REVIEW

Quantitative Aspects of Bilirubin Metabolism for Hematologists

By Nathaniel I. Berlin and Paul D. Berk

Techniques now available for the study of plasma disappearance kinetics of isotopically labeled unconjugated bilirubin have led to new insights into the factors that determine the plasma unconjugated bilirubin concentration (BR). This variable can be shown to depend in turn on five other parameters: the total circulating red cell volume (TRVC), the mean corpuscular hemoglobin concentration (MCHC), the mean red cell lifespan (RBCLS), plasma volume (PV), and the hepatic extraction coefficient for unconjugated bilirubin (k.). Of these, three clearly reflect varying aspects of erythrokinetics and red cell physiology, while only one is reflective of liver function. It is not surprising, therefore, that knowledgeable interpretations of studies of the metabolism of bilirubin and related bile pigments are historically closely linked to studies of the red blood cell.1-3 Within the past decade, however, interest in bilirubin metabolism among hematologists has waned, as reflected in part by the disappearance of the session on pigment metabolism from the annual meetings of the American Society for Hematology. During this same period, newer studies of bilirubin metabolism have made it possible to interpret measurements of the plasma bilirubin concentration in terms of several variables of particular interest to the hematologist. The amount of information derivable from simple measurements of the plasma unconjugated bilirubin concentration had not previously been appreciated, and this measurement is still not well utilized by clinicians interested in hematologic disease.

It is the purpose of this article to examine current concepts of those aspects of bilirubin metabolism of relevance to hematology, to describe a technique for clinical studies of bilirubin metabolism, and to review the data that have been obtained by the application of this technique to clinical problems. It is hoped that this will increase the usefulness to hematologists of simple measurements of the plasma bilirubin concentration.

REVIEW OF BILIRUBIN METABOLISM

Bilirubin IXa, the naturally occurring isomer in man, is derived from the enzymatic opening of the protoporphyrin IX ring of heme at the alpha bridge carbon by microsomal heme oxygenase (Fig. 1).4 Biliverdin, the initial tetrapyrrolic product of the ring opening reaction, is rapidly reduced to bilirubin by a second enzyme, biliverdin reductase.5 During the enzymatic opening of the heme ring, the alpha bridge carbon is oxidized to carbon monoxide, so that one

molecule of bilirubin and one of CO are produced for each molecule of heme degraded.\textsuperscript{4,6} This pathway represents the only source of both bilirubin and CO in man and most other species.

Heme is the prosthetic group of a number of proteins involved in oxygen transport and metabolism, including hemoglobin, myoglobin, and a variety of enzymes such as cytochrome P450, which are present particularly but not exclusively in the liver. The principal source of bilirubin, commonly reported to represent 85\% of the total,\textsuperscript{2,3,6} is the catabolism of the hemoglobin of circulating red blood cells. Ineffective erythropoiesis in the bone marrow constitutes an additional hematopoietic source. The degradation of hemoglobin in the marrow in association with erythropoiesis constitutes an additional hematopoietic source. The degradation of hemoglobin in the marrow in association with erythropoiesis may result either from the loss of a small fraction of the hemoglobin from each normoblast in association with nuclear extrusion\textsuperscript{7} or from the destruction of a fraction of erythroid precursors during their development within the marrow,\textsuperscript{8} but in either case, some degree of ineffective erythropoiesis is a normal phenomenon.

The catabolism to bilirubin of hemoglobin derived from senescent red cells, from extravascular hemolysis and from ineffective erythropoiesis occurs within the reticuloendothelial cells of the liver, spleen, and bone marrow. In the presence of intravascular hemolysis, a minor fraction of the released hemoglobin heme may be transported to the hepatic parenchymal cells, which selectively take up free hemoglobin, hemoglobin-haptoglobin complex, methemalbumin, and heme-hemopexin.\textsuperscript{9,10} The heme of each of these compounds is also converted to bilirubin, since hepatocytes and renal tubular cells, as well as reticuloendothelial cells, contain the necessary enzymes.\textsuperscript{9,11} The normal substrate for the hepatocellular heme-degrading enzymes is the heme derived from the turnover of various hepatic microsomal heme enzymes.\textsuperscript{12} Renal
tubular cells effect the catabolism of any hemoglobin filtered by the kidney during intravascular hemolysis.11

Bilirubin produced in the periphery is transported to the liver tightly bound to albumin.13,14 Uptake from the circulation into the liver cell displays the kinetic properties of carrier-mediated transport,15,16 and a distinct bilirubin binding protein, recently described as a component of liver cell plasma membranes, may represent a part of the carrier system.17 Within the liver cell, bilirubin is tightly bound to two small soluble proteins, one of which, ligandin, appears to play a major role in the transport of many organic anions by both the liver and kidney.18 While not directly involved in the initial uptake of bilirubin into the liver cell, ligandin increases the net efficiency of bilirubin extraction by retarding its reflux to plasma.19

Unconjugated bilirubin within the hepatocyte is converted to bilirubin mono- and diglucuronides prior to excretion into the bile. Conversion to bilirubin monoglucuronide is catalyzed by the microsomal enzyme bilirubin UDP-glucuronyl transferase through a reaction requiring UDP-glucuronic acid as a cofactor.20 Whether the conversion of bilirubin monoglucuronide to diglucuronide is catalyzed by the same enzyme21 or by a distinct enzyme located in the canalicular plasma membrane22 is uncertain.

Bilirubin conjugates enter the bile against an appreciable apparent concentration gradient. Although this gradient is effectively reduced by incorporation of conjugated bilirubin into mixed micelles,23 the kinetics of its biliary excretion support the concept of a carrier-mediated excretory process.24

Within the gastrointestinal tract, bilirubin is further degraded by bacterial action to a series of urobilinogens and a number of other products.25 Although the conversion of heme to bilirubin and carbon monoxide is quantitative, the further degradation of bilirubin to urobilinogens is not. Hence, measurements of fecal urobilinogen excretion may appreciably underestimate total heme degradation.26

QUANTITATION OFHEME TURNOVER AND
BILIRUBIN PRODUCTION

The plasma concentration of unconjugated bilirubin represents a balance between the rate at which newly synthesized bilirubin enters the plasma unconjugated bilirubin pool and the rate at which the liver irreversibly extracts unconjugated bilirubin from this pool. In the steady state, the quantity of newly synthesized bilirubin entering the plasma pool is equivalent to the amount removed. This parameter, designated plasma bilirubin turnover (BRT), is conveniently expressed in terms of milligrams per kilogram body weight per day. Similarly, hepatic bilirubin clearance (C_{BH}), a quantitative measure of hepatic function, may be expressed in units of milliliters per minute per kilogram. Plasma bilirubin turnover closely approximates but is not identical to total bilirubin production. The difference between these two variables results from the fact that a small portion of the bilirubin produced within the hepatocytes themselves from the turnover of hepatic microsomal heme enzymes is excreted directly into the bile and never appears in the plasma. For this reason, techniques that quantitate total bilirubin production or total heme turnover should provide estimates exceeding those for plasma bilirubin turnover by approximately 5%–10%. It should be emphasized that plasma bilirubin turnover, rather than total bilirubin production, is the critical determinant of the plasma concentration of unconjugated bilirubin.

Plasma bilirubin turnover can be calculated by one of two methods, each of which requires the availability of radiolabeled unconjugated bilirubin of high specific activity.27,28 These are: (A) from the plasma disappearance curve of an intravenously injected tracer dose of unconjugated radiolabeled bilirubin29 and (B) from the steady-state plasma bilirubin specific activity during a constant infusion of radiolabeled unconjugated bilirubin.30 Although these two methods have not been directly compared in the same individual, they appear to provide similar values for normal rates of plasma bilirubin turnover.

Total bilirubin production can be determined from: (A) the dilution in fecal urobilinogen of an intravenously injected tracer dose of labeled bilirubin;31 (B) from a measurement of the rate of excretion of carbon monoxide;32 and (C) under certain assumptions, from measurements of total red cell volume and red cell lifespan.33 There are, in addition, two other ways that have been used for estimating bilirubin production: measurement of fecal urobilinogen excretion or of the amount of bilirubin appearing in biliary tract-T-tube drainage. Neither is satisfactory. As noted earlier, the conversion of bilirubin to urobilinogen is not quantitative. Measurements of bilirubin obtained from T-tube drainage have varied widely, due to both methodological problems and to the non-steady-state situation that results from biliary drainage in the jaundiced subject. It is, in any case, not a practical method for physiologic study in intact man.

The most intensively studied of the above methods involves the calculation of plasma bilirubin turnover (BRT) and hepatic bilirubin clearance (C_{BH}) from the plasma disappearance curve of a tracer dose of unconjugated radiobilirubin.4,29 If, following the injection of
such a tracer dose, plasma samples are obtained for approximately 30 hr and the unconjugated bilirubin extracted, the plasma unconjugated radiobilirubin disappearance curve \( X(t) \), in units of dpm/ml plasma, may be determined (Fig. 2). \( X(t) \) can be closely approximated by a sum of three exponential functions of the form:

\[
X(t) = X_0(Ae^{-kt} + Be^{-kt} + Ce^{-kt})
\]

In this formulation, \( X_0 \) equals the extrapolated value at zero time and \( A, B, \) and \( C \) represent the coefficients of the individual exponential components, normalized such that \( A + B + C = 1 \). Using this notation, the function \( P(t) \), defined simply as \( X(t)/X_0 \), represents the fraction of injected radiobilirubin remaining in the plasma at time \( t \).

If the experimental plasma bilirubin disappearance curve is fitted by computer to the function shown in equation 1, a number of basic calculations can be performed, based only on the assumption that the behavior of the injected tracer is identical to that of endogenous pigment.\(^{29,35} \) From the injected dose of radiobilirubin in dpm and the extrapolated value of the curve at zero time, the initial volume of distribution of the injected bilirubin (VDBR) can be calculated as:

\[
\text{VDBR (ml)} = \frac{\text{injected dose (dpm)}}{X_0 \text{(dpm/ml)}} \quad (2)
\]

Not surprisingly, in view of the very tight binding of bilirubin to albumin, VDBR has been found to be virtually identical to the plasma volume when the latter is determined simultaneously with radioiodinated albumin preparations.\(^{34} \)

The fraction of the plasma bilirubin pool that is irreversibly extracted per unit time by the liver has been designated \( k_e \). Using the plasma curve integral technique described by Nosslin,\(^{35} \) it can be shown that \( k_e \) is equal to the reciprocal of the area under the plasma bilirubin disappearance curve \( P(t) \). That is:

\[
k_e = \frac{1}{\int_0^t P(t)dt} = \frac{1}{\frac{A}{k_1} + \frac{B}{k_2} + \frac{C}{k_3}} \quad (3)
\]

If both VDBR and \( k_e \) are known, the product of these two parameters denotes the net volume of plasma from which bilirubin is irreversibly extracted per unit time. This is, by definition, hepatic bilirubin clearance (\( C_{BR} \)), a quantitative test of hepatic function entirely analogous to renal creatinine clearance. Thus,

\[
C_{BR} \text{ (ml/min)} = k_e \text{ (min}^{-1}) \times \text{VDBR (ml)} \quad (4)
\]

Finally, if the plasma unconjugated bilirubin concentration, \( BR \), is known, then the quantity of bilirubin irreversibly extracted from plasma per unit time simply equals \( C_{BR} \times BR \). In the steady state, the amount extracted per unit time equals the amount of newly synthesized bilirubin entering the plasma pool. When expressed in units of milligrams per day, this variable is equivalent to plasma bilirubin turnover, i.e.,

\[
\text{BRT (mg/day)} = C_{BR} \text{ (ml/min)} \times \frac{BR \text{ (mg/ml)}}{1440 \text{ (min/day)}} \quad (5)
\]
Rearranging equation 5 we have:

\[ \text{BR} = \frac{\text{BRT}}{C_{\text{BR}}} \]  

(6)

This equation indicates that the plasma unconjugated bilirubin concentration varies directly with the daily plasma bilirubin turnover and inversely with hepatic bilirubin clearance. This basic equation describes much of the clinical physiology of bilirubin metabolism to be outlined below.

The validity of the calculations presented thus far is most readily demonstrated by comparing calculated values for plasma bilirubin turnover with alternative approaches to measuring bilirubin production, recalling that bilirubin production should slightly exceed the daily quantity of bilirubin passing through the plasma. The simplest approach to estimating bilirubin production involves the fecal isotope dilution principle. If, following the injection of radiolabeled bilirubin, all feces were collected for a sufficient time to recover the entire injected dose, then the amount of bilirubin excreted during the time in question could be calculated as:

\[ \text{Bilirubin excreted (mg)} = \frac{\text{Injected dose (dpm)}}{\text{Bilirubin specific activity (dpm/mg) in the pooled fecal sample}} \]

(7)

In practice, 6–10 days is sufficient to recover the administered radioactivity in individuals with normal hepatic function. Although the feces contain virtually no bilirubin as such, the specific activity of bilirubin entering the gut can be estimated from that of stercobilin isolated from the fecal sample. Hence:

\[ \text{Bilirubin excreted (mg/day)} = \frac{\text{Injected dose (dpm)}}{\text{SA} \times n \times (594/585)} \]

(8)

In this formulation, SA is the specific activity of stercobilin crystallized from the collected fecal sample, n equals the number of days in the stool collection and 594 and 585 are the molecular weights of stercobilin and unconjugated bilirubin, respectively. In the steady state, bilirubin excretion is equal to bilirubin production. Employing this technique in 14 individuals, the ratio of bilirubin production to simultaneous studies of plasma bilirubin turnover was 1.03 ± 0.04 (SEM).31

An alternative approach to validation of measurements of bilirubin turnover involves the determination of carbon monoxide excretion (see Fig. 1). When expressed in micromolar units, normal plasma bilirubin turnover was found to average 6.6 ± 1.2 μM/kg/day.29,36 Three separate studies reported corresponding normal values for carbon monoxide production of 6.6 ± 1.3,37 6.6 ± 0.9,38 and 6.5 ± 2.1 μM/kg/day.39 Similarly, simultaneous studies of bilirubin turnover and carbon monoxide production in 37 individuals with a wide range of hemolytic rates revealed excellent correlation and a slope that was not significantly different from one (Fig. 3).36 As expected, carbon monoxide production exceeded plasma bilirubin turnover by a small amount. The agreement between the values for plasma bilirubin turnover and two independent techniques for estimating heme catabolism strongly supports the validity of the plasma bilirubin kinetic methodology.

**BILIRUBIN TURNOVER AND RED CELL SURVIVAL**

Since traditional early labeled peak studies suggested that approximately 85% of bilirubin is derived from the catabolism of the hemoglobin of adult circulating erythrocytes,21 it is possible to derive a simple relationship between bilirubin turnover and red cell survival. The approach is as follows.35

Since the degradation of 1 g of hemoglobin yields 36.2 mg of bilirubin, the destruction of 1 ml of red cells will produce 36.2 × MCHC mg of bilirubin, where MCHC is the mean corpuscular hemoglobin concentration in g/ml. The volume of red cells destroyed per day may be then calculated as:

\[ V_d = \frac{(0.85 \times \text{BRT})}{(36.2 \times \text{MCHC})} \]

(9)

In the steady state, the mean red blood cell lifespan is equal to the red cell volume (TRCV) divided by the volume of red cells produced or destroyed daily, that is:

\[ \text{RBCLS} - \text{TRVC} = \frac{\text{TRCV} \times 36.2 \times \text{MCHC}}{0.85 \times \text{BRT}} \]

(10)

The total red cell volume can be rapidly determined with 51Cr-labeled erythrocytes. Determination of plasma bilirubin turnover requires approximately 30 hr for blood sampling and an additional 24–36 hr for processing of samples and analysis of the data. Hence, mean red cell survival can be determined by this technique within approximately 3 days. Furthermore, since bilirubin is available labeled with either tritium28 or 14C,27 such studies can be repeated as often as twice a week. In the therapy of patients with, for example, autoimmune hemolytic anemia, this approach would permit the clinician to titrate red cell survival against steroid dose. Specific applications of this methodology will be described below.

It should be noted that the mean red cell survival can only be calculated from equations 9 and 10 in situations in which 85% of bilirubin production is, in
fact, associated with the catabolism of circulating adult red cells. In conditions characterized by increased ineffective erythropoiesis (including the megaloblastic anemias, erythropoietic porphyria and protoporphyria, the thalassemia syndromes, refractory sideroblastic anemia, the rare “shunt” hyperbilirubinemia syndromes, including the various types of congenital dyserythropoietic jaundice, and in lead poisoning), the marked increase in the fraction of bilirubin turnover not derived from circulating red cells renders this approach invalid.6'33 It is both valid and highly useful in the most common varieties of hemolytic anemia, which are associated with normoblastic erythropoiesis and a proportionally normal degree of ineffective erythropoiesis.

The validity of this technique for estimating mean red cell lifespan is illustrated in Fig. 4. In particular, the mean red cell lifespan determined with radiobilirubin was found to be highly correlated with the T'2 of 51Cr-labeled erythrocytes,33 according to the following empirical equation:

\[
\text{RBCLS} = 3.8 \times \text{T}'2 - 11.8
\]

BILIRUBIN IN PLASMA: MEASUREMENT AND NORMAL RANGES

Plasma bilirubin concentration is a frequently ordered laboratory determination. Unless otherwise specified, the total bilirubin concentration is the only determination usually reported. When requested, the direct reacting bilirubin concentration may also be measured in most laboratories, and the indirect reacting bilirubin calculated as the difference between the total and direct reacting pigment. These terms (direct and indirect) are derived from the process by which bilirubin concentration is measured. In the majority of clinical laboratories, this involves some form of the diazotization reaction originally described by Van den Bergh in 1916.40 In this reaction the bilirubin tetrapyrrole is cleaved at its central bridge carbon, resulting in the formation of dipyrrolic azo pigments having a characteristic violet color that is readily measured spectrophotometrically. The amount of color developed within a specified brief period (most often 1 min) after the addition of the appropriate reagents to plasma is a measure of the direct reacting bilirubin fraction. After the addition of methanol, or a number of alternative “accelerators” that expose the central bridge carbon to attack by disrupting the internal hydrogen bonding of the molecule, more of the bilirubin is diazotized and the amount of color increases. The maximum color produced after a specified time period (often 30 min) is a measure of the total plasma bilirubin concentration.41

The indirect reacting bilirubin, representing the difference between the total and direct reacting fractions, is widely equated clinically with unconjugated
Fig. 4. (A) Comparison of mean RBCLS, calculated from bilirubin turnover studies, with data obtained with DFP-3H. Shaded area includes ±2 standard errors of the estimate about the regression line. (B) Comparison of mean RBCLS, calculated from measurements of bilirubin turnover, with the half-life of 51Cr-labeled autologous erythrocytes in 50 patients and normal volunteer subjects. Symbols are as follows: acute intermittent porphyria (○); sideroblastic anemia (□); replicate studies in a normal volunteer (△); a patient with Gilbert’s syndrome (■); and a patient with congenital spherocytosis studied before and after splenectomy (†); all other patients and normal volunteer subjects (□). Shaded area represents ±1 standard error of the estimate about the regression line. (Reproduced by permission from the Journal of Laboratory and Clinical Medicine.™)
bilirubin, whereas the direct reacting fraction is often interpreted as representing conjugated bilirubin. This is clearly an oversimplification, as indicated by the observations that: (A) solutions made from pure crystalline unconjugated bilirubin commonly contain as much as 10% of direct reacting pigment; and (B) despite the presence of appreciable and readily measurable quantities of direct reacting pigment, normal human plasma contains only trace amounts of true conjugated bilirubin as determined by elaborate isotopic or high pressure liquid chromatographic techniques. Nevertheless, the usual clinical interpretation is a useful one, in that the indirect reacting fraction is the predominant fraction elevated in the presence of hemolysis and other conditions resulting in a true unconjugated hyperbilirubinemia. Similarly, the direct reacting fraction is characteristically elevated in conditions in which true plasma conjugated hyperbilirubinemia results from a reflux of conjugated bilirubin from the liver or biliary tract to the blood. Conjugated hyperbilirubinemia indicates either a physiologic or mechanical obstruction to the flow of bile, which may be located at any point from within the hepatocyte itself to the duodenum. Hepatocellular injury of any type substantially reduces the capacity of the hepatocyte to transport bilirubin into bile. Hence, hemolysis occurring in the setting of liver disease frequently results in a combination of conjugated as well as unconjugated hyperbilirubinemia. While there are many other causes of conjugated hyperbilirubinemia, none of them is of primary interest to the hematologist.

Normal ranges for most laboratory analytical procedures are usually determined empirically. This is also true for plasma bilirubin. However, equation 6 provides the opportunity for calculating the normal range for the plasma unconjugated bilirubin concentration from experimentally determined normal ranges for plasma bilirubin turnover and hepatic bilirubin clearance, the two parameters that determine the plasma bilirubin level. Equation 6 suggests that the distribution of bilirubin concentrations in the normal population will be skewed positively. Thus, absolute increments in the plasma unconjugated bilirubin concentration will be progressively greater for successive equal decrements in $C_{BR}$. Similarly, increases in bilirubin turnover will produce greater absolute changes in bilirubin concentration in individuals having lower clearance rates. As a result, the fraction of the normal population whose bilirubin concentrations exceed the normal mean will have a wider range of values than individuals whose concentrations are less than the normal mean. In fact, a positive skew for the plasma total bilirubin concentration (consisting predominately of unconjugated bilirubin) has been observed in three large series of 849, 719, and 18,450 healthy individuals. A convenient method of analyzing skewed distribution data involves the use of a log normal function. When tested, the distribution of the total serum bilirubin concentration in normal individuals, as reported by Butt et al., could be closely approximated by a log normal distribution (Fig. 5). Application of a log normal distribution function to equation 6, using values for plasma bilirubin turnover and hepatic bilirubin clearance determined in normal subjects, indicates that the plasma concentration of unconjugated bilirubin will be between 0.2 and 0.9 mg/dl for 95% of a normal population, and that 99% of such a population will have a value less than 1.0 mg/dl. The cumulative distribution function for plasma unconjugated bilirubin calculated in this manner (Fig. 5) closely parallels that observed clinically for total bilirubin concentration, while the calculated upper limit of normal (1.0 mg/dl) is identical to that determined experimentally in the Clinical Chemistry Laboratory of the National Institutes of Health.

The normal range for direct reacting bilirubin is less well established, in part because this measurement reflects both the very small plasma content of conjugated bilirubin and the tendency of a small but variable proportion of unconjugated bilirubin to give a prompt direct reaction. The upper limit of normal for direct reacting bilirubin in the presence of a normal total plasma bilirubin concentration ($\leq 1.2$ mg/dl) has been empirically set at 0.2 mg/dl. Values greater than 0.2 mg/dl are very rarely seen in normal subjects. In contrast, values in excess of 0.2 mg/dl are not uncommonly seen in patients with established hepatobiliary disease despite a normal total bilirubin concentration. In one study, such values were invariably associated with abnormal 45-min sulfobromophthalein retention in excess of 9%. In a reliable laboratory, an elevated value for the direct reacting bilirubin concentration is a very sensitive and specific test for hepatic and/or biliary dysfunction, particularly if the total bilirubin concentration is normal. When the total bilirubin concentration is increased, precise interpretation of the direct reacting fraction is even more difficult. A useful guide is to consider any direct reacting fraction less than 10% of the total bilirubin to be within normal limits and any value in excess of 15% as abnormal, or at least in need of investigation. Because the value for direct reacting bilirubin varies more with the method employed than does that of the total, it is particularly important that the reference
FACTORS INFLUENCING THE PLASMA UNCONJUGATED BILIRUBIN CONCENTRATION

Plasma Bilirubin Turnover

From equation 6 it is apparent that, for an individual with a constant hepatic bilirubin clearance or for a population (e.g., the normal population) for which hepatic bilirubin clearance remains within a relatively narrow range, the plasma unconjugated bilirubin concentration will increase linearly with increasing plasma bilirubin turnover. This relationship is illustrated in Fig. 6, which includes the regression line relating plasma bilirubin turnover and the plasma unconjugated bilirubin concentration. This figure also
Fig. 6. Relation between plasma bilirubin turnover and the plasma concentration of unconjugated bilirubin. When hepatic bilirubin clearance is within the relatively narrow normal range, the plasma unconjugated bilirubin concentration will increase linearly with increasing rates of bilirubin production, as indicated by the regression line. Stippled area represents two standard errors of the estimate about the regression line. Extrapolation of the regression line to the maximum achievable rate of bilirubin production, or approximately 40 mg/kg body weight/day, indicates the highest value for the plasma unconjugated bilirubin concentration that can occur as the result of sustained hemolysis in an individual with normal hepatic bilirubin clearance, corresponding to approximately 4 mg/100 ml. (Reproduced by permission from Ann Intern Med 82:552, 1975.)

indicates the physiologic rationale for the well known clinical observation that unconjugated bilirubin concentrations in excess of 4 mg/dl do not result from hemolysis per se, but imply a concomitant reduction in hepatic function (i.e., CBR). The basis for this is as follows. Since the bone marrow can only increase erythrocyte production approximately eightfold under a chronic hemolytic stress, the maximum physiologically achievable steady-state bilirubin turnover is eight times normal, or 40 mg/kg/day. Extrapolation of the regression line in Fig. 6 to 40 mg/kg/day thus indicates the highest value for the plasma unconjugated bilirubin concentration that can occur as the result of sustained hemolysis in individuals with normal hepatic function. This corresponds to approximately 4 mg/dl. Note that this observation does not apply to acute massive hemolytic episodes as may occur, for example, with G6PD deficiency or during a hemolytic transfusion reaction. In these non-steady-state situations, the rate of red cell destruction and bilirubin production may, temporarily, greatly exceed the maximum ability of the marrow to produce new red cells.

Hepatic Bilirubin Clearance

Referring again to equation 6, it is apparent that, for a given rate of bilirubin turnover, the plasma unconjugated bilirubin concentration and hepatic bilirubin clearance are linked by an equation of the form \( X \times Y = \text{a constant} \). This is the equation of a rectangular hyperbola, and a family of rectangular hyperbolae relating \( C_{\text{BR}} \) to \( BR \) for a number of different rates of plasma bilirubin turnover are illustrated in Fig. 7. The hyperbolic relationship between \( BR \) and \( C_{\text{BR}} \) is entirely analogous to that relating the serum creatinine concentration to creatinine clearance. The slope of such curves is shallow in the region of normal clearance but very steep in the region of reduced clearance, indicating that large changes in hepatic function in initially normal persons will produce only small absolute changes in the plasma unconjugated bilirubin concentration, whereas in
patients with initially reduced hepatic function, further small changes in $C_{BR}$ will produce large changes in BR. The absolute increase in bilirubin concentration produced by either a given increment in bilirubin turnover or a given reduction in hepatic bilirubin clearance will depend on the initial starting point on the concentration/clearance curve. However, it is evident from equation 6 that the fractional change in plasma bilirubin concentration will occur during any perturbation will always be proportional to the fractional change in the fundamental determining parameters BRT or $C_{BR}$. These phenomena are further illustrated by an example in Table 1.

**Effects of Changes in Total Red Cell Volume and Red Cell Lifespan**

Combining equations 9 and 10, solving for BRT, and substituting the resulting expression in equation 6, one obtains the relationship:

$$BR = \left( \frac{36.2 \times MCHC}{0.85} \right) \cdot \left( \frac{TRCV}{RBCLS \cdot C_{BR}} \right)$$

(12)

Since MCHC varies relatively little compared to other variables, this relationship can be simplified as shown below to indicate that the plasma unconjugated bilirubin concentration varies directly with the total red cell volume and inversely with the mean red cell lifespan and hepatic bilirubin clearance, i.e.,

$$BR = k \left( \frac{TRCV}{RBCLS \cdot C_{BR}} \right)$$

(13)

The three-dimensional relationship between these four parameters is illustrated in Fig. 8. Before examining this figure in detail, it is important to ask how clinical hematologists use the plasma unconjugated bilirubin concentration. One important use of this parameter is as a screening test for hemolysis or hepatic dysfunction. It is commonly assumed that individuals who have normal values for BR have neither hemolysis nor hepatic dysfunction, either of which would be suggested by a value for BR in excess of the upper limit of normal. However, since BR varies directly with the total red cell volume, the upper limit of normal for BR will be an increasingly sensitive screening test for either hemolysis or hepatic dysfunction if this upper limit of normal is, itself, corrected for variations in the circulating red cell mass. The simplest relationship that will accomplish this correction is indicated in equation 14, which is similar in form to the well known reticulocyte correction:

$$\text{Upper limit of normal for } BR = 1.0 \times \left( \frac{\text{Patient's Hct}}{45} \right)$$

(14)

An example of the value of equation 14 is as follows.

Table 1. Influence of Changes in Hepatic Bilirubin Clearance ($C_{m}$) or Plasma Bilirubin Turnover (BRT) on the Plasma Unconjugated Bilirubin Concentration (BR)

<table>
<thead>
<tr>
<th>BRT (mg/kg/day)</th>
<th>Increment</th>
<th>$C_{m}$ (ml/min/kg)</th>
<th>Decrement (%)</th>
<th>BR (mg/dl)</th>
<th>(%)</th>
<th>(mg/dl)</th>
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<tr>
<td>3.9</td>
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<td>-</td>
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<td>-</td>
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<td>100</td>
<td>0.41</td>
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<td>50</td>
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<td>1.64</td>
<td>100</td>
<td>0.82</td>
</tr>
<tr>
<td>0.08</td>
<td>50</td>
<td>0.66</td>
<td>3.28</td>
<td>3.28</td>
<td>100</td>
<td>1.64</td>
</tr>
<tr>
<td>0.04</td>
<td>50</td>
<td>0.66</td>
<td>6.56</td>
<td>6.56</td>
<td>100</td>
<td>3.28</td>
</tr>
</tbody>
</table>

(B) Starting from a normal BRT (3.9 mg/kg/day), a 100% increase produces the following changes in BR, as a function of $C_{m}$:

$$\text{BRT (mg/kg/day)} \quad 3.9 \quad \quad 7.8 \quad \quad \text{Increment}$$

<table>
<thead>
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<th>$C_{m}$ (ml/min/kg)</th>
<th>BR (mg/dl)</th>
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<tr>
<td>0.66</td>
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</tbody>
</table>
An anemic patient with a hematocrit of 22.5 is suspected of possible hemolysis. His plasma unconjugated bilirubin concentration is 0.9 mg/dl, which is within the conventional normal range. However, when the normal range is corrected, in accord with equation 14, the patient is seen to have unconjugated hyperbilirubinemia relative to his circulating red cell mass, supporting the possibility of hemolysis. Many patients with low-grade hemolytic anemia will be found to have unconjugated bilirubin concentrations of less than 1 mg/dl, which are conventionally interpreted as normal. In the majority of such patients, however, correction of the upper limit by equation 14 will demonstrate unconjugated hyperbilirubinemia. Figure 8 illustrates the variation in the upper limit of normal for unconjugated bilirubin as a function of red cell mass. Equation 14 has made the simplifying assumption that red cell mass scales linearly with hematocrit, an assumption that holds within broad limits for hematocrits up to approximately 55%.52

Equation 14 also applies to increases in the circulating red cell mass. Patients with polycythemia are frequently seen to have plasma unconjugated bilirubin concentrations either at or slightly above the conventional upper limit of normal. In particular, among the initial cohort of 431 patients entered into the Polycythemia Vera Study Group, the average red cell volume of 45 ml/kg and the average total bilirubin concentration of 0.9 mg/dl33 were both increased to approximately the same extent above the corresponding normal means.

THE INTERPRETATION OF A SINGLE MEASUREMENT OF THE PLASMA UNCONJUGATED BILIRUBIN CONCENTRATION

The relationships developed above indicate a number of useful guides to the interpretation of a single measurement of the plasma unconjugated bilirubin concentration. As noted, in the presence of normal hepatic bilirubin clearance, chronic hemolysis cannot elevate the plasma unconjugated bilirubin concentration to more than 4 mg/dl. Hence, any chronic unconjugated hyperbilirubinemia more severe than this clearly indicates some impairment of hepatic function, with or without concomitant hemolysis. In the presence of a normal total red cell volume, the upper limit for a normal plasma unconjugated bilirubin concentration is 1.0 mg/dl. Hence, values between 1.0 and 4.0 mg/dl may result either from increased bilirubin production (hemolysis), decreased hepatic bilirubin clearance, or some combination of both. Since the plasma concentration of unconjugated bilirubin is determined by three major parameters—the red cell volume, mean red cell lifespan, and hepatic bilirubin clearance—abnormalities in one or more of these variables can be compensated for, to some extent, by alterations in others. The possibility of an abnormality in one or more of these basic parameters, despite an unconjugated bilirubin concentration within the normal range, decreases as the measured value recedes from the upper limit of normal. This is indicated schematically in Fig. 8 by a logarithmic scale labeled “Probability of Disease” on the XZ plane.

We have concentrated on the relationship of the plasma unconjugated bilirubin concentration to three
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principal parameters, total red cell volume, mean red blood lifespan, and hepatic bilirubin clearance. For the sake of completeness we should recognize that $C_{br}$ itself can be shown to depend on two additional variables, $k_e$ and the volume of distribution of bilirubin, which is in turn equivalent to the plasma volume. Changes in plasma volume do indeed occur in certain disease states. There are characteristic increases of plasma volume in fluid retention states, notably liver disease with ascites,54 and reductions of plasma volume in other conditions including certain of the relative erythrocytoses55 and acute intermittent porphyria.56 Nevertheless, the variation in plasma volume is small compared to the very large fluctuations that may occur in red cell volume, red cell mass, and $k_e$. With respect to $k_e$, under most circumstances, this parameter reflects primarily the intrinsic capacity of the liver to extract bilirubin from plasma. Profound hypoalbuminemia or high concentrations of anions that compete with bilirubin for albumin binding may lead to a more efficient hepatic bilirubin extraction, as reflected in an increase in both $k_e$ and $C_{br}$.

Differential Diagnosis of Unconjugated Hyperbilirubinemia

When faced with a patient with chronic unconjugated hyperbilirubinemia, it is apparent from equation 6 that the initial differential diagnostic distinction to be made is between those cases due to increased plasma bilirubin turnover, those due to reduced hepatic bilirubin clearance, and those due to some combination of the two.57 Although increased production of bilirubin from hepatic sources has been reported in animals,58 the only documented source of increased bilirubin turnover in humans at the present time is hemolysis. Reduced hepatic bilirubin clearance associated with structural liver disease is almost inevitably accompanied by other biochemical evidence of hepatic dysfunction beyond unconjugated hyperbilirubinemia.40 When unconjugated hyperbilirubinemia is unaccompanied by such other abnormalities, the diagnosis of Gilbert’s syndrome must be considered. Gilbert’s syndrome is a common hereditary abnormality of bilirubin metabolism that affects approximately 5% of the white population. Studies of radiobilirubin kinetics provide for a definitive diagnosis of such cases and also permit the detection of those cases that have both Gilbert’s syndrome and hemolysis.59 For the most part, the diagnosis of Gilbert’s syndrome can be made adequately by exclusion and by watchful waiting to eliminate the possibility of occult structural liver disease. Alternatively, if both total red cell volume and the $^{51}$Cr red cell half-life are determined simultaneously following a single injection of chromium-tagged erythrocytes, hepatic bilirubin clearance can be calculated from equation 15:

$$C_{br} = \frac{(2.9575)}{(BR \times (3.8 \cdot T/2 - 11.8))}$$ (15)

This relationship, which has been empirically derived from equations 6 and 9–11 above,60 provides highly accurate estimates of $C_{br}$ in patients with normoblastic erythropoiesis (Table 2). The correlation between values for bilirubin clearance determined from studies of radiobilirubin kinetics and those estimated by the simpler $^{51}$Cr technique is highly significant ($r = 0.9, p < 0.01$). Once both hepatic bilirubin clearance and the $^{51}$Cr red cell half-life are known, the patient with unconjugated hyperbilirubinemia can be appropriately classified as having hemolysis, reduced hepatic clearance, or both.

THE INTERPRETATION OF CHANGES IN PLASMA BILIRUBIN CONCENTRATION

Changes in the plasma unconjugated bilirubin concentration over time can be an extremely useful indicator of altered physiology. It is likely that the first evidence of a decrease in the rate of hemolysis in a patient with hemolytic anemia will be a fall in the plasma bilirubin concentration. That this is so was initially suggested by studies in which the bilirubin concentration was artificially increased up to tenfold by injecting a loading dose of unconjugated bilirubin. In individuals with normal hepatic function, the plasma bilirubin concentration will return to baseline, or to within 10% of baseline within 4 hr. Such studies were the basis of a now discarded liver function test.6 In contrast to the rapid changes in serum unconjugated bilirubin concentration that follow alterations in the load presented to the liver, the reticulocyte count in the hemolyzing patient reflects not only the rate of

Table 2. Comparison of Hepatic Bilirubin Clearance Estimated From Studies With $^{51}$Cr-Labeled Erythrocytes [$C_{br}$ (Estimated)] With Direct Measurements Employing Radiolabeled Bilirubin [$C_{br}$]

<table>
<thead>
<tr>
<th>Group</th>
<th>$C_{br}$ (Estimated)</th>
<th>$C_{br}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects (n = 21) mean ± SEM</td>
<td>0.68 ± 0.05</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Gilbert’s syndrome (n = 19) mean ± SEM</td>
<td>0.20 ± 0.01</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Crigler-Najjar (n = 2) mean ± range</td>
<td>0.011 ± 0.002</td>
<td>0.010 ± 0.003</td>
</tr>
</tbody>
</table>

Ratio: $C_{br}$ (estimated)/$C_{br}$ = 1.01 ± 0.03 (mean ± SEM, n = 61).
Regression: $C_{br}$ (estimated) = 1.0008 $C_{br}$ + 0.0007 ($r = 0.9, n = 61$).

*ml/min/kg body weight.
berlin and berk

Table 3. Changes in Bilirubin Metabolism During Steroid Treatment of Autoimmune Hemolytic Anemia

<table>
<thead>
<tr>
<th>Days of Treatment</th>
<th>0</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRCV (ml/kg)</td>
<td>15.3</td>
<td>16.0</td>
<td>24.0</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>23</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>14.0</td>
<td>12.3</td>
<td>2.8</td>
</tr>
<tr>
<td>BR (mg/100 ml)</td>
<td>1.9</td>
<td>0.45</td>
<td>0.58</td>
</tr>
<tr>
<td>Cw (ml/min/kg)</td>
<td>0.53</td>
<td>0.56</td>
<td>0.62</td>
</tr>
<tr>
<td>BRT (mg/kg/day)</td>
<td>14.4</td>
<td>3.6</td>
<td>5.2</td>
</tr>
<tr>
<td>RBCLS (days)</td>
<td>15</td>
<td>62</td>
<td>65</td>
</tr>
</tbody>
</table>

days after the cessation of hemolysis, until the anemia has been corrected. The usefulness of measurements of the plasma bilirubin concentration as indicators of the cessation of hemolysis is illustrated in Table 3. Three studies of red cell volume and bilirubin kinetics were performed in a patient with an autoimmune hemolytic anemia on the day before and on the 7th and 14th days of corticosteroid therapy. Note that on the 7th day of therapy only changes in the parameters of bilirubin metabolism, including a fall in the plasma unconjugated bilirubin concentration, clearly indicated a significant beneficial effect from therapy. In contrast, the elevated reticulocyte count and persistent anemia suggested to the clinicians that therapy was unsuccessful and that splenectomy should be considered. While measurement of bilirubin kinetics can be used to monitor the response to steroid therapy only in a research setting, serial measurements of the plasma unconjugated bilirubin concentration will provide early and unequivocal evidence of beneficial therapeutic response that may obviate the need for excessive doses of steroids, or for unwarranted and potentially hazardous immunosuppressive or surgical therapy.

NEWER STUDIES ON THE SOURCES OF BILIRUBIN IN MAN

Many of the expressions derived above were based on the assumption that 15% of daily bilirubin turnover results from sources other than the hemoglobin of adult erythrocytes. The figure of 15% derives from the original studies of the early labeled peak of bilirubin production,23 in which the quantity of label recovered in fecal bile pigment, or stercobilin specific activity, was measured at various times after administration of labeled glycine as a heme precursor. Extrapolation from recovery of label to relative rates of heme synthesis in various tissues requires the critical assumption that all of the heme synthesized anywhere in the body at a particular time after administration of labeled precursor will have the same specific activity. In fact, as discussed in detail elsewhere,61 the specific activity of heme synthesized in the liver is likely to be lower than that synthesized in the marrow, leading to an underestimate of hepatic heme synthesis by classical early labeled peak techniques.

The ability to determine plasma bilirubin turnover in man now allows quantitative measurements of the bilirubin produced from those processes that give rise to the early labeled bile pigment peak, which are independent of the critical assumptions of these earlier "early labeling" studies. The quantity of bilirubin derived from senescent red blood cells (BRRbc), in mg/kg/day, can be calculated from the circulating red cell mass, the mean corpuscular hemoglobin concentration, mean red cell lifespan, and the relative molecular weights of hemoglobin and bilirubin.61 Early labeled peak bilirubin synthesis (ELP), also expressed in mg/kg/day, can be calculated as:

\[
ELP = BRT - BR_{Rbc} \tag{16}
\]

In a series of 33 hematologically normal individuals (26 were healthy volunteers), ELP averaged 1.0 mg/kg/day, representing 27% of BRT.61

Assuming that the early labeled peak processes consisted of a component independent of erythropoiesis, principally of hepatic origin, and an erythropoietic component representing ineffective erythropoiesis, BRT and BRRbc can be shown to be related linearly by the expression:

\[
BRT = C + (1 + k) BR_{Rbc} \tag{17}
\]

In this expression, \(k\) represents a proportionality constant between ineffective and effective erythropoiesis. Analysis of experimental values for BRT and BRRbc for 20 subjects with normoblastic erythropoiesis indicated that \(C\) had a mean value of 0.8 mg/kg/day and \(k\) a value of 0.089. These data suggest, therefore, that 21% of normal daily bilirubin turnover is derived from processes independent of erythropoiesis, presumably representing principally the catabolism of hepatic heme enzymes. In addition, the results suggest that for every 100 mg of bilirubin derived from the breakdown of hemoglobin from circulating red blood cells, an additional 8.9 mg are produced as the result of ineffective erythropoiesis.61

Jones et al. have calculated the rate of bilirubin production derived from hepatic heme enzymes in
patients with acute intermittent porphyria.\textsuperscript{62} Values ranged from 0.42 to 0.98 mg/kg/day and averaged 17% of daily BRT. Other data suggest that these values do not differ from those that would be observed in normal humans.\textsuperscript{63} Finally, from studies of endogenous carbon monoxide production in the rat, Landaw has determined values for $C$ and $k$ virtually identical to those reported in man.\textsuperscript{64} These and other studies suggest that appreciably more than the usually quoted figure of 10\%-20\% of total bile pigment production is normally derived from sources other than senescent circulating red cells.

The derivation of several of the equations presented earlier in this review did not take into account the dual sources of early labeled bilirubin. While it has been possible to derive explicit expressions that do take these sources into account, the increasing algebraic complexity of the results is not accompanied by any appreciable increase in the accuracy of physiologic predictions. Accordingly, the assumption that 85\% of bilirubin turnover is derived from adult circulating erythrocytes remains a useful first approximation and permits the derivation of a number of mathematically simple and conceptually understandable guidelines for the interpretation of the plasma unconjugated bilirubin concentration in hematologic disease states. The publication of more complex analytical expressions would be self-defeating and would serve merely to confuse rather than enlighten.

We have reviewed the various physiologic factors that influence the plasma unconjugated bilirubin concentration in order that hematologists may derive the maximum amount of information from such measurements. The usefulness of these concepts will be limited by the accuracy with which the relevant clinical measurements are made. While the impression is widespread that routine measurements of plasma bilirubin are not highly accurate, there are substantial data to show that both accuracy and reproducibility are achievable.\textsuperscript{65,66} Coefficients of variation of $\pm$ 5\% have been reported for samples containing bilirubin concentrations of $\approx$ 1.0 mg/dl,\textsuperscript{67} and reproducibility of $\pm$ 10\% can be attained even in the range of 0.5--1.0 mg/dl. Demands by informed clinicians are the most certain way to ensure that high laboratory standards are, in fact, achieved.

REFERENCES


Quantitative aspects of bilirubin metabolism for hematologists

NI Berlin and PD Berk

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