, is believed to exist either as a colloidal sol (micropolymer) or a flocclulant (macropolymer). It is proposed that it is in the colloidal sol form (micropolymer) that bilirubin toxicity to brain, kidney, intestinal mucosa, erythrocyte, and other organs initially develops by a process of colloid to surface interaction.

Spectrophotometric analysis of bilirubin has been utilized primarily for the determination of bilirubin concentrations (13, 21) and for the study of bilirubin-albumin binding in the evaluation of risk for development of kernicterus (26, 37). Although the principle has been generally accepted that the height of the molar extinction coefficient of bilirubin in chloroform at 453 nm is an index of purity of the sample (35), widely varying values have been reported for the maximal visible light absorption wave length and molar absorptivity of bilirubin in aqueous solution (10, 19, 37, 38), human serum albumin solution (14, 19, 32, 33, 39), and human sera (2, 19, 26, 32). Similarly, reported values for the "solubility" of bilirubin in aqueous solution have varied widely (6, 7, 19, 29). The reasons for this marked variability in maximal absorption wave length and absorptivity from one laboratory to another remain unclear.

Brodersen and his associates (3, 5, 6) extensively studied bilirubin colloid formation in both aqueous and albumin solutions. They reported that the true solubility of bilirubin in 0.1 M phosphate buffer at pH 7.4 was about 0.1 μ M (0.0058 mg/100 ml). At concentrations exceeding this value, they reported that bilirubin existed as a colloidal suspension (3, 5, 6). The formation of large sized bilirubin aggregates resulted in a spectral shoulder at 480–560 nm.

Oxidation of bilirubin by molecular oxygen and light results in spectral alterations characterized by reduction of maximal absorption at 440 nm and shift of the spectral peak to shorter wave lengths (8, 20, 27, 28).

The instability and uncertain physical states of bilirubin have comprised a serious problem in investigations involving this compound (37, 39). Despite the recognition of this problem, there has been no systematic study of the characteristics of bilirubin in relation to the physical environment used in preparing bilirubin for study.

This communication reports on the spectrophotometric characteristics of bilirubin in aqueous media, crystalline albumin solution, and human sera after mechanical agitation, oxidation, inhibition of oxidation, and alteration of pH. This study also extends the spectral observations of Brodersen and his associates (3, 5, 6) on the colloidal state of bilirubin and the possible mechanism of bilirubin toxicity *in vivo*.

METHODS

Bilirubin used was purchased from Eastman Organic Chemicals and its molar extinction coefficient in chloroform, $E_{453}^{1M, 1cm}$, as determined by the method recommended by the Standards Committee of the College of American Pathologists (35), was 5.96×10^4 . A double beam recording spectrophotometer (Perkin-Elmer) and quartz cuvettes with a 1-cm light path were used for most spectral measurements. For bilirubin concentrations lower than 0.05 mg/100 ml, cuvettes with 10-cm light path were used.

BILIRUBIN IN PHOSPHATE BUFFER

Aqueous bilirubin, 0.005–2.500 mg/100 ml 0.1 M phosphate buffer solution (buffer), pH 7.4, was prepared by dissolving 5 mg bilirubin in 10 ml 0.1 N NaOH and adding 1 to 5 ml of the above bilirubin solution to 99 to 95 ml of the buffer at pH 7.4 to which 1 to 5 ml of 0.1 N HCl equivalent to the volume of 0.1 N NaOH bilirubin solution was added to the buffer before adjustment to final volume of 100 ml. Bilirubin in buffer solution was mixed in a beaker with gentle swirling movements or in a graduated cylinder with inversion two to three times, avoiding stirring or shaking unless otherwise stated. If stirring bilirubin in buffer was performed, a magnetic stirrer was utilized at a fixed rate and for a fixed time interval, as stated, in the dark at room temperature ($24 \pm 2^\circ$). Spectrophotometric analysis of bilirubin was performed at wave lengths between 380 and 600 nm and completed within 10 min after initial dissolution of bilirubin, unless otherwise stated.

Bilirubin-buffer solutions at pH ranging from 4 to 12 were prepared by adding bilirubin in 0.1 N NaOH to the previously adjusted buffer solution to obtain the desired final pH.

AQUEOUS BILIRUBIN WITH L-ASCORBIC ACID AND EDTA

Aqueous bilirubin (0.05 mg/100 ml) containing L-ascorbic acid (Sigma) and EDTA (Sigma) at pH values ranging from 4 to 12 was prepared similarly by adding bilirubin in 0.1 N NaOH to the previously prepared and pH-adjusted buffer solution containing 5 mM L-ascorbic acid and 2 mM EDTA. The pH of the final solution was determined with a glass electrode pH meter from Corning Scientific Instruments (model 10) and all specimens were found to have the expected pH.

BILIRUBIN IN NITROGEN GAS-ENRICHED BUFFER

Nitrogen gas-enriched bilirubin-buffer solutions of pH 7.4 were prepared by saturating the degassed buffer solutions with bubbled nitrogen gas (Matheson) and mixing bilirubin under a nitrogen atmosphere.

BILIRUBIN IN SERA OR HUMAN SERUM ALBUMIN SOLUTIONS

Bilirubin in human serum albumin (HSA) (Sigma) solution was prepared by first preparing 4 g crystalline albumin in 100 ml 0.1 M phosphate buffer, pH 7.4, and then adding bilirubin in 0.1 N NaOH to the HSA solution with appropriate addition of 0.1 N HCl to maintain the pH at 7.4. Naturally occurring unconjugated bilirubin in serum was obtained from human newborn infants with unconjugated hyperbilirubinemia and a patient with congenital nonhemolytic unconjugated hyperbilirubinemia, type I (1).

Bilirubin in HSA solution with increasing molar ratio of bilirubin to albumin (B:A) from 0.5 to 10 was prepared by mixing a volume of 0.2 mg bilirubin/100 ml buffer, pH 7.4, with an equal volume of HSA of varying concentrations (11.8–236.0 mg/100 ml buffer, pH 7.4) to yield the desired molar ratios. Bilirubin in human cord sera or human adult sera were prepared similarly by diluting sera with buffer such that the equivalent molar amount of native albumin was added to the bilirubin-buffer solution to maintain a fixed bilirubin concentration of 0.1 mg/100 ml. Calculations were based on an assumed molecular weight for HSA of 69,000. Serum albumin concentrations were determined by the measurement of total protein (18) and percentage of albumin by agarose gel (2%) electrophoresis in a Spectrophor I (Bausch and Lomb, Inc.) in 0.01 M phosphate buffer, pH 8.5, with 300 V applied for 90 min. Densitometric readings of protein fractions were carried out at 205 nm through the quartz-plate bed and with electronic integration. Total and direct-reacting bilirubin in sera were determined by the method of Jendrassik and Grof (16).

METHOD OF PRESENTATION OF SPECTRAL DATA

Spectral curves (380–600 nm) are presented both as recorded and as normalized data. Normalization of curves was achieved by plotting the maximal optical density (OD) value for each curve as 1.0 on the ordinate and expressing all other points on the curve in their corrected relation to the maximum value. The normalization calculation was performed by dividing the OD value at each wave length by the maximum OD value for that curve and expressing each point on the curve other than the maximum as a fractional equivalent of unity in decimal equivalents. This permits comparison of OD values between different curves at each wave length.

RESULTS

BILIRUBIN IN PHOSPHATE BUFFER

Immediate spectral scanning of aqueous bilirubin, 0.5 mg/100 ml buffer, pH 7.4, gave a 440 nm maximal absorption and an $E_{440}^{1M, 1cm}$, of $4.72 \times 10^4 \pm 0.15$ (SE) ($n = 4$) (Fig. 1). After the

preparation stood for 10 min, was shaken vigorously 10 times, or was stirred for 5 min, the maximal absorption shifted from 440 nm to shorter wave lengths, the absorption at 440 nm decreased and a shoulder formed between 480 and 560 nm, maximal between 490 and 500 nm (Fig. 1). Coincident with the formation of the 480–560 nm shoulder, the bilirubin-buffer solution, which had previously appeared entirely clear, became visibly cloudy. The longer the agitation or standing, the greater the degree of shift and formation of shoulder.

On immediate spectral scanning, bilirubin in aqueous media, 0.5 mg/100 ml buffer at all pH values from 4 to 12, demonstrated maximal spectral absorption at 440 nm. At all pH values below 12, the maximal absorption OD was lower and the shoulder formation was wider and higher even before agitation or standing. This was

Fig. 1. Spectral absorption curves of bilirubin, 0.5 mg/100 ml, 0.1 M phosphate buffer, pH 7.4. *a*: immediate reading; *b*: reading after stirring for 15 min; *c*: reading after 30 min. *A*, *B*, and *C* are normalized spectral curves for *a*, *b* and *c*, respectively.

most striking at pH values below 8. After stirring aqueous bilirubin solutions at pH 12 for 15 min, there was a reduction and shift of maximal absorption to shorter wave lengths (420 and 415 nm) but without any shoulder formation at 480–560 nm (Fig. 2). In contrast, at pH 6.0 after stirring for 15 min, there was a shift in the absorption peak to a longer wave length, 445 nm, and formation of wider and higher shoulder at 480–560 nm than was the case with the similar treatment of the same solution at pH 7.4 (Fig. 2).

After either mechanical agitation or exposure to light of aqueous bilirubin, the initial maximum OD (at 440 nm) of bilirubin decreased progressively and to a greater degree at the lower concentration (Tables 1 and 2). At the higher bilirubin concentration, mechanical agitation brought an increase in spectral shoulder formation at 500 nm (Table 1). At the lower concentration, the exposure of aqueous bilirubin to light without mechanical agitation demonstrated an increase in OD at 420 nm (Table 2).

AQUEOUS BILIRUBIN WITH L-ASCORBIC ACID AND EDTA

Aqueous bilirubin, 0.5 mg/100 ml buffer with L-ascorbic acid and EDTA at pH 7.4, demonstrated a maximal spectral absorption at 440 nm and an $E_{440}^{1\text{cm}}$ of $4.72 \times 10^4 \pm 0.14 \times 10^4$ (SE) ($n = 4$), identical with that of aqueous bilirubin without L-ascorbic acid and EDTA (Figs. 1 and 3). After 30 min of stirring or shaking of the above bilirubin-buffer solution, progressive shoulder formation (480–560 nm) was noted but without any shift of the peak to the shorter wave lengths (Fig. 3). In contrast, at pH 12, after stirring for 60 min, bilirubin-buffer solution containing

Fig. 2. Spectral absorption curves of bilirubin, 0.5 mg/100 ml, 0.1 M phosphate buffer at pH 12 and 6. *a*: immediate reading; *b*: reading after stirring for 15 min.

Table 1. Effect of mechanical agitation¹ on spectral absorption of bilirubin in 0.1 M phosphate buffer, pH 7.4¹

Bilirubin, mg/100 ml	Duration of agitation, sec	Optical density ratios		% optical density, reduced at 440 nm
		420 nm/440 nm	500 nm/440 nm	
0.01	0	0.94 ± 0.03 ²	0.16 ± 0.04	75.2 ± 12.6 59.1 ± 13.4 $P < 0.05$
	5	0.91 ± 0.03	0.14 ± 0.05	
	10	0.91 ± 0.05	0.13 ± 0.04	
1.00	0	0.94 ± 0.01	0.21 ± 0.02	87.8 ± 1.2 84.5 ± 3.2 $P < 0.05$
	5	0.95 ± 0.01	0.26 ± 0.02	
	10	0.95 ± 0.01	0.28 ± 0.03	

¹ Low mode, vortex test tube mixer, Scientific Industries, Inc.

² Mean ± SD, $n = 4$ for each.

Table 2. Effect of light exposure¹ on spectral absorption of bilirubin in 0.1 M phosphate buffer, pH 7.4

Bilirubin, (mg/100 ml)	Duration of light exposure, sec	Optical density ratios		% optical density, reduced at 440 nm
		420 nm/440 nm	500 nm/440 nm	
0.01	0	0.94 ± 0.01 ²	0.14 ± 0.01	79.6 ± 6.6 65.2 ± 6.4 P < 0.01
	5	0.96 ± 0.01	0.14 ± 0.01	
	10	0.99 ± 0.02	0.12 ± 0.02	
1.00	0	0.95 ± 0.01	0.21 ± 0.01	92.5 ± 1.2 85.1 ± 1.3 P < 0.01
	5	0.96 ± 0.01	0.21 ± 0.02	
	10	0.95 ± 0.01	0.21 ± 0.02	

¹ Exposure distance: 45 cm from the light source which was eight 20-W daylight fluorescent lamps (Westinghouse) housed in phototherapy unit, model no. PT-53-1. Air-Shields, Inc.

² Mean ± SD, *n* = 4 for each.

Fig. 3. Spectral absorption curves of bilirubin, 0.5 mg/100 ml, 0.1 M phosphate buffer containing 5 mM L-ascorbic acid and 2 mM EDTA at pH 7.4. *a*: immediate reading; *b*: reading after 30 min of stirring; *c*: after stirring for 60 min. *A*, *B*, and *C* are normalized spectral curves for *a*, *b*, and *c*, respectively.

L-ascorbic acid and EDTA exhibited neither a shift of the spectral peak nor any shoulder formation.

In the presence of L-ascorbic acid and EDTA, bilirubin, 0.05 mg/100 ml buffer, at pH ranging from 4 to 12, demonstrated less shift to the shorter wave lengths at each pH than that of aqueous bilirubin without L-ascorbic acid and EDTA. At this low bilirubin concentration (0.05 mg/100 ml), the $E_{440}^{1M, 1cm}$ of bilirubin increased progressively with each increase in pH up to pH 12 and decreased with each decrease in pH down to 4.0 (Fig. 4). The greatest percentage of increase in $E_{440}^{1M, 1cm}$ was observed at pH between 7 and 8 (Fig. 4). The $E_{440}^{1M, 1cm}$ at pH 12 was $6.35 \times 10^4 \pm$

Fig. 4. Molar extinction coefficients of bilirubin in 0.1 M phosphate buffer (0.05 mg/100 ml) at pH 4–12.

0.21×10^4 (SE) (*n* = 3), a value 6.5% greater than the $E_{453}^{1M, 1cm}$ of the same bilirubin in chloroform. Over a range of bilirubin concentrations from 0.005 to 2.500 mg/100 ml buffer, pH 7.4, the $E_{440}^{1M, 1cm}$ did not change significantly and the mean was $4.61 \times 10^4 \pm 0.13 \times 10^4$ (SE) (*n* = 40).

BILIRUBIN IN NITROGEN GAS-ENRICHED BUFFER

Bilirubin, 0.5 mg/100 ml, prepared in degassed, nitrogen gas-enriched buffer at pH 7.4, had spectral characteristics identical with those of aqueous bilirubin with L-ascorbic acid and EDTA, indicating that the addition of antioxidant chemicals had no effect on spectral characteristics, other than inhibition of oxidation. No shift of maximal absorption peak was observed even after 30 min of stirring, but progressive shoulder formation (480–560 nm) did occur, as in the L-ascorbic acid-EDTA experiment at pH 7.4.

BILIRUBIN IN SERA OR HUMAN SERUM ALBUMIN SOLUTIONS

Bilirubin in HSA solution (bilirubin 0.5 mg/100 ml; B:A molar ratio = 1) at pH 7.4 gave a maximal absorption at 460 nm with $E_{460}^{1M, 1cm}$ of $4.70 \times 10^4 \pm 0.13 \times 10^4$ (SE) (*n* = 4). Human adult sera with added unconjugated bilirubin, sera from patients with congenital nonhemolytic unconjugated hyperbilirubinemia, type I

(1), and neonatal physiologic hyperbilirubinemia demonstrated the same spectral absorption peak at 460 nm.

In the presence of reduced concentrations of HSA (bilirubin 0.5 mg/100 ml; B:A molar ratios = 5 and 10), a shift of maximal absorption peaks to the shorter wave lengths was observed with immediate scanning, approaching that of aqueous bilirubin (Fig. 5). After stirring for 60 min, there was a progressive reduction in maximal absorption with shoulder formation at 480–560 nm but the spectral changes were less pronounced than those of aqueous bilirubin (Fig. 6).

DISCUSSION

The study defines the spectral characteristics of bilirubin undergoing various changes of physical state and demonstrates the influence of the interactions of these variables on spectral absorptions of bilirubin.

With great care taken in the preparation of aqueous bilirubin solutions, so that oxidation and excessive agitation are avoided, the wave length of the maximal absorption is 440 nm and $E_{440}^{1\text{M}, 1\text{cm}}$ of this material is 4.67×10^4 at pH 7.4. With exposure to air over a period of 5–30 min, a progressive reduction and shift of the maximal absorption to the shorter wave lengths occur. These spectral alterations could be prevented by addition of antioxidants to the buffer or by removing oxygen from the buffer. Thus, these spectral alterations appear to be due to oxidation of bilirubin, as has been shown by others (8, 20, 27, 28). These alterations were progressively more accentuated by a progressive decrease in bilirubin concentration and/or an increase in pH positively associated with the proportion of bilirubin solubilized in the buffer.

The other observed spectral alterations were reduction and shift of the maximal absorption to the longer wave lengths with concomitant shoulder formation between 480 and 560 nm. These

Fig. 5. Spectral absorption curves of bilirubin (immediate reading), 0.5 mg/100 ml, 0.1 M phosphate buffer, pH 7.4, containing human serum albumin. *a*: at a bilirubin to albumin molar ratio of 0.5; *b*: ratio of 5.0; *c*: ratio of 10.0. *A*, *B*, and *C* are normalized spectral curves for *a*, *b*, and *c*, respectively.

Fig. 6. Spectral absorption curves of bilirubin, 0.5 mg/100 ml, 0.1 M phosphate buffer, pH 7.4, containing human serum albumin after stirring for 1 hr. *a*: at a bilirubin to albumin molar ratio of 0.5; *b*: ratio of 5.0; *c*: ratio of 10.0. *A*, *B*, and *C* are normalized spectral curves for *a*, *b*, and *c*, respectively.

spectral alterations coincided with the development of turbidity and bilirubin-staining of the wall of a cuvette or a beaker. This finding is similar to Brodersen and Theilgaard's observation (6) of 490 nm shoulder formation by bilirubin aggregates. These spectral characteristics, in contrast with those due to oxidation, were progressively accentuated as the concentration of bilirubin increased and/or the pH was lowered. Increase in pH in the alkaline range progressively reduced spectral shoulder formation. As suggested by Brodersen (3), bilirubin appears to exist as a colloidal material whenever its very limited aqueous solubility is exceeded.

Thus, bilirubin in aqueous media at the concentrations used in this study would exist in several states with some molecules truly in solution, whereas the great majority are in a finely dispersed colloidal state (colloidal sol). As a finely dispersed colloid, they could enter into solution resulting in an increasing molar extinction coefficient, as demonstrated with increasing pH of the media. Increased solubility of bilirubin in alkaline pH has been suggested to be due to its decreased ability to form hydrogen bonding of propionic acid side chains, intramolecularly or between molecules, and removal of hydrogen ions from these side chains (4). With agitation of the bilirubin preparation at pH 7.4, the generated kinetic energy would be expected to overcome the repulsion between finely dispersed colloidal micropolymers of bilirubin and form larger aggregates (floculants) producing the 480–560 nm spectral shoulder. When these floculants achieve sufficient size they would then precipitate. This observation fits well with modern concepts of the behavior of hydrophobic colloids (36).

If, in the preparation of bilirubin solutions, excessive agitation and exposure to light or oxygen takes place, new physical or chemical states of the pigment will develop. Many of these changes produce new spectral characteristics, the final appearance of which may represent a composite of the characteristics of four possible

types of pigment: (1) bilirubin truly in solution with a 440 nm maximal absorption; (2) finely dispersed bilirubin colloid, with spectral characteristics indistinguishable from those of soluble bilirubin; (3) bilirubin flocculant with maximal absorption between 480 and 560 nm; and (4) oxidation products of bilirubin with shorter wave lengths of maximal absorption. The proportion of each type of pigment in a preparation depends on concentration, pH, time, degree of mechanical agitation, and presence of oxidants, including molecular oxygen and trace metals. These types of changes probably account for the widely varying reports as to the wave length of maximal absorption, ranging from 420 to 440 nm, as well as the variously reported molar extinction coefficients for bilirubin in aqueous media (10, 19, 37, 38).

The solubility of bilirubin in aqueous solution as reported in the literature also varies widely (6, 7, 19, 29). Utilizing a dialysis method, Martin (19) reported that 0.1 mg/100 ml bilirubin was soluble in 0.1 M phosphate buffer at pH 7.4. Burnstine and Schmid (7) gave the solubility of bilirubin as approximately 7 mg/100 ml by an ultracentrifugation method and Brodersen and Theilgaard (6) reported 0.1 μ M (0.0058 mg/100 ml) by the evidence of colloid formation at the same ionic strength and pH. By potentiometric titration, Overbeek *et al.* (29) estimated that less than 0.003 μ g/100 ml was soluble in solutions with ionic strengths ranging from 0.003 to 0.006.

We anticipate that the molar extinction coefficient would decline with increasing bilirubin concentration if solubility were exceeded at some specific concentration. However, at bilirubin concentrations ranging from 0.005 to 2.500 mg/100 ml, no statistically significant change in molar extinction coefficient was observed. With increasing concentrations of bilirubin, there appears to be an increase in the amount of bilirubin in "colloidal sol." In contrast, significantly higher extinction coefficient ($E_{440}^{1\text{cm}}$) was obtained by increasing the pH, suggesting that increasing solubility is associated with increasing light absorptivity of the pigment. At pH 7.4, decreasing the concentration of bilirubin to the lowest concentration detectable with present equipment did not result in a significant increase in the molar extinction coefficient, suggesting that even at 0.005 mg/100 ml, the majority of the pigment was not in true solution. Moreover, shoulder formation was observed, indicating that the true solubility of bilirubin in 0.1 M phosphate buffer at pH 7.4 must be lower than 0.005 mg/100 ml.

Bilirubin in HSA solution (B:A = 1) at pH 7.4 gave a spectral peak at 460 nm with $E_{460}^{1\text{cm}}$ of 4.70×10^4 , not different from that of aqueous bilirubin at the same pH, suggesting that albumin may stabilize finely dispersed bilirubin colloid although not increasing the solubility of bilirubin.

This study has implications for both laboratory and clinical studies of bilirubin. Most investigators (6, 20, 27, 29) recognize the instability of bilirubin preparations and guard against exposure to light and heat in order to minimize oxidation. The importance of duration and degree of agitation in the preparation of bilirubin in aqueous media has not been recognized, however. In studies of bilirubin binding and of spectral characteristics this may be of enormous importance. Details of procedures used in preparation of bilirubin solutions should be described. Prolonged stirring of concentrated bilirubin preparation before its addition to albumin for studies of albumin binding will seriously alter the subsequent binding determinations. Failure to adhere to strict routines will result in poor reproducibility.

It has been suggested that the transfer of unbound unconjugated bilirubin into the central nervous system is responsible for kernicterus (9, 24, 26). The nature and amount of unbound bilirubin in whole blood should therefore be a critical factor in the development of kernicterus. Various investigators have estimated unbound bilirubin concentrations in newborn infant sera at from 0.05 to 3.00 mg/100 ml (15, 34, 40). These concentrations of unbound bilirubin in sera exceed the true solubility of bilirubin and, therefore, most of the bilirubin would be expected to exist as colloid bilirubin. Brodersen (3) postulated that colloidal bilirubin may cocrystallize with albumin to form macroaggregates (floc-

ulants) and thus prevent the transfer of bilirubin to the central nervous system. This may be true for bilirubin flocculant, but may not be so for finely dispersed bilirubin colloid.

At the present time, two alternative speculations for bilirubin transport to the central nervous system can be formulated on the basis of this study and others (9, 24, 26). The first speculation is that although the concentration of solubilized bilirubin in plasma water may be extremely small, a large amount of truly soluble bilirubin may be able to enter the brain as a result of a rapid rate of equilibration between colloidal and solubilized forms. The second postulate would be that unbound bilirubin in serum at the concentrations exceeding albumin binding capacity and its aqueous solubility must exist either as a colloidal sol or a flocculant; therefore, finely dispersed bilirubin colloid may enter cell membranes, where, either in colloidal state or after solubilization in cellular membrane lipids, it may be transported to subcellular organelles (mitochondria) where its toxic effect on cellular metabolism may result in impaired cellular function or cell death.

Bilirubin impairs the function of erythrocyte (30) and brain mitochondria (22, 31). Congenitally hyperbilirubinemic Gunn rats manifest impaired renal function (25) as well as decreased protein synthesis in the brain compared with nonicteric, heterozygote Gunn rats (12). The reported spectral curves of bilirubin associated with red cell membrane (17), phospholipid fraction of mitochondria (23), and unesterified fatty acids (11) are identical with that of bilirubin flocculant described in this communication.

Further delineation of states of bilirubin *in vivo* and *in vitro* may expand our understanding of the pathophysiology of bilirubin toxicity to various organs, including the brain.

SUMMARY

Spectrophotometric characteristics of bilirubin have been studied under various physical conditions. Mechanical agitation of bilirubin preparation at pH 7.4 progressively reduced and altered the maximal spectral absorption (440 nm) of bilirubin in aqueous media. The reduction of maximal absorption and its shift to the shorter wave lengths are attributed to oxidation of bilirubin, whereas the reduction and spectral shoulder formation at 480–560 nm are attributed to the formation of large size bilirubin flocculate. The formation of flocculants when a "solution" is agitated indicates that significant portions of the pigment were, in fact, not in solution and must have existed previously as a finely dispersed colloidal sol or supersaturated solution which progressed to a colloidal sol.

Spectral curves of bilirubin may represent a composite resulting from four physical states of bilirubin: (1) bilirubin truly in solution; (2) bilirubin in fine colloidal dispersion; (3) bilirubin flocculant; and (4) oxidation products of bilirubin.

Bilirubin in colloid form may exist *in vivo* and be responsible for bilirubin toxicity to various tissues.

REFERENCES AND NOTES

1. Arias, I. M., Gartner, L. M., Cohen, M. I., Ben Ezzer, J., and Levi, J.: Chronic nonhemolytic unconjugated hyperbilirubinemia with hepatic glucuronyl transferase deficiency: Clinical, biochemical, pharmacologic evidence for heterogeneity. *Amer. J. Med.*, 47: 395 (1969).
2. Bourrillon, R.: Analyse spectrophotométrique de la bilirubine plasmatique. *Bull. Soc. Chim. Biol. (Paris)*, 40: 111 (1958).
3. Brodersen, R.: Supersaturation with bilirubin followed by colloid formation and disposition. With a hypothesis on the etiology of kernicterus. *Scand. J. Clin. Lab. Invest.*, 29: 447 (1972).
4. Brodersen, R., Flodgaard, H., and Krogh Hansen, J.: Intramolecular bonding in bilirubin. *Acta Chem. Scand.*, 21: 2284 (1967).
5. Brodersen, R., Funding, L., Pedersen, A. O., and Roigaard-Petersen, H.: Binding of bilirubin to low-affinity sites of human serum albumin *in vitro* followed by co-crystallization. *Scand. J. Clin. Lab. Invest.*, 29: 433 (1972).
6. Brodersen, R., and Theilgaard, J.: Bilirubin colloid formation in neutral aqueous solution. *Scand. J. Clin. Lab. Invest.*, 24: 395 (1969).
7. Burnstine, R. C., and Schmid, R.: Solubility of bilirubin in aqueous solution. *Proc. Soc. Exper. Biol. Med.*, 109: 356 (1962).
8. Cremer, R. J., Perryman, P. W., and Richards, D. H.: Influence of light on the hyperbilirubinemia of infants. *Lancet*, i: 1094 (1958).
9. Diamond, I., and Schmid, R.: Experimental bilirubin encephalopathy: The mode

- of entry of bilirubin-¹⁴C into the central nervous system. *J. Clin. Invest.*, **45**: 678 (1966).
10. Fog, J., and Bakken, A. F.: Absorptivity of alkaline bilirubin and bilirubin in serum. *Scand. J. Clin. Lab. Invest.*, **20**: 70 (1967).
 11. Gartner, L. M., and Lee, K. S.: Bilirubin transport, free fatty acids and a new concept for the pathogenesis of kernicterus. Presented at the International Symposium on Bilirubin Metabolism in the Newborn, Jerusalem, Israel, April 1974. (In press.)
 12. Greenfield, S. S., and Majumdar, A. P. N.: Bilirubin encephalopathy: Effect on protein synthesis in the brain of the Gunn rat. *J. Neurol. Sci.*, **22**: 83 (1974).
 13. Herz, H., and Dybkaer, R.: Molar absorption coefficients for bilirubin in adult and infant serum with determination of an isosbestic point. *Scand. J. Clin. Lab. Invest.*, **29**: 217 (1972).
 14. Jackson, S. H., and Hernandez, A. H.: A new "bilirubinometer" and its use in estimating total and conjugated bilirubin in serum. *Clin. Chem.*, **16**: 462 (1970).
 15. Jacobsen, J., and Fedders, O.: Determination of non-albumin-bound-bilirubin in human serum. *Scand. J. Clin. Lab. Invest.*, **26**: 237 (1970).
 16. Jendrassik, L., and Grof, P.: Vereinfachte photometrische Methoden zur Bestimmung des Blut-Bilirubins. *Biochem. Z.*, **297**: 81 (1939).
 17. Kapoor, C. L., Kishna Murti, C. R., and Bajpai, P. C.: Toxic effect of bilirubin on human red blood cell and its reversal with lipids. *Indian J. Med. Res.*, **60**: 918 (1972).
 18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265 (1951).
 19. Martin, N. H.: Preparation and properties of serum and plasma proteins. XXI. Interactions with bilirubin. *J. Amer. Chem. Soc.*, **71**: 1230 (1967).
 20. McDonagh, A. F.: The role of singlet oxygen in bilirubin photooxidation. *Biochem. Biophys. Res. Commun.*, **44**: 1306 (1971).
 21. Michaelsson, M.: Evaluation of method for determination of bilirubin in serum using direct spectrophotometry. *Scand. J. Clin. Lab. Invest.*, **30**: 387 (1972).
 22. Mustafa, M. G., Cowger, M. L., and King, T. E.: The effect of bilirubin on mitochondrial reactions. *J. Biol. Chem.*, **244**: 6403 (1969).
 23. Mustafa, M. G., and King, T. E.: Binding of bilirubin with lipids: A possible mechanism of its toxic reactions in mitochondria. *J. Biol. Chem.*, **245**: 1084 (1970).
 24. Odell, G. B.: Studies in kernicterus. I. The protein binding of bilirubin. *J. Clin. Invest.*, **38**: 823 (1959).
 25. Odell, G. B., Bolen, J. L., Poland, R. L., Seungdamrong, S., and Cukier, J. O.: Protection from bilirubin nephropathy in jaundiced Gunn rats. *Gastroenterology*, **66**: 1218 (1974).
 26. Odell, G. B., Cohen, S. N., and Kelly, P. C.: Studies in kernicterus. II. The determination of the saturation of serum albumin with bilirubin. *J. Pediatr.*, **74**: 214 (1969).
 27. Ostrow, J. D., and Branham, R. V.: Photodecomposition of bilirubin and biliverdin in vitro. *Gastroenterology*, **58**: 15 (1970).
 28. Ostrow, J. D., Hammaker, L., and Schmid, R.: The preparation of crystalline bilirubin-C¹⁴. *J. Clin. Invest.*, **40**: 1442 (1962).
 29. Overbeek, J. T. G., Vink, C. L. J., and Denstra, H.: The solubility of bilirubin. *Rec. Trav. Chim. Pays-Bas*, **74**(4): 81 (1955).
 30. Petrich, V., Gemp-Friedrich, W., and Gobel, U.: Comparative measurements of enzyme activities and 2,3-diphosphoglycerate in the erythrocytes of newborns with and without transitory hyperbilirubinemia. *Acta Paediat. Stockh.*, **62**: 596 (1973).
 31. Rose, A. L., and Johnson, A. B.: Bilirubin encephalopathy: Neuropathological and histochemical studies in the Gunn rat model. *Neurology*, **22**: 420 (1972).
 32. Sioni, R., Sauwalder, H., and Richterich, R.: Ein vergleich physikalischer und chemischer Methoden zur Bestimmung des Plasmabilirubins. *Schweiz. Med. Wschr.*, **99**: 1784 (1969).
 33. Shinowara, G. Y.: Spectrophotometric studies in blood serum and plasma. The physical determination of hemoglobin and bilirubin. *Amer. J. Clin. Pathol.*, **24**: 696 (1954).
 34. Schiff, D., Chan, G., and Stern, L.: Sephadex G-25 quantitative estimation of free bilirubin potential in jaundiced newborn infants' sera: A guide to the prevention of kernicterus. *J. Lab. Clin. Med.*, **80**: 445 (1972).
 35. Standards Committee: A uniform bilirubin standard. Recommendations of the College of American Pathologists. *Amer. J. Clin. Pathol.*, **39**: 90 (1963).
 36. Vold, M. J., and Vold, R. D.: *Colloid Chemistry*, p. 9 (Van Nostrand Reinhold Co., New York, 1964).
 37. Wennberg, R. P., and Cowger, M. L.: Spectral characteristics of bilirubin bovine albumin complexes. *Clin. Chim. Acta*, **43**: 55 (1973).
 38. With, T. K.: Spectral absorption of bilirubin. Measurements in pure aqueous solution and in solutions containing human sera. *Acta Physiol. Scand.*, **10**: 172 (1945).
 39. Woolley, P. V., III, and Hunter, M. J.: Binding and circular dichroism data on bilirubin-albumin in the presence of oleate and salicylate. *Arch. Biochem. Biophys.*, **140**: 197 (1970).
 40. Zamet, P., and Chunga, F.: Separation by gel filtration and microdetermination of unbound bilirubin. II. Study of sera in icteric newborn infants. *Acta Paediat. Scand.*, **60**: 33 (1971).
 41. Dr. Gartner is a Career Development Awardee from the National Institute for Child Health and Human Development.
 42. This work has been supported by grants from the National Institute for Child Health and Human Development NIH 5 R01-HD 03783 and the Gail I. Zuckerman Foundation for Research in Chronic Liver Diseases in Children.
 43. Requests for reprints should be addressed to: L. M. Gartner, M.D., Department of Pediatrics, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, N.Y. 10461 (USA).
 44. Accepted for publication March 15, 1976.

Copyright © 1976 International Pediatric Research Foundation, Inc.

Printed in U.S.A.

Pediat. Res. **10**: 788-791 (1976)

Bilirubin
hyperbilirubinemia
liver

promethazine-HCl
Rh erythroblastosis

The Effect of Promethazine Hydrochloride on Bilirubin Metabolism in the Rat

SERGIO L. VAISMAN, KWANG-SUN LEE, AND LAWRENCE M. GARTNER⁽²²⁾

Division of Neonatology, Department of Pediatrics, The Rose F. Kennedy Center for Research in Mental Retardation and Human Development and the Liver Research Center, Albert Einstein College of Medicine, Bronx, New York, USA

Extract

The effect of 21 days of promethazine-HCl administration on hepatic bilirubin metabolism and transport was studied in adult rats. A significant increase in mean cumulative hepatic bilirubin uptake (84.5 ± 7.6 (SE) $\mu\text{g}/100 \text{ g}/\text{min}$ in controls vs. 110.0 ± 4.3 in treated rats), mean hepatic glucuronide conjugation ($1,330 \pm 86$ (SE) μg bilirubin conjugated/g liver/40 min in controls vs. $1,713 \pm 61$ in treated rats), and mean maximal hepatic excretion (47.2 ± 4.9 (SE) $\mu\text{g}/100 \text{ g}/\text{min}$ vs. 63.5 ± 2.7) was observed in

treated animals. Mean total liver weight and total hepatic protein also increased significantly.

These observations suggest that promethazine is an inducer of protein and enzyme synthesis in rat liver and is capable of significantly stimulating the three major steps in hepatic disposal of bilirubin.

Speculation

The stimulatory effect of promethazine on bilirubin metabolism and transport introduces a second or alternate explanation for the