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Determination of Unbound Bilirubin in the Serum of Newborns

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An enzymatic assay is described for non-albumin-bound bilirubin in the serum of newborn infants. Unbound bilirubin is oxidized to colorless compounds by ethyl hydroperoxide in the presence of horseradish peroxidase (EC 1.11.1.7), while albumin-bound bilirubin is protected from oxidation. Because the equilibrium between albumin and bilirubin occurs rapidly, the oxidation step is rate limiting, and the initial oxidation velocity of total bilirubin is proportional to the unbound bilirubin concentration. By titrating serum with bilirubin in vitro, the association constant and binding capacity of high-affinity sites for albumin binding can be determined. Normal human serum albumin tightly binds 1 mole of bilirubin per mole of albumin (binding constant, 2–4 × 10^8 liter/mol). Although weaker secondary binding occurs, the unbound bilirubin fraction increases rapidly after the high-affinity binding sites are saturated. Compromised newborns may have a decreased apparent binding capacity and (or) binding affinity. The method can be used to assess the risk of a jaundiced infant for bilirubin encephalopathy.

Additional Keyphrases: *bilirubin encephalopathy • free and bound bilirubin • binding affinity and capacity • perinatal chemistry • effects of drugs • kernicterus • erythroblastosis • enzymatic assay*

Unconjugated bilirubin can poison many vital cell functions (*1, 2*) but is normally detoxified by binding firmly to serum albumin (*2–4*). A wealth of experimental and clinical evidence indicates that the unbound bilirubin concentration is probably the most critical parameter in establishing the risk for bilirubin encephalopathy (*2–9*).

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Until recently, there has been no practical method for measuring the low concentrations of unbound bilirubin that normally exist in icteric sera, and risk for bilirubin encephalopathy has been estimated by indirect assessments of albumin binding. Principles involved in these methods include competitive displacement of a dye by bilirubin [e.g., HBABA (*10*), PSP (*11*), bromphenol blue (*12*)], displacement of bilirubin by high concentrations of a competing substance [e.g., salicylate (*13*)], and competition with albumin for bilirubin binding [e.g., erythrocyte binding (*14*), Sephadex filtration (*15, 16*)]. Of these tests, Sephadex gel filtration most directly measures the unbound bilirubin concentration, but has the limitation of being insensitive to low concentrations of unbound bilirubin. Furthermore, Sephadex partially binds bilirubin in competition with albumin and cannot be expected to reflect accurately the concentration of unbound pigment (*17*).

Unbound bilirubin has been determined and albumin binding characterized by use of dialysis (*18*) and spectrophotometric techniques (*19*), but these methods are difficult and unsuitable for routine clinical analysis. A sensitive enzymatic method for measuring low concentrations of unbound bilirubin was recently developed by Jacobsen (*20*), based on the observation of Brodersen and Bartels (*21*) that bilirubin can be oxidized by hydrogen peroxide in the presence of a peroxidase. Excess albumin greatly inhibits the oxidation, because of albumin–bilirubin complex formation. Jacobsen (*20*) concluded that bilirubin and albumin equilibrate very rapidly compared with the rate at which unbound bilirubin is oxidized. Thus, the concentration of unbound bilirubin could be determined from the initial oxidation rate of *total* bilirubin, measured as the decrease in chloroform-extractable bilirubin after a timed exposure to peroxidase and H_2O_2 (Figure 1). More recently, Jacobsen and Fedders (*22*) analyzed the unbound

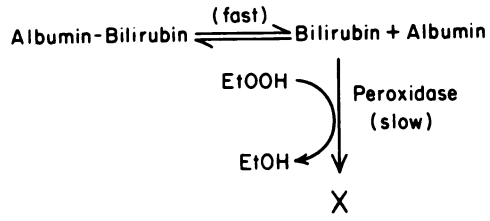

Fig. 1. Chemical reactions involved in the peroxidase assay
The oxidation step is rate limiting

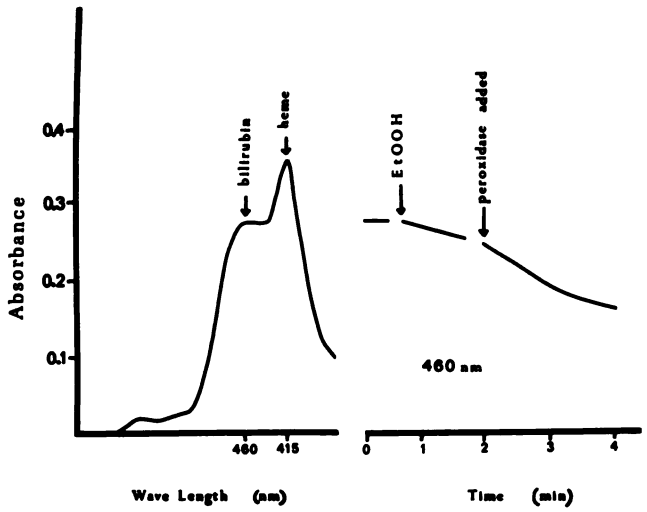

Fig. 2. Example of peroxidase assay
The absorption spectrum of the diluted serum sample is scanned and absorbances at 460 nm and 415 nm are noted. With the wavelength set at 460 nm, the decrease in absorbance is measured after adding ethyl hydroperoxide and again after adding horseradish peroxidase

bilirubin in human sera, substituting ethyl hydroperoxide for H_2O_2 to avoid the effects of catalase activity in serum. Unfortunately, their method failed to correct for the oxidation of bound bilirubin and thus yielded erroneously high values for unbound bilirubin.

Here, we examine the biochemical basis of the peroxidase method in more detail and describe modifications that make the assay more practical for evaluating serum from newborns. Because the oxidation products are colorless and only minute concentrations of conjugated bilirubin usually exist in newborn sera, the oxidation rate is determined by direct spectrophotometry and the chloroform-extraction step is eliminated.

Materials and Methods

Materials

Crystalline bilirubin (Sigma grade), human serum albumin (fraction V), and horseradish peroxidase (type 1) (EC 1.11.1.7) were obtained from Sigma Chemical Co., St. Louis, Mo. 63178. The bilirubin had a molar absorptivity (ϵ) in chloroform of 60 100 at 452 nm. Ten percent ethyl hydroperoxide was obtained from A. B. Ferrosan, S 210-10 Malmo, Sweden.[3] Sulfisoxazole diethanolamine (Gantrisin) was produced by Roche Laboratories. Serum samples from patients were usually analyzed within 24 h of collection.

Reagents

Phosphate buffer, pH 7.4, 55 mmol/liter, containing ethylenediaminetetraacetate, 1.0 mmol/liter.

Bilirubin, 1 mg dissolved in 10 μl of NaOH, 1.0 mol/liter, and diluted with 1.0 ml of demineralized water and 10 μl of ethylenediaminetetraacetate, 0.1 mol/liter. This stock solution, which is stable for several hours, was diluted 15-fold with water just before using.

Horseradish peroxidase, 1.0 mg dissolved in 1.0 ml of demineralized water.

Ethyl hydroperoxide, prepared freshly each day by diluting the purchased stock solution 10-fold with demineralized water.

[3] Distributed in the United States through Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y. 11514.

Procedure

Serum, 25 μl, was added to 1.0 ml phosphate buffer in a semi-micro cuvette of 10-mm pathlength and the absorption spectrum was recorded between 610 and 400 nm (Figure 2). Ethyl hydroperoxide, 5 μl, was added to the sample and the decrease in absorbance at 460 nm (the absorbance maximum of albumin-bound bilirubin) recorded for 1 min. Peroxidase solution, 10 μl, was then added and the decrease in A_{460} again recorded. Assays were conducted at room temperature with a Cary Model 16 recording spectrophotometer.

Calculations. Total bilirubin concentration was determined by direct spectrophotometry. The molar absorptivity of bilirubin in the presence of albumin was determined to be 48 500 at 460 nm. The contribution of heme contamination to absorbance at 460 nm was estimated by the formula:

$$A_{460}^{(\text{Hemoglobin})} = \left(A_{415} - \frac{A_{460}}{2} \right) \times 0.1 \qquad (1)$$

The unbound bilirubin concentration was calculated from the oxidation velocity. The decrease in A_{460} after adding ethyl hydroperoxide was due to the peroxidase activity of hemoglobin (Figure 2). This reaction rate was subtracted from the initial rate observed after adding the horseradish peroxidase to determine the oxidation velocity caused by peroxidase alone (V_o). An additional correction was made for the slow oxidation rate of bound bilirubin; this was necessary for accurate determinations of very small concentrations of unbound bilirubin.

Because first-order reaction conditions exist, the unbound bilirubin concentration was calculated as follows:

$$Vo = K\ [\text{substrate}] \times [\text{enzyme}] \quad (2)$$

Therefore,

$$[\text{Unbound bilirubin}] = \frac{V_0}{K\ [\text{peroxidase}]} \quad (3)$$

The peroxidase rate constant, K, was determined by measuring the oxidation velocity of bilirubin in the absence of albumin. If V_0 is corrected to a standard peroxidase activity, the formula may be simplified to

$$[\text{Unbound bilirubin}] = \frac{Vo}{K_p} \quad (4)$$

Peroxidase rate constant. Since the bilirubin solution is supersaturated in the experiment (*23*), care must be taken in mixing samples to minimize bilirubin aggregation. The following procedure proved satisfactory:

(1) A 5 μmol/liter bilirubin solution, pH 8.5–9.5, was prepared using 0.1 mol/liter NaOH and degassed deionized water.

(2) 2.0 ml bilirubin solution was added to 2.0 ml of phosphate buffer (0.11 mol/liter, pH 7.4) in a test tube and mixed by pouring gently into a 4-ml cuvette.

(3) The bilirubin concentration was determined from the absorbance at 440 nm, the absorbance maximum of aqueous bilirubin. The molar absorptivity of aqueous bilirubin was determined to be 47 500 at 440 nm.

(4) The procedure was then repeated except that 25 μl of ethyl hydroperoxide and 10 μl of diluted peroxidase (0.5 mg/100 ml) were mixed with the buffer just before bilirubin was added. The reaction rate was determined from the initial decrease in absorbance at 440 nm.

This procedure was also followed to determine V_{max} and K_m for the reaction, except that the bilirubin concentration varied.

Titration of albumin. Serum binding properties could be ascertained by adding increasing amounts of bilirubin to diluted serum samples, repeating the peroxidase assay, and plotting the data on a Scatchard plot. Each 5 μl of bilirubin solution added was equivalent to about a 2.0 mg/100 ml increase in serum bilirubin concentration.

Other methods. Serum albumin concentrations were assayed by the bromcresol green method (*24*). Conjugated bilirubin concentrations were determined by the method of Weber and Schalm (*25*). Gel filtration of bilirubin–albumin solutions was performed by using 5-cm columns of Sephadex G-25 gel in Pasteur pipettes. The columns were equilibrated with phosphate buffer (55 mmol/liter, pH 7.4). Protein applied to the column (50 μl) was completely recovered in a 2.0-ml eluate. Bilirubin bound to the Sephadex was removed by a second elution with albumin.

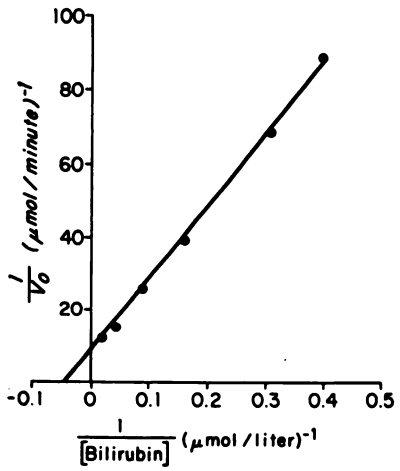

Fig. 3. Lineweaver–Burk plot describing the oxidation of aqueous biirubin in the presence of ethyl hydroperoxide and horseradish peroxidase

Results

Rationale and Standardization

Reaction kinetics. The kinetics of bilirubin oxidation with ethyl hydroperoxide and horseradish peroxidase are illustrated on a double-reciprocal plot in Figure 3. Variation in reaction rate with bilirubin concentration obeyed the Michaelis–Menten law (even though the bilirubin solution was supersaturated). V_{max} was 2.3 μmol/min and the K_m was 20 μmol/liter.

The peroxidase rate constant, K_p, was determined with use of 2.5 μmol/liter bilirubin and was calculated for a peroxidase concentration of 10 μg/ml (used in most clinical assays). K_p was approximately 3.8 ΔA/min per micromole of bilirubin per liter. Repeated determinations of K_p for a given peroxidase preparation varied only slightly when a single bilirubin standard solution was used (CV ranged from 0.0–3.7%). Greater variation in K_p occurred when more than one standard bilirubin solution was used. Since the latter variation would contribute to day-to-day variation of the method, it was studied by repeating the K_p determination with 3–5 bilirubin solutions daily for 14 days (using a freshly prepared peroxidase each day). The within-day coefficient of variation ranged 2.3–19.2%; the average CV was 6.7%. The reason for the variation was not clear, but may be related to micro-aggregation in some solutions.

Oxidation of bound bilirubin. The addition of albumin (fraction V) greatly inhibited the reaction, especially with a molar ratio of bilirubin:albumin less than one (Figure 4). Extrapolation to a molar ratio of zero indicated that bound bilirubin was oxidized at a slow rate. The oxidation velocity of bound bilirubin could best be estimated by measuring V_o at various concentrations of bilirubin with a fixed bilirubin:albumin molar ratio (0.10) and plotting V_o vs. total bilirubin concentration (Figure 5). Since the

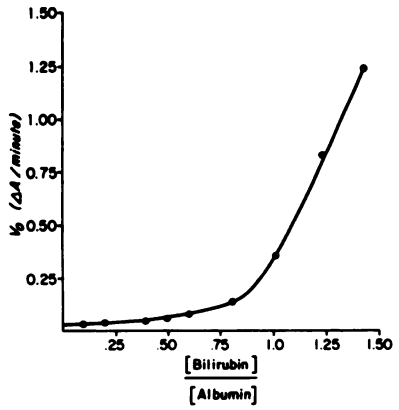

Fig. 4. Inhibition of bilirubin oxidation by albumin (Cohn fraction V)

Bilirubin:albumin molar ratio extrapolated to zero does not intercept at zero velocity, indicating slow oxidation of albumin-bound bilirubin. The concentration of unbound bilirubin increases rapidly when the molar ratio exceeds 1.0

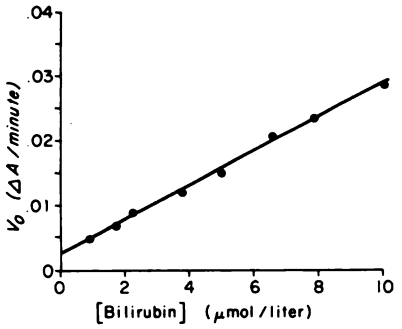

Fig. 5. Oxidation of albumin-bound bilirubin

The bilirubin:albumin (fraction V) molar ratio is 0.10. Because the association constant is very high, the unbound bilirubin concentration does not change with increasing concentration of total bilirubin; the slope, 0.025 ΔA/min per μmol of bilirubin per liter, represents the oxidation rate of bilirubin bound to albumin

association constant of the complex is very high, the concentration of unbound bilirubin remains essentially constant at all concentrations of total bilirubin, and the slope of the line represents the oxidation rate of bound bilirubin. At a peroxidase concentration of 10 μg/ml, the correction was 0.0025 ΔA per minute per μmol/liter bound bilirubin for fraction V albumin and 0.0010 ± 0.0003 (±SD) for infant sera (6 samples). The unbound bilirubin concentration was calculated after subtracting the oxidation rate of bound bilirubin from the observed V_o.

Precision

Two serum controls were prepared from a commercial lyophilized serum reference.[4] The reference served as Control I (bilirubin:albumin molar ratio = 0.23). Control II was prepared by adding bilirubin to Control I (molar ratio = 0.52). The control samples were divided and frozen until used. For 18 days, unbound bilirubin concentrations were determined each day from duplicate or triplicate samples. Within-day variation was slight (Table 1). Day-to-day variation was tested by one or more samples daily for 18–24 days, and was considerably greater than the within-day variation (Table 1). The greater day-to-day variation can be partially explained by variations in K_p determination.

Table 1. Precision of the Assay

Within-day variation

Sample	Molar ratio	Detns./ day	No. days	Coefficient of variation, % Range	Av
I	0.23	3	18	1.42–10.54	3.94
II	0.52	2–3	18	0.00–8.27	3.28

Day-to-day variation

Sample	Molar ratio	No. days	Mean unbound bilirubin (μmol/liter)	CV (%)
I	0.23	18	.00202	10.7
II	0.52	24	.00755	11.0

Clinical Observations

Unbound bilirubin concentration. We have analyzed 170 sera from 117 normal or sick newborns, within the first week of life. Total bilirubin concentration ranged from 1.5 to 31.0 mg/100 ml, unbound bilirubin concentrations ranged 1.5 to 31.0 mg/100 ml, and unbound bilirubin concentrations ranged from 0.06 to 6.49 μg/100 ml. The mean unbound bilirubin concentration in six infants with total bilirubin concentrations exceeding 20 mg/100 ml was 2.40 ± 1.97 (±SD) μg/100 ml. Seventeen infants with total bilirubin concentrations ranging from 9.5 to 19.8 mg/100 ml had unbound bilirubin concentrations within this range, emphasizing the wide variation in binding among infants.

We have insufficient data to establish what critical concentration of unbound bilirubin reflects neurotoxicity, although one full-term erythroblastotic infant with a total serum bilirubin concentration of 26.3 mg/100 ml and an unbound bilirubin of 6.5 μg/100 ml developed early clinical signs of kernicterus, which improved after exchange transfusion.

Determination of albumin binding. After the high-affinity binding sites are saturated, the unbound bilirubin fraction increases dramatically, so that a small increment in total bilirubin will place the infant at a substantially increased risk for developing bilirubin encephalopathy. Knowledge of a patient's albumin-binding properties, therefore, would allow the physician to predict when an infant will be at increased risk before the total bilirubin reaches a dangerous concentration. The serum albumin-binding properties can be determined by titrating albumin or

[4] Versatol A.; Warner-Chilcott Laboratories, Morris Plains, N.J.

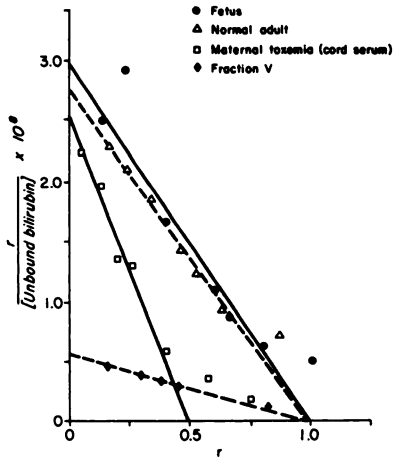

Fig. 6. Scatchard plot describing the binding of bilirubin by serum albumin obtained from various sources

The intercept with the abscissa represents the bilirubin:albumin molar ratio at which high-affinity albumin-binding sites become saturated (n). The intercept with the ordinate is nk, where k is the association constant of the bilirubin:albumin complex. The data deviate from a straight line as the molar ratio approaches 1.0 because of weaker secondary binding (*20*)

serum with bilirubin in vitro and measuring the concentration of unbound bilirubin at various total bilirubin:albumin molar ratios. Data so obtained can be plotted on a Scatchard graph (*26*) and will be linear until the high affinity binding sites approach saturation.

Examples of bilirubin titrations are shown in Figure 6. Fraction V albumin had a single high-affinity binding site with an apparent association constant of 5.6×10^7 liter/mol. Serum obtained from a normal adult had an albumin-binding capacity similar to fraction V albumin (n = 1), although the binding constant was considerably higher (2.5×10^8 liter/mol). Albumin obtained from a normal human abortus at 16 weeks of gestation bound bilirubin as well as did serum from adults, suggesting that the *intrinsic* binding affinity of serum from the premature infant is not relatively low. However, serum albumin from infants who had undergone various stresses in the perinatal period often had lower apparent association constants or decreased binding capacities (apparent saturation of high-affinity binding sites before a 1:1 molar ratio of bilirubin:albumin was attained). We have observed apparent decreases in the binding capacity, where (n) ranged from 0.42 to 0.85, in 17 newborns, most of whom were prematurely born. A decrease in binding capacity is presumed to be caused by binding competitors that have an association constant in the same order of magnitude as that of bilirubin. However, no such competitor has yet been identified. Apparent binding constants (nk) ranged from 4.2×10^7 to 4.3×10^8 liter/mol. Assuming that the intrinsic binding constant for bilirubin is uniform, the depressed binding affinity can best be explained by the presence of relatively high concentrations of weak competitors.

Comparison of Peroxidase and Sephadex Methods

Purified human albumin (280 mmol/liter) was titrated with bilirubin, and the unbound bilirubin concentration in each sample was determined by Sephadex G25 gel filtration and by the peroxidase method. Data obtained from the peroxidase method were plotted on a Scatchard graph, and Sephadex data were plotted according to the method of Schiff et al. (*27*) (Figure 7). Good agreement was found in establishing the binding capacity, but Sephadex filtration was insensitive to low unbound bilirubin concentrations and therefore provided no information regarding binding affinity of the primary binding site. When unbound bilirubin could be detected by Sephadex gel filtration, the calculated concentrations were 30- to 50-fold higher then values obtained by the peroxidase method, presumably because of Sephadex binding of bilirubin in competition with albumin (*17*). Only the peroxidase method provided information regarding the serum binding characteristics within the bilirubin concentration range usually encountered in clinical situations.

Potential Limitations

Effects of sample dilution. To perform the peroxidase test conveniently it is necessary to make a 40-fold dilution of serum. Dilution would have a negligible effect on bilirubin binding to the high-affinity sites, but should facilitate dissociation from secondary binding sites. When the first sites were not saturated, the unbound bilirubin in diluted serum was essentially the same concentration as in the undiluted serum (measured in a cuvette with a 1-cm pathlength).

A more important consideration is that drugs or endogenous substances may compete with bilirubin for albumin binding, and this effect could be obscured by dilution. Such would occur when the sub-

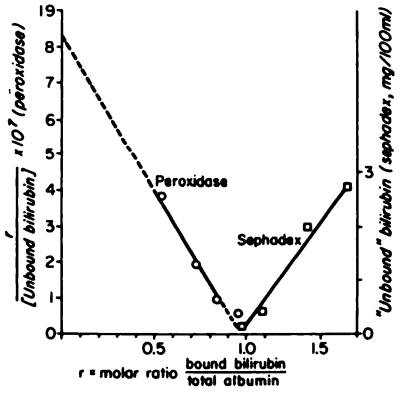

Fig. 7. Comparison of peroxidase and Sephadex methods

Peroxidase data are presented on a Scatchard plot; Sephadex data are plotted according to Schiff et al. (*27*). There is close agreement in the determination of the binding capacity, but gel filtration is insensitive to low bilirubin concentrations existing below a 1.0 molar ratio

stance has a relatively low intrinsic binding constant but competes effectively by virtue of a high concentration, e.g., after an intravenous injection of a sulfonamide or sodium benzoate.

The sensitivity of the peroxidase method was compared with the sensitivity of the Sephadex method for detecting drug displacement of bilirubin. Increasing amounts of sulfisoxazole (Gantrisin) were added to a solution containing, per liter, 140 μmol of bilirubin and 280 μmol of albumin. Samples were analyzed undiluted on a Sephadex column and, after diluting 40-fold, with the peroxidase assay. Bilirubin did not bind to the Sephadex column until the sulfisoxazole concentration reached about 10 mmol/liter, whereas the oxidation velocity increased significantly when the undiluted serum drug concentration was 0.4 mmol/liter.

Conjugated bilirubin. An additional problem was encountered when the concentration of bilirubin conjugates was abnormally high. Such sera (obtained from adults with hepatic cirrhosis) were found to have high oxidation rates, suggesting that these pigments form a less-stable complex with albumin than does unconjugated bilirubin. Because the assay does not differentiate conjugated from unconjugated bilirubin, the binding status of the toxic unconjugated bilirubin in these sera was difficult to interpret. This problem was not confined to the peroxidase method; gel filtration of sera with elevated conjugated bilirubin resulted in bilirubin staining of the column.

Fortunately, bilirubin conjugates are only rarely present in significant concentrations in sera from newborns (*28*). In the unusual case where the concentration of conjugated bilirubin is very high (e.g., the inspissated bile syndrome), the binding of unconjugated bilirubin can be ascertained by the chloroform extraction method described by Jacobsen and Fedders (*22*). A simpler approach to differentiate conjugated from unconjugated bilirubin in sera with high oxidation velocities is to add bilirubin in vitro and repeat the assay. Only a slight increase in oxidation velocity should occur if the initial high reaction rate is due to conjugated bilirubin.

Discussion

The peroxidase method offers the first practical clinical assay for unbound bilirubin under usual clinical conditions (i.e., before the high-affinity albumin-binding sites are saturated).

Determination of the unbound bilirubin concentration is important for both patient management and clinical investigation. If the unbound bilirubin concentration is assayed at increasing total bilirubin concentrations, both the binding affinity and binding capacity of the serum albumin can be determined. Early evaluation of serum binding in a newborn who is susceptible to jaundice (e.g., hemolytic disease or prematurity) will allow the physician to predict the approximate concentration of total bilirubin at which the high affinity sites will become saturated and plan his clinical management for that particular infant accordingly. Characterization of albumin binding can also provide essential information for clinical investigation. The lack of a sensitive assay for unbound bilirubin has greatly impeded investigations dealing with the pathogenesis of kernicterus, such as the relative importance of albumin binding vs. tissue susceptibility, the etiology of kernicterus in small prematures, the relationship of unbound bilirubin to minimal brain damage, drug–bilirubin interaction, identification of endogenous competitors, etc.

The method is relatively easy to perform and a single determination requires only 5–10 min. Serum titration and calculation of binding characteristics is more cumbersome and requires some data interpretation. We anticipate that the use of centrifugal analyzers will greatly facilitate this procedure. In following an infant at risk for kernicterus (e.g., a sick premature or erythroblastotic infant), we have performed titrations in the first hours of life and subsequently managed the patient with single determinations of unbound bilirubin to ascertain whether the binding characteristics have changed.

It has been assumed that the unbound bilirubin fraction is responsible for kernicterus (*8*), but the hypothesis has never been proven, and the critical concentration of unbound bilirubin that will cause kernicterus or minimal brain damage is not known precisely. The peroxidase test provides the first opportunity to accurately measure unbound bilirubin in infants and should permit meaningful clinical laboratory correlations to establish what are toxic concentrations under various clinical conditions. Although such values are not now known, it is clear that the risk for bilirubin encephalopathy increases rapidly once the high-affinity binding sites become saturated.

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