Fluorometric Determination of "Albumin-Titratable Bilirubin" in the Jaundiced Neonate

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We report a fluorometric technique for determination of albumin-titratable bilirubin in the jaundiced neonate. Although bilirubin alone has very little native fluorescence, considerable emission is observed in the presence of albumin under acid conditions. Analysis of the plasma sample alone and in the presence of excess human serum albumin solution appears to reflect the bilirubin tightly bound to albumin and the total serum bilirubin, respectively. The difference between these two values has been designated as "albumin-titratable bilirubin." Where the concentration of albumin-titratable bilirubin is considerable, a typical saturation effect is observed. In samples where the circulating bilirubin is strongly bound to endogenous albumin, no change in fluorescence is seen when exogenous albumin is added. Results correlate well with the clinical picture.

Additional Keyphrases: pediatric chemistry • liver disease • diagnostic aid • kernicterus

While a causal relationship between hyperbilirubinemia during the neonatal period and kernicterus in infancy is well established, cases of kernicterus at relatively low concentrations of bilirubin have been reported (1–6). Odell et al. (7) emphasized that total bilirubin concentration is not necessarily the most important criterion of toxicity, but rather the portion of bilirubin that is not firmly bound to albumin and is thus free to cross the cell membrane.

Measurement of this non-albumin-bound bilirubin is difficult, because the concentrations are low and unconjugated bilirubin, by virtue of its lipid solubility, is never "free" in plasma, but binds to erythrocyte (8) and platelet surfaces (9). Methods (10–14), with the possible exception of the peroxidase technique described by Jacobsen and Wennberg (15), aimed at assessing those infants at risk from bilirubin toxicity are relatively difficult to perform, time consuming, and usually require large amounts of serum, so that they are not applicable to routine investigation.

It would appear that some of these difficulties could be overcome by using the simple, rapid method described by Roth (16), who noted that bilirubin would fluoresce in the presence of albumin under acid conditions. Roth suggested that the loss of linear relationship between bilirubin concentration and fluorescence at bilirubin concentrations in serum exceeding 120 mg/liter could be due to saturation of endogenous serum proteins with bilirubin. Samples with high bilirubin or low albumin concentration could only be measured accurately by the fluorescence technique in the presence of added albumin, i.e., in some sera the bilirubin could be "titrated" with albumin. In anticipation that this "titratable bilirubin" could provide an index of the bilirubin not firmly bound to albumin, we have further investigated Roth’s method.

Materials and Methods

Materials

Blood samples. Blood samples, obtained from umbilical cord veins or by heelprick, from newborn infants, were collected into heparinized tubes. Where possible, plasma was separated within 1 h and stored at 4 °C in the dark. Assays were usually done within 2 h and invariably within 24 h of collection.

Reagents. Elevated bilirubin calibration sera used were "Versatol Pediatric" (General Diagnostics Division, Warner Chilcott Laboratories, Morris Plains, N. J. 07950) or that supplied by Dade (Dade Division, American Hospital Supply Corp., Miami, Fla. 33152), or "Q-pak Chemistry Control Serum II (Hyland, Division of Travenol Laboratories, Costa Mesa, Calif. 92626).

Phosphoric acid (85%, d^20 = 1.71) was obtained from Merck, Darmstadt, Germany. Aqueous ascorbic acid (10 g/liter) was prepared just before use and protected from light. Sodium chloride, 9 g/liter in bicarbonate-free water, was adjusted to pH 7.4 with NaOH, 1 mol/liter, if necessary and stored at 4 °C.

Acridine orange was obtained from Eastman Kodak, Rochester, N. Y. 14650. Just before use, a
stock aqueous solution (1000 µg/liter) was diluted in dimethyl formamide to 0.2 mg/liter. This diluted solution was stable for 1 h.

Rabbit antihuman serum albumin was obtained from Behringwerke, Germany. Horse antihuman serum was obtained from Institut de Pasteur, Paris, France.

Albumin. Human serum albumin concentrate (250 g/liter) obtained from Commonwealth Serum Laboratories, Parkville, Victoria, Australia, was diluted with sodium chloride solution (9 g/liter) to give a final concentration of approximately 40 g/liter. The pH was adjusted to 7.4 with NaOH and the solution was stored at 4 °C. This albumin solution was used originally in assays of patients’ plasma. A fresh solution was made up weekly.

Human serum albumin obtained from a number of other sources was also tested in the assay procedure: Human serum albumin RHA 21, dried and purified to 100% electrophoretic purity, was obtained from Behringwerke, Germany, and used as a 40 g/liter solution in physiological saline (NaCl, 9 g/liter); the pH of this solution was 7.5. Human serum albumin fraction V (crystalline) was obtained from Commonwealth Serum Laboratories, Parkville, Victoria, and used as a 40 g/liter solution, by weight, in saline. The pH was adjusted to 7.4 with NaOH. Human serum albumin, crystallized, obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio 44128, was used as a 40 g/liter solution, in saline. The pH was adjusted to 7.4 with NaOH. In addition, a 100 g/liter aqueous solution of this albumin was defatted by the method of Chen (17).

Neonatal albumin. Human neonatal albumin was prepared by the method of Cohn et al. (18) from pooled cord blood, collected at time of delivery. The fraction V precipitate was resuspended in saline. The suspension (70 g/liter) was further purified by elution from a diethylaminoethyl-cellulose-containing column [2.5 cm by 40 cm, equilibrated with Tris-HCl (20 mmol/liter, pH 8.0) containing, per liter, 0.5 mmol of ethylenediaminetetraacetate and 14 mmol of mercaptoethanol] by use of a sodium chloride gradient (0–0.4 mol/liter). Fractions containing the albumin peak were pooled and concentrated in a Diaflow apparatus (Amicon Corp., Lexington, Mass. 02173). The concentrate was checked for purity by electrophoresis on cellulose acetate, immunoelctrophoresis, and radial immunodiffusion on Ouchterlony plates.

Normal human serum. Blood from normal healthy women volunteers was collected by venipuncture into plain glass tubes and allowed to clot at room temperature. The serum was then stored at 4 °C and used within three weeks.

Procedures

Assay of albumin. Albumin in the plasma samples was determined by the modified bromcresol green method of McPherson and Everard (19), or, in the case of albumin solutions, by its absorption at 279 nm, with use of an absorptivity of 5.30 for a 10 g/liter solution (20).

Total serum proteins. These were measured by refractive index, with an Hitachi/Perkin-Elmer (Perkin-Elmer Corp., Norwalk, Conn. 06856) hand refractometer (21).

Fluorometry. The procedure follows:

1. Four glass tubes were set up for each blood sample, two containing 40 µl of saline solution (NaCl, 9 g/liter) and two containing 50 µl of normal human serum or a serum albumin solution (40 g/liter).
2. 10 µl of plasma was pipetted into each of the four tubes.
3. A standard bilirubin curve was set up in the range 0–200 mg/liter, with use of 40-µl aliquots of serial dilutions in saline of the Dade bilirubin control (bilirubin 200 mg/liter, albumin 55 g/liter).
4. 40 µl of normal human serum was added to a tube containing 10 µl of saline. This tube provided a correction factor for the endogenous bilirubin and albumin present in the normal human serum. (All additions in steps 1–4 were made with Pedersen glass micropipets.)
5. Phosphoric acid, 0.6 ml, was added to all standards and samples and mixed on a vortex-type mixer.
6. At least 1 min after mixing, 3 ml of ascorbic acid reagent was added to each tube and again mixed.

Fluorescence short-scan measurements were made within 2 h in quartz cuvets with an Hitachi/Perkin Elmer Model MPF-2A fluorescence spectrophotometer at excitation 435 nm, excitation slit 6 mm, emission between 480 and 520 nm, and emission slit 12 mm. The fluorescence maximum was set on either the highest standard or the acridine orange solution (0.2 mg/liter).

A tube containing 40 µl of saline and 10 µl of the plasma sample was treated as a reagent blank. This was diluted with the ascorbic acid solution before phosphoric acid was added. The fluorescence blank for plasma samples was less than one fluorescence unit, and varied only ±5% between samples, so that it was only necessary to set up one plasma blank for each series of samples. High blanks have, however, been observed in cases of rhesus incompatibility when conjugated bilirubin concentrations are abnormally high. Separate blanks were necessary for each concentration of bilirubin in the standard curve. Corrected standard values were plotted on a graph, and the corrected plasma sample values were read from the graph.

Because samples are diluted fivefold in the assay, results from the graph have to be multiplied by 5 to give mg of bilirubin per deciliter (or 50 for mg/liter). The initial value of bilirubin in serum in the absence of exogenous albumin was considered to correspond (see below) to the bilirubin that is tightly bound to albumin in the patient’s serum, in such a way that it is able to fluoresce, while the final value, in the presence of 40 µl of exogenous albumin, gave the total bilirubin concentration (16). The difference between
these two values was the "albumin-titratable bilirubin".

Total bilirubin concentrations were also measured by the Evelyn-Malloy diazo method (22) in the Department of Chemical Pathology, Royal Prince Alfred Hospital.

Error of method. The bilirubin standard curve produced by the fluorescence method is linear in the range 0–200 mg/liter if the Dade Bilirubin calibration material is used. Interassay variability of diluted plasma samples or standards was determined as ±4 mg/liter.

Results

Albumin-titratable Bilirubin

Initially, sera were assayed fluorometrically after adding increasing amounts of an albumin solution to 10-μl aliquots of sample, the total volume being adjusted to 50 μl with saline.

Subsequently, two simplifications have been introduced as indicated above (Materials and Methods). First, because varying results were obtained with some batches of albumin, normal human serum was used instead (see below). Second, we found that two fluorescence measurements were sufficient, one with no addition of normal serum, the other with the addition of sufficient normal serum to saturate all the bilirubin, i.e., the initial and end point of the "titration."

However, in order to describe the development of the method, we present results obtained from titration experiments. A typical saturation effect was seen in most elevated samples. For example, patient C (Figure 1) had a total serum bilirubin of 230 mg/liter, although the fluorescent method in the absence of added albumin indicated a value of only 140 mg/liter. Exogenous albumin caused a steady increase in the fluorescence of bilirubin/albumin. In other words, only a portion of the total bilirubin in the original sample was bound to (or interacted with) albumin in such a way that it fluoresced under the experimental conditions. When excess albumin was added, the intensity of fluorescence corresponded to the total bilirubin present. Because the difference can only be measured after addition of albumin, we have designated this portion of the bilirubin, "albumin-titratable bilirubin." In Figure 1, sample C therefore has an albumin-titratable bilirubin of 90 mg/liter, while samples from patients F and H had no detectable change in fluorescence of bilirubin after addition of albumin.

In Figure 2, another example of albumin-titratable bilirubin is shown (upper curve). This sample was taken before a third exchange transfusion of a baby with rhesus incompatibility. The sample represented by the bottom curve was taken from the same baby two days later, when no albumin-titratable bilirubin was found. No further transfusion was required for this infant.

Total Bilirubin

Bilirubin concentrations measured fluorometrically in fivefold albumin-diluted samples correlated well with total bilirubin values obtained by the microadaptation of the Evelyn-Malloy diazo technique (22). The 95% confidence limits for r were 0.88 and 0.95. This confirms the findings of Roth, who reported a
good correlation \( r = 0.96 \) between his method and the Michaelsson modification \( (23) \) of the Jendrassik and Grof method \( (24) \).

**Albumin Solutions**

Various albumin solutions were substituted for the human serum albumin, 40 g/liter, which had been used routinely in the Fluorescence method. All had low fluorescence blanks except for the Nutritional Biochemicals Corp. crystalline albumin, which had been defatted \( (17) \). Before defatting there was a low fluorescence blank for this albumin solution. Almost identical saturation effects with the elevated calibration serum sample were observed initially for all the albumin samples tested except for the Behringwerke RHA 21, which failed to saturate but gave increasing emission with increasing aliquots of albumin added to the hyperbilirubinemic serum. The results after the final addition of albumin indicated a bilirubin greater than that in the calibration solution. There was no obvious explanation for this inconsistent result.

Difficulties were experienced, however, with subsequent batches of albumin from Commonwealth Serum Laboratories and Nutritional Biochemicals Corp. in that fluorescence was severely quenched when these albumin solutions were added to bilirubin calibration solutions. This problem would make the use of these albumin solutions impracticable in the routine situation. Normal human serum can be substituted for albumin solution and no quenching has been observed to date. Its routine use is recommended.

**Neonatal Albumin**

Neonatal albumin, purified as described above, produced a single albumin band on cellulose acetate electrophoresis. The albumin was immunologically pure, giving a single precipitin line vs. rabbit antihuman serum albumin or horse antihuman serum in immunoelectrophoresis or Ouchterlony gel.

A solution of the neonatal albumin, diluted to 40 g/liter with saline, as estimated by the bromcresol green dye-binding method, was used instead of the human serum albumin in the fluorometric assay. The fluorescence maxima were the same as for human serum albumin from adults. When the saturation effect of neonatal human serum albumin was compared with that of serum albumin from adults on an elevated bilirubin sample, no difference was observed.

**Correlation between Albumin-titratable Bilirubin and Bilirubin/Albumin Ratio**

Albumin-titratable bilirubin, total bilirubin, and serum albumin concentrations were measured in 44 samples from 23 individuals. The group included infants with neonatal jaundice associated with ABO and Rh incompatibility, urinary tract infection, and respiratory distress. There was a significant correlation between albumin-titratable bilirubin and the total bilirubin/albumin ratio. 95% confidence limits for \( r \) were 0.71 and 0.90. The equation for the regression line was \( y = 0.89x - 0.81 \) (Figure 3).

**Correlation between Albumin-titratable Bilirubin and Bilirubin/Total-Protein Ratio**

Total protein was measured in all jaundiced newborns in this hospital; albumin was estimated in only a few cases. Because there is a fairly constant relationship between albumin and the total serum protein concentration, we decided to compare albumin-titratable bilirubin with bilirubin/total protein ratio, and 92 samples from 64 infants were assayed (Figure 4). Once again, the group included infants with Rh and ABO incompatibilities, before and after exchange transfusions. A significant correlation existed between the two variables; 95% confidence limits for \( r \) were 0.81 and 0.87. The equation for the regression line was \( y = 1.99x - 1.98 \).

**Discussion**

The method we describe appears to provide a useful supplement to methods currently available to the clinician for assessment of the jaundiced neonate. It is easy to perform and requires only small amounts of plasma so that a heelprick blood-sample is sufficient.\(^2\) The one experiment provides an estimate of both total bilirubin and of a variable that we call “al-

\(^2\) Uete \( (27) \) has recently described a modification of Roth’s method to measure total bilirubin in the neonate.
albumin-titratable bilirubin,” which appears to reflect the clinical condition of the patient.

The in vitro experiments of Jacobsen (25) indicate that one molecule of albumin can bind as many as three molecules of bilirubin, one very tightly (K_D approx. $7 \times 10^{-9}$ mol/liter) and two less tightly (K_D approx. $10^{-7}$ mol/liter). However, the in vitro situation does not appear to correspond to that in vivo. For example, Jacobsen and Fedders (13), using the peroxidase method, demonstrated that the bilirubin-binding capacity in the newborn is considerably less than one molecule of bilirubin per molecule of albumin. Further, Nakamura and Lardinois (26), using Sephadex gel filtration, detected “free” bilirubin at a bilirubin/albumin ratio of 0.4, while above 0.6 “unbound” bilirubin could be detected in 13 of 14 cases.

In the current experiments, the highest bilirubin/albumin molar ratio observed was 0.68. However, albumin-titratable bilirubin could be detected at bilirubin/albumin molar ratios as low as 0.1 in some infants suffering from very mild neonatal jaundice. Therefore we believed it was important, if possible, to assess the significance of various concentrations of albumin-titratable bilirubin without waiting for a followup examination of central nervous system function in the infants at five years of age.

Odell et al. (7) reported such a followup study in connection with their saturation-index method. They found that infants with saturation-index values >8 demonstrated significant central nervous system damage at five years of age. They also demonstrated a close correlation between the saturation index and the bilirubin/total-protein ratio, if values from Rh-incompatibility-affected infants were excluded.

There is evidently a close correlation between both the bilirubin/total-protein ratio and albumin-titratable bilirubin (Figure 4). The correlations held, even when values from cases of Rh and ABO incompatibility were included. They could be used to obtain a relationship between the saturation index and the albumin-titratable bilirubin, because the regression lines associated with them were parallel. From this relationship, the value of albumin-titratable bilirubin corresponding to a saturation index of 8 was found to be 55 mg/liter.

We considered the values for albumin-titratable bilirubin in Figure 4; those infants who could be affected by bilirubin toxicity would be a number of Rh-incompatibility-affected infants, one with ABO incompatibility, a case of urinary tract infection, an infant of a diabetic mother, and a premature infant with prolonged low-grade jaundice.

Most of the values obtained lie close to the regression lines relating albumin-titratable bilirubin to the bilirubin/total-protein and bilirubin/albumin ratios. In most cases, therefore, these ratios could provide as much useful information regarding the danger of bilirubin toxicity as would the measurement of albumin-titratable bilirubin. In practice, however, cases of bilirubin toxicity have been observed with low values of the bilirubin/total-protein ratio.

This is emphasized in Figure 4 by those samples that differ markedly from the expected value. In these cases there may be factors that influence the binding of bilirubin to albumin that would not be detected by using the bilirubin/albumin ratio. Infants suffering from hemolytic disease are in danger of bilirubin toxicity because the high turnover of erythrocytes results in increased unconjugated bilirubin values. In the case of urinary tract infection and the infant of a diabetic mother, factors such as hypoglycemia and acidosis could possibly play a part in impairing bilirubin–albumin binding, so that high concentrations of albumin-titratable bilirubin could be detected at bilirubin concentrations as low as 150 mg/liter. The result for one of the infants with urinary tract infection shown on Figure 4 lies outside the 95% tolerance limit of the relationship between albumin-titratable bilirubin and bilirubin/total-protein ratio, which may indicate the presence of such factors.

Clearly, followup studies are required to assess the newborn in whom albumin-titratable bilirubin was measured. Until these results are available, we think that infants with albumin-titratable bilirubin >50 mg/liter should be considered for exchange transfusion.

It should be stressed that it is not possible at this stage to ascribe a physical meaning to the variable we have designated as “albumin-titratable bilirubin.” While it is reasonable to assume that the fluorescence results from the stoichoimetric binding of bilirubin to
albumin (26, 27), the equilibrium between bilirubin and albumin would be considerably altered on dilution of the sample and reduction of pH. For this reason, it is considered that while the "cut-off" figure of 50 mg/liter may reflect the disturbance of this equilibrium, it should be regarded as an experimentally useful variable that may only partly reflect the true physical situation. Although the physical chemistry is obscure, the method does provide useful information as to the status of the jaundiced neonate.

We thank Dr. P. B. Rowe for bringing the article by M. Roth to our attention. This work was supported by the Special Services Fund of the Royal Prince Alfred Hospital (S.B.McC.) and by the National Health and Medical Research Council of Australia (G.K.B. and W.J.O'S.).

References