SPIN ASSAY AS A GENERAL METHOD FOR STUDYING PLASMA PROTEIN BINDING. BILIRUBIN - ALBUMIN BINDING

D.J. Wood and J.C. Hsia

Department of Pharmacology
Faculty of Medicine
University of Toronto
Toronto, Canada M5S 1A8

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Summary. The binding of a spin label to pre-existing binding sites of a protein can be monitored by electron spin resonance spectroscopy and can be interpreted in terms of a spin assay for any ligand which shares these sites. As an example of this procedure, a method is presented which evaluates the reserve capacity of the bilirubin high affinity binding site of human serum albumin for additional bilirubin binding. Hyperbilirubinemia in pre-mature or low-birth-weight term infants can result in severe neurological damage. The maturation of this technique will provide a needed clinical assay in the management of jaundiced newborn infants.

Hyperbilirubinemia (jaundice) in low-birth-weight or premature neonates can result in neurological damage such as hearing loss and motor retardation, and in severe cases, death (1,2). Bilirubin (see Figure 1) is produced by the catabolism of haem and normally is detoxified in the circulating blood by binding to the predominant protein in the serum, human serum albumin (HSA) (3). The binding of bilirubin to HSA is thought to involve one high affinity site ($Ka \sim 10^8$ litres/mole) and number of secondary sites ($Ka \sim 10^5$ litres/mole) (4); the capacity of the high affinity site is taken as a measure of the capacity of HSA to bind, and hold, bilirubin due to its much greater affinity for bilirubin, compared to the body tissues and the secondary sites (5). The saturation of the high affinity site with bilirubin results in bilirubin being available for uptake into the tissues and thus the critical parameter for bilirubin detoxification is the reserve binding capacity of the bilirubin high affinity site. This is generally termed the reserve bilirubin binding capacity (RBBC).

There have been a number of methods proposed to measure the bilirubin

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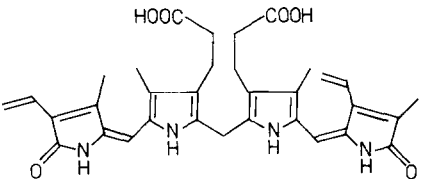
available for binding to the tissues (6-12) (i.e. not contained by the high affinity bilirubin binding site) and also methods which evaluate an "RBBC index"(13-17). A number of these procedures have been criticized (4,7,18-20) and many are experimentally complex and/or require large volumes of serum. In addition, optical density measurements are usually required to quantitate the amount of bilirubin which is present or to measure the concentration of any dyes used in simulated binding studies. These measurements tend to be complex due to the insensitivity of the spectrum to the binding state of the species. Also, spectra from other species in the samples can variably augment the observed absorbances. It has recently been suggested that the spectral characteristics of bilirubin are a very complicated and poorly understood area (21). Because of these difficulties, we present here a method to measure the reserve capacity of the bilirubin high affinity site using electron spin resonance spectroscopy (esr) and the spin labeling technique. We propose that a spin label probe can be designed which will sense the capacity of the high affinity bilirubin binding site and yet be easily quantifiable.

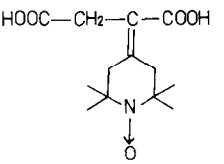
There are a number of unique advantages to the esr method of determining binding parameters. The spin label probe can be tailor-made for a particular application, requiring only that the compound contains a nitroxyl moiety (see Figure 1) so that the label is paramagnetic. The amount of label which is free or bound to macromolecule can be determined without physically separating the free from bound label. The spectra of the free and bound labels are distinct and their concentrations can be easily determined through a calibration of the free peak height with standards (22,23). The esr instrument itself, is straightforward, requiring no day-to-day adjustments, 10-25 ul samples, a detectable concentration range down to 10^{-7} - 10^{-6} Molar and a "sample-in, read, sample-out" procedure.

MATERIALS AND METHODS

Fraction V human serum albumin was obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Bilirubin was also obtained from Sigma and further purified by the method of Fog (24). All procedures involving bilirubin were



BILIRUBIN



TOPS

Figure 1 Structural formula of bilirubin and 2,2,6,6, - tetramethyl-1-oxyl-4-piperidylidene succinic acid (TOPS)

carried-out in reduced light. A spin labeled probe, 2,2,6,6-tetramethyl-1-oxyl-4-piperidylidene succinic acid (TOPS , see Figure 1) was synthesized by the method of Rozantsev (25). The experimental samples (100 ul) were prepared by the combination of aqueous stock solutions of TOPS, HSA and bilirubin disodium salt with a 50 ul Hamilton syringe. After vortex mixing, 25 ul of the sample was transfered to a 25 ul Micropet disposable pipette (Becton, Dickinson and Company, Parsippany, N.J., U.S.A.) which was used for the esr sample. The esr spectra of these samples consists of a sharp first-derivative triplet due to the TOPS which is not protein bound, superimposed on a number of broad resonances near the baseline due to the TOPS which is bound to the protein (22). The concentration of the TOPS which is free in solution is proportional to the height of the high-field peak of the triplet and the proportionality can be determined through the use of standards of TOPS in 100 mM phosphate buffer. Since the total amount of added TOPS is known, the concentration of TOPS that is bound to HSA can easily be determined. The esr spectra were recorded on a Varian E6 X-band esr spectrometer at 20 $\pm$ 1°C. The use of the thin capillary disposable pipettes and a variable temperature Dewar insert (J.F. Scanlon Co.) permits the recording of an entire series of samples without the need to change instrument conditions. The estimated error in these measurements under these conditions is less than 1%. Including pipetting and the other facets of sample preparation, the estimated accuracy of these measurement is $\pm$ 3%.

RESULTS AND DISCUSSION

TOPS was chosen for this study because of the dicarboxylic acid structural similarity with bilirubin. Furthermore, since TOPS is a small polar molecule, it is readily water soluble. This considerably reduces secondary and non-specific binding of the label to HSA.

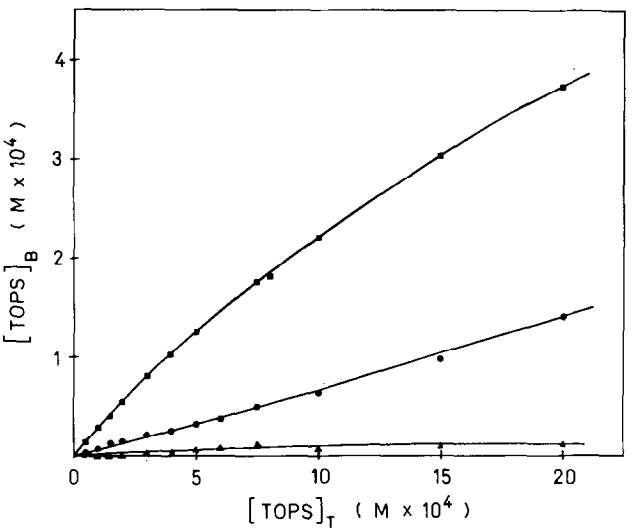


Figure 2 The binding of TOPS and albumin is model sera (5×10^{-4} HSA in 100 mM phosphate buffer at pH of 7.4). Total molar concentration of added TOPS, $[TOPS]_T$, is plotted against the molar concentration of bound $[TOPS]_B$, for model sera with no bilirubin (■), with 1.1 equivalents (bilirubin to HSA) of bilirubin (●) and 2.3 equivalents of bilirubin (▲).

The proposal that TOPS binds to HSA is confirmed by the data given in Figure 2. These data along with a Scatchard analysis (26) of the binding (not shown) are consistent with the interpretation that TOPS binds to albumin with an association constant of $\sim 10^3$ litres/mole and this binding is primarily to one site for total TOPS to HSA ratios of around one. For TOPS to HSA ratios much greater than one, there is a partition binding of the label with the protein. Figure 2 also illustrates that the TOPS binding site(s) is shared with bilirubin as the addition of 2.3 equivalents of bilirubin completely inhibits binding of TOPS to HSA. Considering the differing affinity constants of bilirubin and TOPS, this inhibition is expected.

The competition between bilirubin and TOPS for the same sites on HSA (for a solution of TOPS to HSA of one to one (27))is more clearly seen in the displacement curve given in Figure 3. The addition of ~ 1.4 equivalents of bilirubin (bilirubin to HSA) results in all but a four percent background of TOPS being displaced. This suggests that not only are the same sites

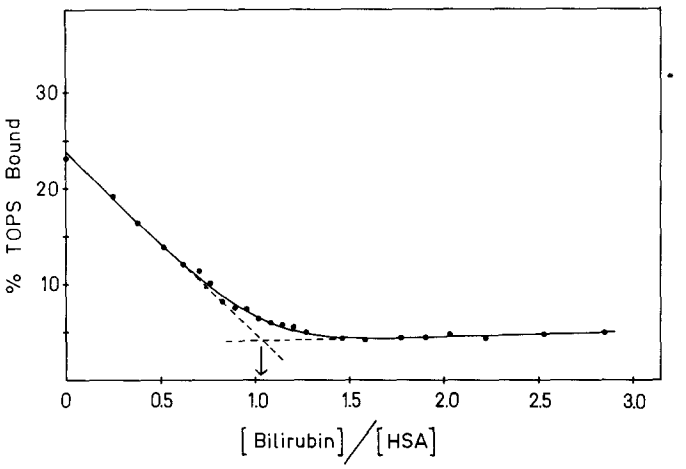


Figure 3 Displacement of TOPS from HSA by bilirubin. The HSA concentration is 4.7×10^{-4}M in 100 mM phosphate buffer at a pH of 7.4. The TOPS concentration is 5×10^{-4}M. The square brackets denote molar concentrations.

shared but the primary shared site is the high affinity bilirubin binding site. Extrapolations of the linear portions of the displacement curve intersect at a bilirubin to HSA ratio of approximately one. This behaviour can be interpreted as the binding of the first added equivalent of bilirubin to its high affinity site and consequently, the exclusion of TOPS from binding to this site. Hence, except for the four percent background binding, the binding of TOPS to HSA is to the bilirubin high affinity binding site.

It is apparent that TOPS is sensing the capacity of the bilirubin high affinity site on albumin and since we can accurately follow the binding state (free or bound) of TOPS, TOPS and molecules of similar structure will be suitable probe molecules for an RBBC assay of newborn sera. We are involved in this investigation at the present time.

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27. A similar result is obtained for solutions of TOPS to HSA of two to one. This collaborates the interpretation of the one to one displacement data and indicates that an exact stoichiometric ratio of TOPS and HSA is not required for the displacement phenomenon.