# BINDING OF BILIRUBIN TO HUMAN SERUM ALBUMIN – DETERMINATION OF THE DISSOCIATION CONSTANTS

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## 1. Introduction

The binding of bilirubin to serum albumin has been known for more than fifty years [1]. The affinity is high and it has not been possible to determine the dissociation constant, since a sensitive method for measuring the very low concentrations of non-bound bilirubin has not been available. In the present work such determinations are carried out using a kinetic method capable of estimating non-bound bilirubin concentrations as low as $10^{-10}$M.

## 2. Experimental

### 2.1. *Material*

Crystalline bilirubin from Sigma (Sigma grade). Human serum albumin, dry "reinst" (Behringswerke AG, Marburg-Lahn). Horse-radish peroxidase (Sigma, type I).

### 2.2. *Method*

Brodersen and Bartels [2] have recently shown that peroxidase and hydrogen peroxide oxidize bilirubin to green products including biliverdin. The process is strongly inhibited by albumin. The albumin-bilirubin complex is not oxidized under these circumstances. As the $K_m$ value for bilirubin is $8 \times 10^{-4}$M (at pH 8.2) and the concentration of non-bound bilirubin is very far below that, the initial velocity must be proportional to the substrate concentration. The concentration of non-bound bilirubin is determined by two measurements. (1). Oxidation in the absence of albumin: 8 ml Tris-HCl buffer, pH 7.4, ionic strength 0.1M, 1 ml 50mM EDTA-solution, pH 8.2 (to avoid spontaneous breakdown of bilirubin), 0.9 ml of water, 0.5 ml 0.3% hydrogen peroxide and 0.1 nl 0.1% bilirubin solution (bilirubin dissolved in sodium hydroxide, diluted with water and pH brought to about 8.2 with hydrochloric acid). Final bilirubin concentration, 10 $\mu$M. One ml portions of this mixture are incubated with variable concentrations of peroxidase (including zero) for three minutes at 37°C. (2). Oxidation in the presence of albumin: buffer, EDTA, and hydrogen peroxide as above, 0.5 ml bilirubin solution, final concentration about 55 $\mu$M and variable volumes of a 2.5mM albumin solution (final concentration range from 20 to 150 $\mu$M). Depending on the albumin concentration the peroxidase concentration has to be varied from 50 to 7500 times the concentration in the albumin free process to get measurable velocities. The time course of the process is followed with fixed enzyme concentration. The processes with and without albumin are stopped by adding 1 ml phosphate-ascorbate buffer, pH 8.2, after which the remaining bilirubin is extracted with 3 ml chloroform [3]. The concentration is calculated from the extinction of the chloroform phase. From the initial velocity of the bilirubin degradation in the two experiments and the ratio between the peroxidase concentrations, the concentration of non-bound bilirubin is calulated.

## 3. Results

A linear time course was found for the process with added albumin. Pigment is released from the protein complex with a velocity which is high



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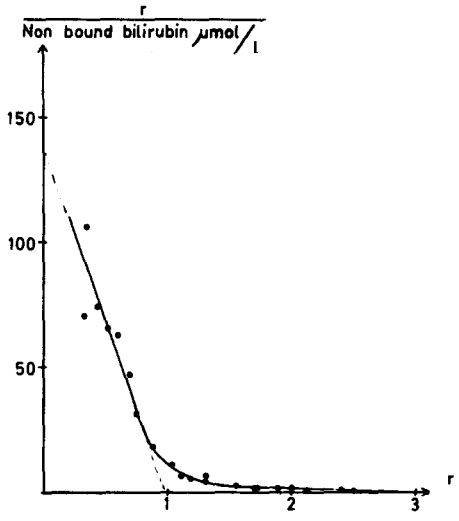


Fig. 1.

Abscissa, $r\left(\frac{[\text{bound bilirubin}]}{[\text{total albumin}]}\right)$.

Ordinate, $\frac{r}{[\text{non-bound bilirubin}]}$.

The left part of the curve is a straight line. The number of binding sites in the first class is determined as the intercept of this line at the abscissa. The first dissociation constant is calculated as the reciprocal of the intercept at the ordinate.

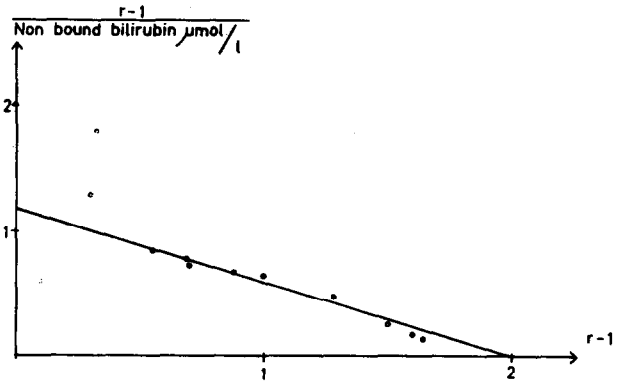


Fig. 2. Investigation of the second class of binding sites.

Abscissa, $r$-1.

Ordinate, $\frac{r\text{-}1}{[\text{non-bound bilirubin}]}$.

Calculations from the intercepts as in fig. 1.

compared to the rate determining process of oxidation. The binding is fast and reversible. The rate of the oxidation is then proportional to the equilibrium concentration. The law of mass action is obeyed, as seen from the following graphs. Fig. 1 is a Scatchards plot. The curve shows that there are more than one class of binding sites. The first part of the curve is a straight line with the intercept at unity at the abscissa. One mole of bilirubin is therefore bound per mole albumin in the first class. The dissociation constant, calculated as the reciprocal of the intercept at the ordinate is $7 \times 10^{-9}$M. According to Scatchard [4] the dissociation at the following class of sites may be determined from a similar plot, using $r$-1 instead of $r$. Two moles are bound in the second class. The dissociation constant is $2 \times 10^{-6}$M. For mole ratios above 2 the concentration of non-bound bilirubin is several μM. According to Brodersen and Theilgaard [5] the solubility of bilirubin at the present pH is about 1.5 μM. Supersaturated solutions may be stable during the few minutes necessary for the kinetic determination. Due to this fact the results of the determinations in the second class are rather inaccurate.

## 4. Discussion

Josephson and Furst [6] have concluded that the binding of bilirubin to albumin is irreversible, as they were unable to demonstrate any bilirubin in the ultrafiltrate from serum, using a diazo method. The present measurements of the concentrations of non-bound bilirubin in an albumin solution, with a more sensitive method, show that at least three moles of bilirubin are reversibly bound to one mole of albumin. Recently Scholton and Gloxhuber [7] have attempted to determine the dissociation constant for the bilirubin-albumin complex by ultracentrifugation. Due to the lack of a sensitive method for the bilirubin determination they have no measurements at mole ratios below unity. They were therefore unable to determine the first dissociation constant. These authors have further examined the changes in specific optical rotation and reduced specific viscosity when bilirubin is bound to albumin. They found that

these two parameters change in one direction, when the first molecule is bound, and in the opposite direction during binding of the next molecules. This indicates different mechanisms of binding in the two classes in agreement with the finding that the first molecule is bound with a much higher affinity than the following. The difference in affinity for the two binding sites has also been found by Bjerrum [8], who has shown that albumin has two classes of binding sites for bromophenol blue with different affinity, and that bilirubin is bound in both classes.

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