Affinity Labeling of the Primary Bilirubin Binding Site of Human Serum Albumin*

(Received for publication, September 8, 1975)

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A label for the bilirubin binding sites of human serum albumin was synthesized by reacting 2 mol of Woodward’s reagent K (N-ethyl-5-phenylisoxazolium-3'-sulfonate) with 1 mol of bilirubin. This yielded a water-soluble derivative in which both carboxyl groups of bilirubin were converted to reactive enol esters. Covalent labeling was achieved by reacting the label with human serum albumin under nitrogen at pH 9.4 and 20°C. Under the same conditions, no covalent binding to the monomers of several other proteins could be demonstrated. The number of binding sites for bilirubin and the label were found to be the same, and competition experiments with bilirubin showed inhibition of covalent labeling. The absorption, fluorescence and CD spectra of the label in a complex with human serum albumin were similar to those of the bilirubin-human serum albumin complex. However, following covalent attachment the spectral properties were changed, indicating loss of conformational freedom of the chromophore. Labeling ratios were selected to result in the incorporation of less than 1 mol of label/mol of human serum albumin. Under these conditions, labeling is thought to occur primarily at the high affinity binding site.

Bilirubin, the end product of heme catabolism in the reticuloendothelial system, is transported to the liver, conjugated mainly with carbohydrates (1), and excreted into the bile. Serum albumin reversibly binds and transports unconjugated bilirubin in the plasma. In newborns with excessive unconjugated hyperbilirubinemia, the bilirubin binding capacity of albumin is saturated, and unconjugated, water-insoluble bilirubin escapes into lipophilic tissues such as the brain, where its inhibitory action on energy metabolism (Ref. 2 and papers cited therein) produces extensive tissue damage, resulting in permanent neurological sequelae or death. The obvious medical importance of bilirubin transport and its general interest as a model for protein-ligand interaction prompted us to undertake the structural analysis of the primary binding site of human serum albumin using an affinity label.

Information on the bilirubin binding sites is limited and controversial. Jacobsen (3) has postulated the presence of two types of binding site, each albumin molecule possessing one high affinity binding site (dissociation constant 7 x 10^-8 M) and two low affinity binding sites (dissociation constants 2 x 10^-4 M). Beaven et al. (4) showed that the number of sites and their binding strengths vary with salt concentration. At low ionic strength they found three binding sites, namely one high affinity binding site (dissociation constant 1.4 to 2 x 10^-7 M) and two sites with lower affinity (dissociation constants 3.3 to 10 x 10^-4 M and 3.3 to 5 x 10^-6 M, respectively). In 0.5 M NaCl, however, there was one high affinity binding site (dissociation constant 1.5 x 10^-7 M) and only one low affinity binding site (dissociation constant 3.3 x 10^-6 M).

Analysis of the groups involved in bilirubin binding has been attempted by modifying certain amino acid residues of human serum albumin. Modification of histidine, arginine, and tyrosine reduces the bilirubin binding affinity, whereas modification of amino, carboxyl, cysteinyl, and tryptophanyl groups does not (5). Evidence has been presented for the presence of at least 1 lysine residue in or close to the high affinity binding site (6). No other structural data on the bilirubin binding sites are available.

In an effort to further characterize the high affinity binding site, we chose to investigate it with the aid of an affinity label and subsequently to analyze the labeled region of the human serum albumin molecule. The affinity label was synthesized by reacting Woodward’s reagent K (N-ethyl-5-phenylisoxazolium-3'-sulfonate) with bilirubin to yield a water-soluble bilirubin analog BW1 with activated carboxyl groups. Reaction of this label with human serum albumin at alkaline pH resulted in covalent binding to the protein. In this communication, evidence will be presented to show that under the chosen conditions, attachment occurred specifically to the high affinity binding site.

The abbreviations used are: BW, label formed by reacting 1 mol of Woodward’s reagent K with 2 mol of bilirubin (label BW); IgG, γ-globulin; PBS, phosphate-buffered saline; 0.01 M sodium phosphate buffer, pH 7.4; containing 0.15 M NaCl; covalent product, bilirubin covalently bound to monomer human serum albumin; azo product, covalent product after azo coupling of the attached chromophore.

* This work was supported by the Fritz Hoffmann-La Roche Foundation, Grant 130.
ity binding site, thus enabling the use of BW as an affinity label.

**EXPERIMENTAL PROCEDURE**

**Materials**

Human serum albumin was obtained from the Central Laboratories of the Swiss Red Cross. Bern. Bilirubin and Woodward’s reagent K were obtained from Fluka. α-Amino-[4-14C]levulinic acid hydrochloride, specific activity 29.8 mCi/mmol, was purchased from New England Nuclear. Rabbit muscle creatine kinase was from Boehringer Mannheim. Human IgG, bovine IgG (Cohn Fraction II), conalbumin (type I), lysozyme (grade I), ovalbumin (grade V), β-lactoglobulin, lysine-rich histone from calf thymus (type III), and bovine serum albumin were obtained from Sigma. Instagel was from Packard. Sephadex and CNBr-activated Sepharose 4B were purchased from Pharmacia. All chemicals used were analytical reagents from Merck.

**Methods**

**Synthesis of Affinity Label BW—Bilirubin** (116.8 mg, 0.2 mmol) and Woodward’s reagent K (151.8 mg, 0.6 mmol) were reacted with stirring at 20° for 45 min in 20 ml of acetonitrile and 0.3 ml (20 mmol) of triethylamine. Following evaporation at 30°, the residue was dissolved in 5 ml of H2O, and the solution was filtered and evaporated to dryness. The residue was dissolved in 5 ml of methanol, and 50 ml of diethyl ether was added dropwise with stirring. The precipitate was collected by centrifugation and dried in a high vacuum. After correction for 13.18% inorganic material (probably 8O2), elemental analysis gave C20 H24 N4 O2 S2 (M− 109.124).

Calculated: C 68.03, H 5.36, N 7.70, S 5.58.

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**Optical spectrum, \( \epsilon_{254} \) (154 nm), 44,500 m−1 cm−1 (in H2O) and 36,700 m−1 cm−1 (in PBS).

Infared spectrum: (KBr) 3440, 2980, 2930; \( 1755 \text{ cm}^{-1} \text{ (amide); } 1665 \text{ cm}^{-1} \text{ (amide); } 1620, 1455, 1200, 1110, 1037, 966 \text{ cm}^{-1} \). The infrared spectrum of the purified BW product is given in Fig. 1.

**Structural Investigation of BW—The label (124 mg) was further purified by thin layer chromatography on silica gel using dichloromethane/methanol (3/1 v/v) as the solvent. A minor yellow spot at Rf 0.75 was separated from the bulk of the colored material traveling at Rf 0.45. The latter was scraped from the plate and eluted with methanol, and the solution was filtered and evaporated to dryness. The residue was dissolved in 5 ml of methanol, and 50 ml of diethyl ether were added dropwise with stirring. The precipitate was collected by centrifugation and dried in a high vacuum. After correction for 13.18% inorganic material (probably 8O2), elemental analysis gave C20 H24 N4 O2 S2 (M− 109.124).

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**Covalent Attachment of Label to High Affinity Binding Site in Human Serum Albumin—Human serum albumin was defatted according to Chen (8). On the basis of the specific absorbance at 278 nm, it was calculated that the preparations contained on the average 7% water and inorganic salts. This was obtained in all calculations of molar concentration from weighed amounts of protein. Defatted human serum albumin (220 mg, 3 µmol) was dissolved in 14 ml of PBS and BW (3 mg, 2.8 µmol) was added. The mixture was stirred under nitrogen at 20° for 2 hours. After 8 hours, the product was chromatographed at 4° on a column (2.8 × 62 cm) of Sephadex G-200 in PBS at a flow rate of 43 ml/hour. The fractions containing the human serum albumin monomer were collected, dialyzed exhaustively against water, and lyophilized. The yield was 90 mg (1.2 µmol, 40%) of covalent product (ε447 nm, H2O), 38,800 m−1 cm−1).

**Azo Coupling of Bilirubin Chromophore Covalently Attached to Human Serum Albumin—Covalent product (180 mg, 2.4 µmol) was dissolved in 114 ml of water, acidified with 6 ml of 0.1 m sodium acetate, pH 4.0, and the solution was cooled to 0°. The diazo reagent was freshly prepared in an ice bath as follows: NaNO2 (40 mg, 580 µmol) in 2.5 ml of water and anhydrous sulfuric acid (95 mg, 550 µmol) in 7 ml of 1 N HCl were mixed, maintained on ice for 5 min, and supplemented with 40 µg of urea. Two milliliters of this reagent were immediately added to the covalent product at 20° in the dark for 30 min, during which time the reaction mixture turned from yellow to pink. The protein was precipitated with 67 g of solid (NH4)2SO4 (80% saturation), and the precipitate was collected by centrifugation and dissolved in 10 ml of PBS. Following dialysis against the same buffer, the solution was chromatographed on Sephadex G-200 as above. The effluent was monitored at 280 nm, and the monomer fraction was collected, dialyzed exhaustively against water, and lyophilized. Yield was 45 mg (0.6 µmol, 25%) of azo product.

**Synthesis of [14C]BW—A bile fistula was fashioned surgically as described by Barrett et al. (10) in a female mongrel dog weighing 15 kg. After a 24-hour fast, a cannula was inserted into a small mesenteric vein, its tip extended into the portal vein, and 1 mCi of α-[4-14C]levulinic acid hydrochloride (specific activity 29.8 mCi/mmol) is 300 ml of plasma expander (Haemaccel, Behringwerke) was infused over the 1st postoperative hour. Bile was collected over 45 hours into a plastic bag containing 40 mg of ascorbic acid. Bile secretion was stimulated by two intravenous injections of 5 ml of 20% sodium deoxycholate 24 and 28 hours after operation.

Bilirubin was isolated from the collected bile by an abbreviated method of Ostrow et al. (11). The unconjugated bilirubin generated by alkaline hydrolysis was extracted once with 4 volumes of CHCl3 and reprecipitated by evaporation at 30°. The yield was 157 mg (0.27 mmol) of purified [14C]bilirubin, specific activity 1.98 mCi/mmol. Using this preparation as a starting material, radioactive BW of specific activity 1.96 mCi/mmol was prepared as described above. Radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer model 3380 using Instagel as scintillator.

**Demonstration of Covalent Binding of Chromophore to Human Serum Albumin**—Samples of 22 mg each of either covalent product or azo product were dissolved in 2 ml of 0.05 M Tris-HCl, pH 9.1, containing 0.2 m mercaptoethanol and 8 m urea, and dialyzed against 10 ml of the same buffer at 4° for 24 hours. The absorbance at 447 nm (covalent product) or 520 nm (azo product) was measured in retardant and dialysate.

**Determination of Maximum Labeling of Human Serum Albumin by BW—BW was introduced into each of six samples of defatted human serum albumin (22 mg in 1 ml of PBS) to yield molar ratios of label to protein of 0.6, 0.9, 1.35, 2.5, 5.0, and 10.0. Covalent coupling and removal of reagents by Sephadex G-200 chromatography was performed on each sample as previously described. Following collection of the monomeric, noncovalently bound BW was removed by affinity chromatography on a column (0.5 × 5 cm) of human serum albumin-Sepharose. This was prepared from CNBr-activated Sepharose 4B (10 g) and human serum albumin (600 mg) according to the manufacturer’s instructions. The human serum albumin and chromophore concentrations in the eluates were determined spectrophotometrically. In the determination of protein concentration, the absorbance at 280 nm was used for interference by chromophore. The correction factor (15% of the absorbance at 447 nm) was based on the observation that the absorbance of bilirubin at 280 nm is 15% of its maximum absorbance.

**Labeling Attempts on Nonalbumin Proteins—Each protein (22 mg) was subjected to the standard coupling procedure described above. Following the reaction, three different methods were used to remove noncovalently bound BW. (a) To the reaction mixtures containing human and bovine IgG, lysozyme, ovalbumin, and β-lactoglobulin, 22 mg of human serum albumin were added, and stirring was continued overnight at 2-4°. Gel chromatography was then performed on columns (1.8 × 90 cm) of Sephadex G-75 in PBS at a flow rate of 1.8 ml/hour. (b) To the reaction mixtures containing covalent bilirubin and creatine phosphokinase 0.1 N HCl was added to lower the pH to 7.4, and stirring was continued overnight at 2-4°. Affinity chromatography on human serum albumin-Sepharose was then performed as described above, followed by chromatography on Sephadex G-75 in as in (a). (c) To the reaction mixture containing histidine-rich histone, glacial acetic acid was added to lower the pH to 4.5, and stirring was continued overnight at 2-4°. Affinity chromatography was performed as in (b) but in 0.05 M sodium acetate, pH 4.5. The eluate was concentrated to 1.5 ml by vacuum dialysis and, following addition of sodium dodecyl sulfate to a final concentration of 0.1%, was heated at 100° for 5 min and chromatographed on Sephadex G-75 (1.8 × 90 cm) in 0.05 M sodium acetate, pH 4.5, containing 0.1% sodium dodecyl sulfate. The eluates from the final Sephadex chromatographic steps from (a), (b), and (c) were monitored at 580 and 480 nm. All columns were calibrated.
Inhibition by Bilirubin of Covalent Labeling with BW—Samples of 22 mg of human serum albumin in 3 ml of PBS were exposed for 2 hours at pH 8.5 in the dark to bilirubin (dissolved in 0.02 M NaOH) at molar ratios of bilirubin to human serum albumin of 0.25, 0.5, 1.0, 2.0, 3.0, and 6.0. The pH was lowered to 7.4 and \(^{[14C]}\text{BW}\) was added to an aliquot of each reaction mixture to a final molar ratio of \(^{[14C]}\text{BW}\) to human serum albumin of 0.9. The covalent coupling reaction was performed on the aliquots as described above. The aliquots were stored frozen until chromatographed on Sephadex G-200 in PBS. Human serum albumin monomer fractions were collected, and affinity chromatography was performed on human serum albumin-Sepharose as outlined above. Protein concentrations were determined (in cuvettes of 5-cm light path using a Beckman Acta CIII spectrophotometer).

Radioactivity was determined as described under synthesis of \(^{[14C]}\text{BW}\).

**Spectra**—All absorption measurements were taken with a Beckman DB G spectrophotometer except where otherwise indicated. Fluorescence measurements were carried out using a Perkin-Elmer spectrofluorimeter model MPF-2A, and CD spectra using a Cary 61 spectrophotometer.

**RESULTS AND DISCUSSION**

**Covalent Labeling of Human Serum Albumin by BW**

Various spectral properties of bilirubin-human serum albumin complexes indicate that the specificity of the bilirubin binding site resides in the recognition of the aromatic system (4, 9, 13-21). Furthermore, suitably positioned amino groups of the protein contribute to bilirubin binding (6). Thus, an affinity label in which the chromophore remained intact would be directed into the binding site by noncovalent forces, and specific side chains of the protein could then react with appropriately activated groups of the label. Exploiting the fact that the carboxyl groups of bilirubin are sufficiently remote from the chromophore to allow their modification without effect on the aromatic structure, and reasoning that in the complex with human serum albumin bilirubin carboxyls may be favorably oriented with respect to protein amino groups, with which they would normally form salt bridges, we chose to prepare a derivative of bilirubin with activated carboxyl groups. Of the many reagents available for that purpose Woodward’s reagent \(K\) was selected because its enol ester products are known to be amenable to purification and its sulfonic acid groups could be expected to confer water solubility to bilirubin. The reaction conditions for the synthesis of the label BW were adapted from Woodward’s work (22, 23) on the activation of carboxyl groups by isoxazolium salts. The enol ester structure (Fig. 1) was formulated according to proposals made by the same authors and substantiated by elemental analysis and spectral data (see under “Methods”). The pronounced similarity of the absorption spectra of BW and bilirubin (absorption maxima both at 453 nm) for comparison of the shapes of the spectra see Fig. 2 and Ref. 7) indicated that the chromophore had remained unchanged. Moreover, evidence for the attachment of the substituents at the carboxyl groups of bilirubin was provided by the infrared band at 1755 cm\(^{-1}\), typical of enol esters, and the absence of signals due to carboxylic acid protons in the NMR spectrum.

Preliminary evidence indicated that more than 1 mol equivalent of label could be bound to human serum albumin. In order to restrict the labeling to the high affinity binding site, molar ratios BW to human serum albumin of less than 1 were employed. After mixing BW with human serum albumin, the reactants were allowed to equilibrate at pH 7.4 for 2 hours. Pilot experiments had shown that covalent attachment did not occur under these conditions. After equilibration, nucleophilic substitution was initiated by the addition of imidazol, which raised the pH to 4.4 and, furthermore, acted as a catalyst (24). Although CD spectroscopy showed that labeling was not complete before 16 hours, we reduced the reaction time to 8 hours as a compromise between complete labeling and oxidative degradation of the label. This, and the observation that some hydrolysis of the label occurred as a side reaction to covalent attachment, explains the relatively low labeling ratios obtained. These were usually 0.5 mol of label/mol of human serum albumin (Fig. 8), though occasionally higher ratios up to 0.76 were obtained. Control experiments with bilirubin instead of BW showed that under identical conditions neither binding to human serum albumin nor changes in the chromophore, as evidenced by CD spectroscopy, occurred.

Chromatography of the reaction products on Sephadex G-200 showed the presence of polymers, dimers and monomers, all of which were labeled as judged from their absorbance at 447 nm. The proportion of monomers to polymers was approximately 5 to 1 (Fig. 9). This ratio was similar to the chromatographic pattern of uncovalent human serum albumin indicating
that polymerization, a normal property of the protein, was not a consequence of cross linking by the label.

Evidence for covalent attachment in the monomeric fraction was provided by the results of dialysis under strongly denaturing conditions. No chromophore was detectable by spectrophotometry in the dialysate. Under these conditions bilirubin forming a complex with human serum albumin was totally dissociable. The covalent attachment of the label to human serum albumin was shown to occur through both activated carboxyl groups by exploiting the azo coupling reaction, which is known to cleave bilirubin and its derivatives at the central methylene bridge (Fig. 1, Ref. 25). Azo coupling performed on the chromophore in the covalent product resulted in a pink derivative (absorption maximum 520 nm in water) having the absorption characteristics of model azo pigments (absorption maximum 518 nm in water). Dialysis under denaturing conditions of the azo product released no pigment. This demonstrated that anchoring of the label occurred through two covalent bonds, one in each half of the chromophore.

Specificity of Labeling

Absorption Spectra—On forming complexes with human serum albumin, the absorption maxima of both bilirubin and BW were equally displaced from 453 to 458 nm (Fig. 2, Ref. 7). This might be expected if both the natural ligand and the label occupy the same binding site. However, when the label was covalently bound to human serum albumin, the absorption maximum shifted to 447 nm. This shift may reflect an altered protein conformation, the occurrence of which is supported by the CD spectra (see below), but it cannot be excluded that it represents rearrangements of the chromophore to another binding site during the coupling reaction.

Spectrofluorimetry—Beaven et al. (4) have described an approximately 10-fold enhancement in the fluorescence of bilirubin on complexing with human serum albumin. Similarly, we found that the fluorescence intensity relative to the peak intensity of free BW increased 16-fold on forming a complex and 21-fold following covalent coupling of the label with human serum albumin. Relating the bilirubin-human serum albumin complex to the same standard (free BW), the fluorescent enhancement matched that of the covalent product, the two spectra being almost superimposable (Fig. 3). The emission maxima were at 518 nm for both the bilirubin-human serum albumin complex and the covalent product, while that of the BW-human serum albumin complex was shifted to 522 nm.

The dependence of the relative fluorescence intensity on the molar protein to bilirubin ratio in the presence and in the absence of salt has also been reported (4). Relative fluorescence increased steeply with increasing protein concentration (Fig. 4, inset), the effect being less pronounced in the presence of salt. The BW-human serum albumin complex behaved in a similar, though not identical, manner (Fig. 4), indicating that salt had

Fig. 2. Absorption spectra of various bilirubin chromophore derivatives, all in PBS. ---, free BW; --, bilirubin-human serum albumin complex, molar ratio 0.9; ---, BW-human serum albumin complex, molar ratio 0.9; ---, covalent product, molar chromophore to human serum albumin ratio 0.75. The indicated molar extinction coefficients relate to chromophore concentration.

Fig. 3 (left). Fluorescence spectra of various bilirubin chromophores. ---, free BW; --, bilirubin-human serum albumin complex, molar ratio 1.0; ---, BW-human serum albumin complex, molar ratio 1.0; ---, covalent product, molar chromophore to human serum albumin ratio 0.76. The fluorescence intensity (F/Fmax) is expressed relative to the maximum fluorescence of BW (Fmax). Emission spectra were taken in water at pH 8.5 using 1-cm cuvettes; excitation was at 450 nm. All solutions were prepared as described by Beaven et al. (4), and contained a final concentration of 2.26 μM chromophore. Concentrations of chromophore and protein were determined spectrophotometrically.

Fig. 4 (right). Dependence of relative fluorescence on the protein to bilirubin molar ratio in the presence and absence of sodium chloride. All solutions contained chromophore at a concentration of 2.26 μM. ---, BW-human serum albumin (HSA) complex. Inset (---,), bilirubin-human serum albumin (HSA) complex, data taken from Beaven et al. (4). The fluorescence intensity at 525 nm (F/Fmax) is expressed relative to the fluorescence intensity at the highest protein to chromophore ratio (12/1, not shown). All other conditions were as described in Fig. 3.
similar effects on the protein environment of label and bilirubin.

Circular Dichroism—Bilirubin, upon forming a complex with human serum albumin, exhibits a very strong ellipticity in the visible region of the spectrum (9, 13-19). This has been attributed to an asymmetric structure of bilirubin, imposed upon it by the conformation of the binding site. A helix, similar to hexahelicene, seems most probable (20); it is right-handed at neutral or alkaline pH and left-handed at acidic pH. This is reflected by a pH-dependent change in the sign of the Cotton effect. The pH at which this transition occurs is dependent on the protein to ligand ratio and buffer composition (4, 9, 13, 15, 19). Our CD measurements, performed under conditions identical with those described by Beaven et al. (4), showed qualitatively similar profiles for the bilirubin-human serum albumin complex and the BW-human serum albumin complex (Fig. 5). In the upper pH range both showed a negative Cotton effect at the short wavelength band and a positive one at the long wavelength band, whereas in the lower pH range the signs of the Cotton effects were inverted. However, the BW-human serum albumin complex showed a somewhat smaller ellipticity, and the positive to negative transition did not occur at the same pH. In contrast, the covalent product demonstrated one-fifth of the ellipticity shown by the bilirubin-human serum albumin complex and only minimal changes with pH variations. This behavior is indicative of immobilization of the chromophore produced by covalent bonding at more than one site.

As opposed to human serum albumin, bovine serum albumin when forming a complex with bilirubin displays negative ellipticity between 470 nm and 480 nm and positive ellipticity between 415 nm and 425 nm at all pH values (15, 16, 21). As shown in Fig. 6 qualitatively similar behavior occurred with the BW-bovine serum albumin complex. This suggests that, as with human serum albumin, bilirubin and BW occupy the same binding site.

Maximum Labeling of Human Serum Albumin by BW—Evidence has been adduced for the presence of three specific binding sites for bilirubin in human serum albumin at low salt concentration (3, 4). However, in 0.5 M NaCl the number of detectable binding sites is reduced to two (4). Assuming that the affinity label does, in fact, bind specifically, one should also be able to demonstrate three, or at least two, binding sites with BW. Human serum albumin was exposed to increasing amounts of BW, and following the covalent reaction, affinity chromatography was employed to remove noncovalently bound BW from the monomers. A plot of the molar ratio of covalently bound label versus the molar ratio of BW to human serum albumin in the reaction mixtures gave a rectangular hyperbola which was transformed into the double reciprocal plot shown in Fig. 7. The maximum achievable labeling, calculated as the reciprocal of the intercept at the ordinate, was 3 mol of label/mol of human serum albumin. This confirmed that covalent labeling with BW is indeed saturable and the ratio is consistent with the number of bilirubin binding sites in human serum albumin.

Inhibition by Bilirubin of Covalent Labeling of Human Se-

![Fig. 5. Effect of pH on CD spectra of various bilirubin-chromophore derivatives. ---, bilirubin-human serum albumin complex, molar ratio 1.0 (17 μM); ---, BW-human serum albumin complex, molar ratio 1.0 (17 μM); ---, covalent product, molar chromophore to human serum albumin ratio 0.9 (20.4 μM with respect to chromophore). All solutions were prepared as described by Beaven et al. (4) except for the BW-human serum albumin complex which was equilibrated at pH 7.4. Concentrations of chromophore and protein were determined spectrophotometrically.](http://www.jbc.org/)

![Fig. 6. CD spectra of chromophore-bovine serum albumin complexes. ---, bilirubin-bovine serum albumin complex, molar ratio 0.5 (25 μM bilirubin); ---, BW-bovine serum albumin complex, molar ratio 0.5 (25 μM BW). Conditions were as described in Fig. 5; concentrations of bovine serum albumin were calculated using A278 (278 nm), 6.44 (21).](http://www.jbc.org/)

**High Affinity Bilirubin Binding Site in Human Serum Albumin**

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High Affinity Bilirubin Binding Site in Human Serum Albumin

The results of the six independent tests presented above support the conclusion that BW, a bis-enol ester of bilirubin, serves as a label for bilirubin-binding sites in human serum albumin. Since the number of binding sites for bilirubin and BW are the same, we suggest that under conditions where the molar ratio of BW to human serum albumin is less than 1, incorporation occurs only at the high affinity site. Structural investigations are currently underway to determine the position of covalent attachement. This communication presents the synthesis and the use of the first bilirubin derivative which can be employed for more detailed investigations of the bilirubin binding site of human serum albumin.

**Acknowledgments**—We are indebted to Dr. M. Becker for surgery performed on dogs used for the biosynthetic production of radioactive bilirubin. Thanks are also due to Mrs. C. Ruedin and Mr. A. Schultess for technical assistance.

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Affinity labeling of the primary bilirubin binding site of human serum albumin.
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