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Unbound Bilirubin: A Better Predictor of Kernicterus?

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The peroxidase method described in this paper measures non–protein-bound bilirubin by oxidation of the free fraction. The oxidation rate is slow compared with rapid dissociation of albumin-bound bilirubin. Thus, the unbound bilirubin can be estimated from the initial rate of decline in total bilirubin concentration.

In the 1970s, severe neonatal hyperbilirubinemia and kernicterus were major clinical problems. Kernicterus could usually be prevented by administering an exchange transfusion to infants whose serum unconjugated bilirubin concentration reached 341 μmol/L (20 mg/dL), but the diagnostic specificity of 341 μmol/L (20 mg/dL) was quite poor. Premature infants sometimes developed encephalopathy at very low bilirubin concentrations, especially when receiving sulfonamides.

While engaged in a fellowship in neonatal medicine, I was stimulated by Gerald Odell's 1959 paper, in which he used pharmacological arguments to explain how these drugs produced kernicterus by competitively interfering with bilirubin-albumin binding *(1)*. I was convinced we needed a method to measure unbound unconjugated bilirubin, either directly or indirectly by measuring binding parameters. After failing to determine these parameters using standard techniques, I was able to estimate the binding constant of bilirubin to BSA using difference spectroscopy of very dilute solutions and, by inference from competitive-binding studies with BSA, the binding constant of human serum albumin.

At about the same time, Jørgen Jacobsen, a graduate biochemistry student working with Rolf Brodersen in Denmark, was examining albumin-bilirubin binding using a unique technique. Brodersen had observed that bilirubin was oxidized by hydrogen peroxide, a reaction that was catalyzed by peroxidase and inhibited by albumin. Jørgen further developed the enzymatic reaction and determined the unbound bilirubin at various human albumin concentrations. The resulting Scatchard plot indicated that albumin had 1 high-affinity site and perhaps 2 or more weaker sites *(2)*. The calculated dissociation constant was similar to my estimated value, and the method had promise for clinical application because it directly measured the minute concentration of unbound bilirubin, which Odell's work suggested was the critical parameter for bilirubin entry into the brain.

In 1971, I visited Brodersen's laboratory in Aarhus. A very good personal and scientific relationship developed, and we decided that Jørgen should apply for funding for a 1-year stay at the University of Washington, where we would undertake a collaborative effort to transform the peroxidase principle into a practical method that could be tested in patients and performed in clinical chemistry laboratories. The results of our efforts were published in 1974, and the peroxidase method has since been used extensively, either as originally described or minimally modified *(3, 4)*, for bilirubin toxicity experiments with tissue culture or experimental animals and for structural studies of protein-ligand binding using bilirubin as a high-affinity ligand.

Although several clinical studies have shown that unbound bilirubin measured by the peroxidase method is superior to total bilirubin in identifying patients at risk for early reversible bilirubin toxicity, the method has not been widely adopted for its original purpose, which was to improve clinical management of jaundiced term and premature newborns. There are several reasons for this. Foremost were the discovery and application of anti-Rh immune globulin to prevent hemolytic disease and the introduction of phototherapy to treat hyperbilirubinemia, both of which resulted in a marked decline of severe jaundice and lesser interest in jaundice as a clinical problem. In addition, the conservative intervention guidelines based on total bilirubin concentration essentially eliminated kernicterus in term infants and precluded critical clinical studies. Finally, the transformation of hospital clinical laboratories to automated systems changed their perspective toward offering the manual peroxidase procedure for clinical evaluation.

In recent years, however, there has been an increase in the incidence of brain injury from hyperbilirubinemia and an increased interest in reevaluating appropriate management guidelines *(5)*. Several studies are currently planned, or in progress, to examine the

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[2] This paper has been cited more than 230 times since publication. Jacobsen J, Wennberg RP. Determination of unbound bilirubin in the serum of newborns. *Clin Chem* 1974;20:783–9.

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value of measuring unbound bilirubin in risk assessment of jaundiced infants. Just possibly, after 44 years, the peroxidase method may serve its intended purpose.

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