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Bilirubin

More than just coloring for Bruises

by

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Abbreviations:
CNS – central nervous system
NADPH – reduced nicotinamide adenine diphosphate

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Abstract

Bilirubin is one of the breakdown products of hemoglobin. It is not very soluble, to be transported in plasma it is tightly associated with albumin. The bilirubin-albumin complex is carried to the liver where glucuronate is added which increases the solubility of bilirubin. Bilirubin can also become isomerized in the presence of blue light to form lumirubin, which can also be readily excreted. Bilirubin is able to react with two peroxyl radicals to help keep albumin from becoming oxidized. Its antioxidant activity is similar to vitamin E when tested in an aqueous dispersion of multilamellar liposomes of phosphatidylcholine. Bilirubin can interfere with phosphorylation, but this activity can be restored with the addition of polylysine. Bilirubin is a multifunctional protein, much more than coloring for bruises.

Introduction

A red blood cell has a lifetime of approximately 120 days in adults and 70 days in infants. The active component in a red blood cell is hemoglobin. Hemoglobin is a porphyrin ring with an iron atom in the center. It is able to bind and transport dioxygen to tissues and carbon dioxide to the lungs. When the red blood cell is degraded, iron is recovered but the heme porphyrin ring is degraded into the yellow-orange pigment of bilirubin.

The conversion of heme to bilirubin involves two steps. The first step uses heme oxygenase, a monooxygenease, in the presence of dioxygen and NADPH to cleave the alpha methylene [1]. A carbon from a methene bridge is released as carbon monoxide (CO) and the ring structure is opened to produce biliverdin. Biliverdin is the final step in the breakdown of heme for many species of birds, amphibians, and reptiles and is excreted directly [1]. In humans, the central methene bridge of biliverdin is reduced by biliverdin reductase and NADPH to from bilirubin (please see figure 1) [1].

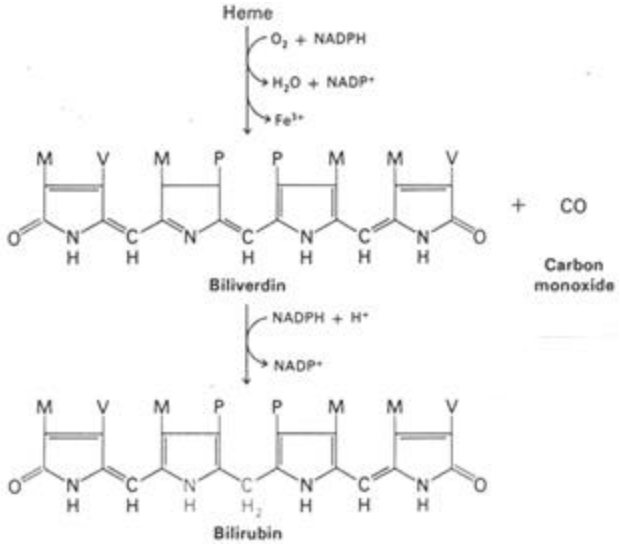


Figure 1: Degradation of heme to bilirubin.

Normal plasma bilirubin concentrations are in the range of 5 to 17 µM, where the majority is unconjugated and bound to albumin [2]. The bilirubin-albumin complex is transported to the liver where glucuronate is covalently attached to the propionate side chains [1]. Glucuronate is similar to glucose but has a COO- at the C-6 position rather than CH_2OH. The conjugate of bilirubin and two glucuronates, called bilirubin diglucuronide, is secreted into the bile (please see figure 2) [3].

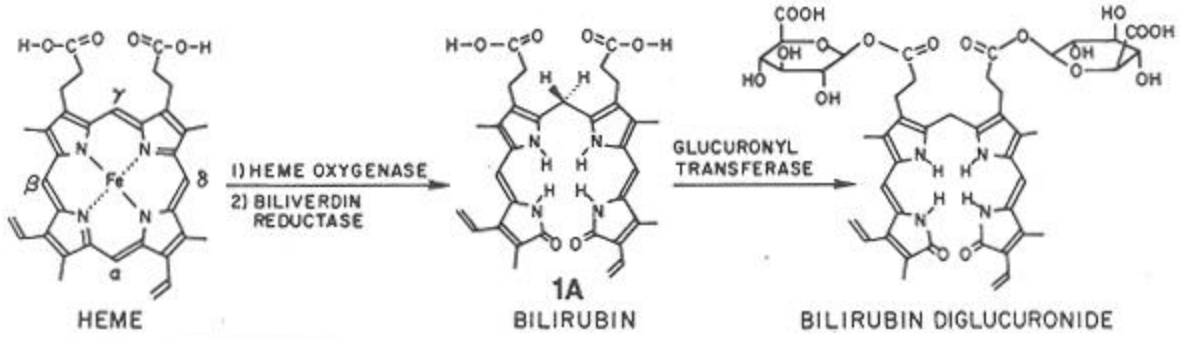


Figure 2. Main steps in bilirubin formation and metabolism. (Monoglucuronides are also formed in addition to the diglucuronide structure shown.)

When the bilirubin-glucuronidating enzyme (glucuronyl transferase) is low, only a single bile species, bilirubin, appears in the blood [3]. If the enzyme activity remains depressed then bilirubin will accumulate. Due to its lipophicity, membrane permeability and its lack of solubility in plasma, bilirubin can be deposited in the tissues, resulting ultimately in clinical jaundice.

Bilirubin can be oxidized by two hydroperoxyl radicals to form biliverdin. Biliverdin can then be reduced back to form bilirubin. On a molar basis, a bilirubin bound albumin is approximately one tenth as effective as ascorbate in affording protection against water-soluble peroxides [1]. In membranes, bilirubin is a highly potent antioxidant similar to vitamin E [1].

This paper will briefly discuss bilirubin in the production of jaundice, removal, antioxidant abilities and the uncoupling of protein phosphorylation. Within these topics sensitive detection methods will be introduced.

Production of Jaundice

Jaundice can occur in neonates and adults, usually under different conditions. Neonatal jaundice is principally transient in nature and can be the result of a deficiency in bilirubin conjugation, hepatic uptake or increased enterohepatic circulation [4]. In adults, jaundice can be a symptom of a larger disease state, for example Crigler-Najjar [4] and cholestatis [5].

Removal of Bilirubin

Bilirubin is only slightly soluble in water at physiological pH and ionic strength. To be transported in the blood, bilirubin must be tightly bound to albumin. When the limited capacity of albumin to bind bilirubin is exceeded; bilirubin is increasingly sequestered in intracellular sites. If plasma concentrations rise above 300 µM, neurological dysfunction called kernicterus or bilirubin encephalopathy may develop [2]. CNS toxicity of bilirubin occurs in two phases: an acute phase reversible by pigment removal and a later phase where problems become irreversible. Neonatal bilirubin removal methods include; exchange transfusion, antibody suppression of hemolysis and inhibition of bilirubin production. Another method of reducing the level of bilirubin is to inhibit production by blocking heme oxygenase by metalloporphyrins [4].

Bilirubin can be removed by photo-oxidation. Photo-oxidation, involving singlet oxygen and blue light, is able to transform bilirubin into the water-soluble 4Z,15E bilirubin (lumirubin) that is easily excreted (please see figure 3) [3].

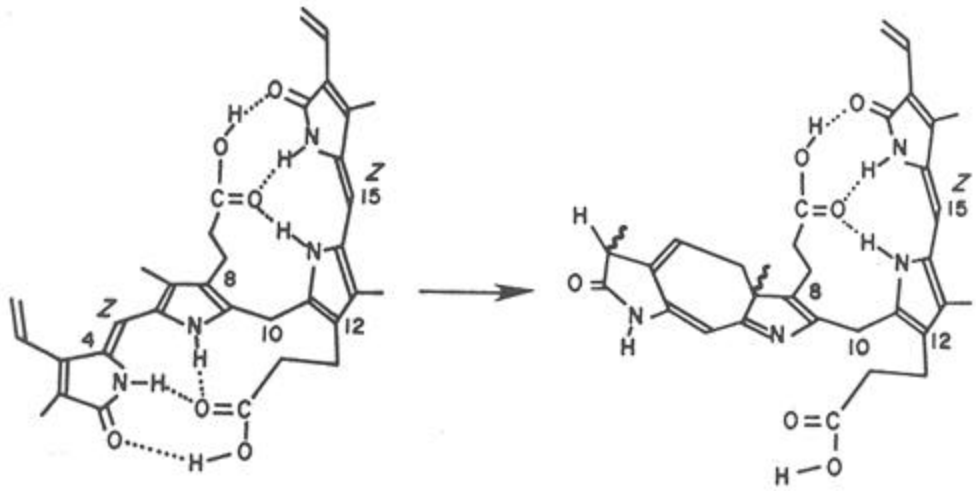


Figure 3. Intramolecular cyclization of bilirubin in presence of light to form lumirubin.

Detection of Bulirubin

The conventional *in vitro* assay procedure for determining serum bilirubin concentration uses Ehrlich's diazotized sulfanic acid [7]. This method measures diazo derivatives formed by the reaction of serum bilirubin and diazotized sulfanic acid. In principle it can determine "direct" reacting (beta and gamma fractions, conjugated with glucuronate) and "indirect" reacting (alpha, unconjugated) bilirubins [7]. In this assay the delta fraction should not react, as it is convalently bound to albumin. However, this procedure is not very sensitive, as the alpha and delta fractions can be read in the direct reaction. This direct reading can lead to falsely elevated measurements of the conjugated bilirubin levels.

A more sensitive method for determining conjugated bilirubin levels is high performance liquid chromatography (HPLC). To determine the sensitivity of this method, rats and guinea pigs were tested under normal conditions and after a bile duct ligation. A bile duct ligation increases the

level of conjugated bilirubin in the circulation. Under normal conditions, serum conjugated bilirubin was undetectable (0.006 mg/dl) in rats and guinea pigs [5]. After the bile duct ligation, the HPLC detected elevated rat conjugated bilirubin levels by 10 minutes, whereas the conventional bilirubin measurement did not become significantly elevated until 120 minutes [5]. For the guinea pig, time before detecting elevated results was 30 and 240 minutes, respectively [5].

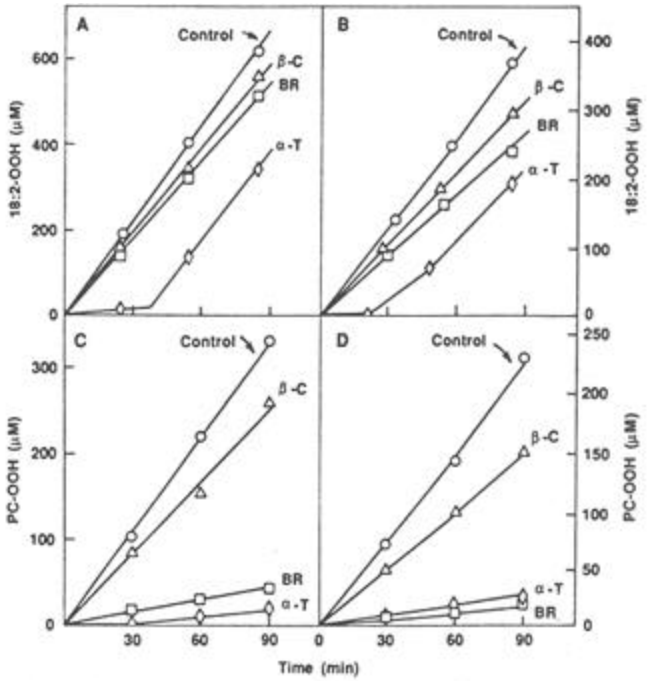
Micellar electrokinetic chromatography (MEKC) can separate electrically neutral serum bilirubin fractions (alpha, beta, gamma and delta) [7]. It can also be used to determine bilirubin oxidase activity and monitor the catalytic reaction [7].

Bilirubin as an Antioxidant

An antioxidant interacts with an oxidizing species, itself becoming reduced and changing the oxidant to a less active molecule. Bilirubin can protect albumin from peroxyl radical oxidation. A single bilirubin can react with two peroxyl radicals, becoming biliverdin [8]. Biliverdin can then become reduced to form bilirubin. Ames et al. have shown that bilirubin bound to albumin can react with peroxyl radicals as well as uric acid but not as effectively as vitamin C [8]. They determined that peroxyl radicals interacted with bilirubin-albumin 3.1 times faster than uric acid and 12.5 times slower than ascorbate [8].

The antioxidant activity of bilirubin is effected by the concentration of dioxygen. When oxygen was at approximately 20%, bilirubin was able to reduce the rate of oxidation of linoleic acid by about 16%, compared to 10% by ß-carotene [2]. However, a-tocopherol was able to inhibit the initial rate by 97% [2]. With the same concentrations of antioxidants in 2% oxygen (comparable

to what is found in tissues) activity of bilirubin increased to 35% [2]. Inhibition by ß -carotene was also increased but a -tocopherol did not change. When these compounds were tested using an aqueous dispersion of multilamellar liposomes of phosphatidylcholine (a more biologically relevant system) ß-carotene, bilirubin and a-tocopherol inhibited initial rates by 22, 87 and 99% (please see figure 4) [2]. Figure 4



Comparison of the rate of oxidation of (**A** and **B**) purified linoleic acid (158 m*M*) in homogeneous solution and (**C** and **D**) purified soybean phosphatidylcholine (PC; 10 m*M*) (*29*) in aqueous dispersion under (A and C) air and (B and D) 2% O_2 and 98% N_2 (v/v); in the absence (○) or presence of 10 μ*M* of each, trans-β-carotene (Sigma, type I) (β-C; △), recrystallized bilirubin (BR; □) and *dl*-α-tocopherol (Supelco) (α-T; ◇). The experimental details for (A) and (B) were as described in the legend of Fig. 1. (C and D) Multilamellar liposomes in water were prepared as described by Niki *et al.* (*6*) with the use of purified soybean PC (10 m*M*) and AMVN (0.25 m*M*). The reaction mixture was agitated in a water bath set at 50°C and at various time points aliquots were removed and analyzed for PC hydroperoxides (PC-OOH) at 234 nm by HPLC on an LC-NH_2 column (Supelco) with methanol and 40 m*M* NaH_2PO_4 (9/1, v/v) (1.5 ml/min) as the mobile phase (*29*). The results shown represent the averages of two to four independent experiments, with a variance of less than 7%.

This data indicates that bilirubin at micromolar concentrations *in vitro* efficiently scavenges peroxyl radicals generated chemically in either homogeneous solution or multilamellar liposomes.

Bilirubin and Protein Phosphorylation

When phosphorylation is inhibited within the cell there can be toxic effects. Phosphorylation of

sugars keeps them within the cell. If these sugars are allowed to freely move out of the membrane then energy reserves and starting material for nucleotide synthesis is lost [1]. Bilirubin may inhibit phosphorylation by binding lysine residues [9].

When bilirubin was tested on isolated liver mitochondria in concentrations of 3×10^{-4} M, there was almost a complete inhibition of protein phosphorylation and a partial inhibition of respiration [10]. The concentration of 300 µM is also the critical *in vivo* level where neurological damage in infants is probable. Walaas et al. tested the ability of a polylysine peptide to restore phosphorylation in the presence of bilirubin. They followed the phosphorylation activity of PKA (cAMP-dependent protein kinase). PKA was able to phosphorylate a substrate normally in the presence of 50 uM bilirubin when 100 uM of polylysine was added [9].

Conclusion:

When heme is broken down, bilirubin is produced. Bilirubin is only sparingly soluble in the plasma and is usually found complexed to albumin. Due to bilirubin's low solubility, it can become sequestered in the cells and can lead to jaundice. Bilirubin is further processed in the liver where glucuronates are added, increasing the solubility of the molecule so it can be excreted. Bilirubin can also become isomerized by blue light to form lumirubin, which can also be readily secreted. The albumin-bilirubin complex can bind two peroxyl radicals. Its antioxidant activity is similar to vitamin E when tested in an aqueous dispersion of multilamellar liposomes of phosphatidylcholine. Bilirubin can interfere with phosphorylation, but this activity can be restored when polylysine is added. Bilirubin is a large multifunctional protein, much more than just coloring for bruises.

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