

BILIRUBIN-ALBUMIN BINDING AT VARYING CONDITIONS OF  
HEMATOCRIT, SODIUM BENZOATE CONCENTRATION, AND  
BILIRUBIN/ALBUMIN MOLAR RATIOS

Submitted in partial fulfillment of the Requirements for Graduation  
with Honors to the Department of Biology Carroll College, Helena,  
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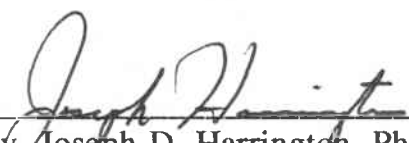
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This thesis for honors recognition has been approved for the Department of Biology.

  
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## ABSTRACT

Hyperbilirubinemia, which causes jaundice, is quite common in newborns. Typically bilirubin is bound to its carrier protein albumin and very little unbound bilirubin is present in the blood. However, in cases of hyperbilirubinemia there is a marked increase in unbound bilirubin, which can be toxic. The two methods traditionally used to treat hyperbilirubinemia are exchange transfusion and phototherapy. Each of these methods have drawbacks.

The Department of Chemical, Bio, and Materials Engineering at Arizona State University is currently developing an extra-corporeal hemoperfusion column that will remove the unbound bilirubin fraction from the blood of jaundiced patients. This column utilizes coated activated charcoal beads, which serves as the sorbent for unbound bilirubin, and sodium benzoate, which serves as the solutizer for unbinding bilirubin from albumin.

The effect of varying hematocrit, sodium benzoate concentration, and bilirubin to albumin molar ratios were studied in relation to bilirubin-albumin unbinding. Specific attention was given to unbound bilirubin concentration. The results showed increasing hematocrits reduced both total bilirubin concentration ([TB]) and unbound bilirubin concentration ([UB]). In fact, a hematocrit of 60 resulted in a [UB] that was less than 10 ug% at any sodium benzoate concentration ([SB]) or bilirubin to albumin molar ratio (B/A).

Sodium benzoate was shown to unbind bilirubin from albumin at low bilirubin to albumin molar ratios and at low to moderate hematocrit levels. Therefore, sodium benzoate is capable of increasing the unbound bilirubin fraction, thereby increasing the hemoperfusion columns efficiency. The results also indicate that an increase in [SB] from 50 to 100 mM did not significantly increase the [UB].

The data suggests as the B/A approaches unity, sodium benzoate in any concentration was not effective in increasing the [UB] when red blood cells were present.

Because of its low concentration, the measurement of unbound bilirubin is difficult. The most accepted method of determining [UB] is the peroxidase method. Also, the automated Labo UB Analyzer UA-1, is used in clinical situations to measure both [TB] and [UB]. The Labo is based on the manual peroxidase test. Both of these methods were compared prior to analyzing serum samples. Results showed that the Labo read [UB] with a much smaller coefficient of variation (8.4%) than the manual peroxidase method (23.1%). Because of this, the Labo was used in analyzing the serum samples that were prepared in order to study bilirubin-albumin binding.

## TABLE OF CONTENTS

Acknowledgments.....	iii
Abstract.....	iv
Table of Contents.....	vi
List of Figures.....	vii
List of Tables.....	viii
Introduction.....	1
Literature Review.....	4
Materials and Methods.....	9
Reagents.....	9
Procedures.....	11
Observations and Results.....	16
Comparison of Manual and Automated Peroxidase Methods....	16
Total bilirubin concentration ([TB]) with respect to Sodium Benzoate Concentration ([SB]).....	17
Unbound Bilirubin Concentration ([UB]) with respect to [SB]....	19
[UB] with respect to Hematocrit.....	20
[UB] with respect to B/A.....	21
Discussion.....	22
Conclusions.....	24
Appendix.....	28
Literature Cited.....	45

## LIST OF FIGURES

Figure 1. The bilirubin molecule.....	28
Figure 2. Rate Dependence on Peroxidase Concentration.....	31
Figure 3.1. [TB] versus [SB], B/A = 0.5.....	32
Figure 3.2. [TB] versus [SB], B/A = 1.0.....	33
Figure 3.3. [TB] versus [SB], B/A = 1.5.....	34
Figure 4.1. [UB] versus [SB], B/A = 0.5.....	35
Figure 4.2. [UB] versus [SB], B/A = 1.0.....	36
Figure 4.3. [UB] versus [SB], B/A = 1.5.....	37
Figure 5.1. [UB] versus HCT, B/A = 0.5.....	38
Figure 5.2. [UB] versus HCT, B/A = 1.0.....	39
Figure 5.3. [UB] versus HCT, B/A = 1.5.....	40
Figure 6.1. [UB] versus B/A, SB = 0 mmole/L.....	41
Figure 6.2. [UB] versus B/A, SB = 20 mmole/L.....	42
Figure 6.3. [UB] versus B/A, SB = 50 mmole/L.....	43
Figure 6.4. [UB] versus B/A, SB = 100 mmole/L.....	44

## LIST OF TABLES

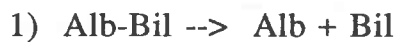
Table 1. Determination of variability in bilirubin-albuminbinding at different B/A ratios, hematocrits, and [SB] using the Labo Analyzer.....	29
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## INTRODUCTION

The hemoperfusion (HP) column being developed by the Department of Chemical, Bio, and Materials Engineering at Arizona State University makes use of coated activated charcoal (CAC) and sodium benzoate, which serves as a solutizer. Brian has shown that CAC effectively removes unbound bilirubin from blood (3). Sodium benzoate is used in to displace bilirubin from albumin, its carrier protein in plasma. Sodium benzoate greatly enhances the removal rate of bilirubin by the HP column. Hemoperfusion was performed on a canine after the infusion of bilirubin in the absence of sodium benzoate. This yielded a 19.8% removal of inlet bilirubin by the column over a two hour period (4). However, in optimum bench-top batch tests with sodium benzoate, Brian has shown 90% bilirubin removal (3). Sodium benzoate is biocompatible and is used as a preservative in many beverages and in pharmaceutical preparations. The effect of sodium benzoate on serum protein electrophoretic patterns is negligible at concentrations as high as 200 mM (3).

The object of the research presented in this thesis is to look at bilirubin-albumin binding at varying conditions of hematocrit, bilirubin/albumin molar ratios and sodium benzoate concentrations. This information will be valuable in determining the efficacy of the experimental column and perhaps in developing protocols for column use. The focus of the research was unbound bilirubin concentrations because it is the unbound, unconjugated bilirubin fraction that gives rise to potentially harmful physiological effects (2).

In order to study the unbinding of bilirubin from albumin the manual peroxidase method utilized by Nakamura (11) and the automated Labo UB Analyzer UA-1, which is based on the manual peroxidase method (15), were used. The peroxidase method has been shown to give good estimates of unbound bilirubin concentration (7,11). The binding of bilirubin to albumin is such that extremely small concentrations of free bilirubin exist at equilibrium in normal serum samples and cannot be measured by direct spectrophotometry, dialysis, ultracentrifugation, or gel filtration as long as the bilirubin to albumin molar ratio is less than one (11). However, unbound bilirubin can be oxidized by peroxidase in the presence of hydrogen peroxide to colorless products. Bilirubin bound to albumin is not oxidized by peroxidase (7). Also, Jacobson concluded that bilirubin and albumin equilibrate very rapidly compared to the rate at which unbound bilirubin is oxidized (11). The reaction scheme is as follows (7):



Because of the rapidity of the first reaction in the scheme, it is assumed that the second reaction is the rate limiting step in the overall reaction. This assumption has been verified (7). According to the reaction conditions that pertain to the oxidation of bilirubin by

peroxidase, the following formula is applicable:

$$V_0 = -dA/dt = k \cdot [\text{Bil}] \cdot [\text{POD}]$$

[Bil], free bilirubin concentration

[POD], peroxidase concentration

$V_0$ , rate of bilirubin disappearance over a measured time interval

k, rate constant

From this equation, the concentration of unbound bilirubin can be calculated from the oxidation velocity (11). The rate constant in this equation is determined by using a standard albumin-free bilirubin solution of known concentration along with peroxidase solutions of known concentration (7). Once the rate constant is determined, the peroxidase method can be used to determine free bilirubin concentration in serum samples. The procedures of this test were carried out according to the method of Nakamura (11).

## LITERATURE REVIEW

Bilirubin is the major breakdown product of heme, which is found in red blood cells, erythroid precursors, myoglobin and certain enzymes (2,13). The destruction of senescent RBCs accounts for 80% of the bilirubin produced (1). The life span of adult RBCs is 120 days while neonatal RBCs have a lifespan of 70 days (10). Under normal circumstances in the adult, 1% of circulating RBCs are destroyed each day (1). RBC catabolism to bilirubin, iron and globin occurs primarily in the liver, spleen, lymph nodes, and bone marrow. The initial products of RBC catabolism are iron, globin, and heme (2). The iron atom is recycled, and the globin is metabolized to its constituent amino acids. Heme undergoes further conversion to form bilirubin which is eventually excreted (13). The conversion of heme to bilirubin involves heme oxygenase and biliverdin reductase enzymes. Biliverdin is an intermediate in the breakdown of heme to bilirubin (1).

A two dimensional representation of the bilirubin molecule shows the presence of hydrophilic -COOH and -OH groups and the molecule appears polar. However, the three dimensional model of bilirubin, which assumes a "bent paper clip" shape, shows the presence of hydrogen bonds between juxtaposed NH/O and OH/O groups. Because of this the -COOH and -OH groups are intimately associated and unavailable for interaction with polar groups in the environment (Figure 1). Therefore, bilirubin is insoluble in H<sub>2</sub>O and methanol but soluble in non-polar solvents due to the hydrocarbon groups on the

perimeter of the molecule. Bilirubin in this unbound, unconjugated form is toxic because it can pass readily across biological membranes such as the placenta and blood-brain barrier and is then difficult to excrete (10).

The plasma protein albumin binds free bilirubin and keeps the amount of free bilirubin low. This prevents or inhibits access of free bilirubin to lipid tissues such as the brain and intracellular membranes (5). Albumin provides a readily and reversibly accessible storage space for bilirubin. Also, albumin allows for transport of free bilirubin through the vascular compartment to sites of further metabolism (1,5). The albumin molecule has multiple binding sites for bilirubin. The primary binding site has an affinity constant of approximately  $1 \times 10^8$ . The two secondary binding sites have affinity constants ranging from approximately  $1 \times 10^5$  to  $1 \times 10^6$  (5). However, as the bilirubin to albumin molar ratio approaches unity, toxic levels of free bilirubin exist (3,10). Albumin and bilirubin interact to form a noncovalently bonded, freely reversible association complex (10). The ability of albumin to bind bilirubin is diminished in the presence of other organic anions, caffeine, and non-esterified fatty acids (1,12).

The bilirubin-albumin complex is then transferred to liver cells which contain Z and Y (ligandin) proteins, which have an affinity for bilirubin that is 5 to 8 times stronger than albumin (5). These proteins facilitate the transport of bilirubin through the hepatic cell membranes and also prevent reflux (1,5). Once in the liver cells, the free bilirubin is detoxified by conjugation with two glucuronic acid

sugars to form bilirubin diglucuronide. This conjugation process is mediated by the enzyme UDP-glycosyl-transferase which is found in the rough endoplasmic reticulum of the hepatocytes (5,10). The glucuronic acid sugar molecule adds several polar -OH and -COOH groups to the bilirubin molecule and generates a new pigment that is more water soluble and polar enough to be excreted from the liver in bile, or from the kidney in urine (10). The system that excretes bilirubin diglucuronide is specific and it is unable to transfer unconjugated bilirubin from hepatic cord cells to the bile ducts (2). Fevery reported that in freshly collected bile of man and rats, unconjugated bilirubin makes up less than 1% of the total bilirubin present (5).

For adults hyperbilirubinemia, which gives rise to jaundice, is a rare occurrence. Normally only trace amounts of unbound bilirubin (1 mmole/L) are present in the serum of adults. However, jaundice occurs in 50% of newborn infants and 5% of newborns require treatment (12). For premature, low birth-weight infants, jaundice is more severe and prolonged (2). Severe jaundice can lead to impairments in intelligence, dyslexia, hyperactivity, cerebral palsy, hearing loss, seizures and death (3). In some cases congenital defects will cause higher levels of free bilirubin in the serum. For example, Crigler-Najjar syndrome results from a deficiency in UDP-glucuronyl transferase (1) and Gilberts disease results from an increased rate of reflux of hepatic unconjugated bilirubin to the plasma (2).

Aside from congenital defects, many additional factors contribute to jaundice in the "normal" neonate. First, neonates produce more

bilirubin than adults; 8.5 versus 3.8 mg/kg/day, respectively. This is because neonates have a greater total hemoglobin mass and shorter RBC lifetimes (5). Second, UDP-glucuronyl transferase activity is low in the neonatal liver (10). Third, fetal albumin seems to have a lower binding capacity for bilirubin (5). Fourth, adult levels of ligandin are not achieved until several weeks after birth. This is important because the unbound bilirubin is denied access to the developing fetal liver. The unbound bilirubin is able to cross the placental membrane and then is excreted by the mother (1). Because of these developmental events, the rate limiting factor for bilirubin excretion in the neonate is bilirubin uptake by the liver and or conjugation by glucuronyl transferase (2). Therefore, there is more bilirubin in the neonate and the turnover rate for bilirubin is slower. Additionally, Watson recovered bilirubin from within RBCs of jaundiced newborns (16). Malik et al reported the binding of bilirubin to RBCs is markedly increased if there is a reduction in the capacity of albumin to bind bilirubin (9).

Neonates who need treatment for hyperbilirubinemia typically undergo phototherapy or exchange transfusion. Phototherapy is used in cases of mild jaundice. This treatment involves placing the newborn under blue lights (i.e. visible light , wavelength approximately 600 nm). The blue light causes the bilirubin near the skin to be photoisomerized/oxidized to forms that are soluble and excretable in the bile (10). Phototherapy can effectively convert approximately 15% of total bilirubin to excretable forms. At elevated and dangerous levels of bilirubin, exchange transfusion is the

treatment of choice. Both of these treatments have drawbacks. With phototherapy, there is the possibility of DNA modifications and growth retardation, especially of cranial circumference. Exchange transfusions can be hazardous for low birth weight infants due to large priming volume and system complexity in relation to the infants' size and blood volume (3,4).

Because of the frequency of the occurrence of jaundice in neonates and the drawbacks associated with the traditional treatment methods, the Department of Chemical, Bio and Material Engineering at Arizona State University began working on an extra-corporeal hemoperfusion column that will remove bilirubin from the blood of jaundiced infants. Thaler et al have demonstrated that a hemoperfusion column can remove bilirubin with an efficiency that is comparable to exchange transfusion. Also, bilirubin removal with a HP column shows little or no bilirubin rebound after treatment. This bilirubin rebound phenomenon is strongly noted with exchange transfusion. HP is superior to exchange transfusion due to the absence of acidosis and hypoglycemia and the stability of blood chemistry, hematology and hormone levels during treatment (14).

## MATERIALS and METHODS

### REAGENTS

*Phosphate buffer:* 0.1 M, pH 7.4

*Bilirubin stock:* Solution #1; 1.87 mg of bilirubin (Sigma) from bovine gall stones was dissolved in 0.5 mL of 5 mM EDTA in 0.1 M NaOH and 4.5 mL of deionized water ([bilirubin] = 0.6397 umole/mL).

Solution #2: 33.33 mg of bilirubin was dissolved in 2.136 ml of 5 mM EDTA in 0.1 M NaOH. This solution was then used to immediately spike serum samples to give desired bilirubin/albumin molar ratios with minimal change in the physiological environment of the serum ([bilirubin] = 26.7 umole/mL).

*Glucose solution:* 0.5 g of glucose was dissolved in 10 mL of deionized water.

*Glucose oxidase solution:* 11.87 mg of glucose oxidase (Sigma) from Aspergillus niger (176.9 U/mg) was dissolved in 1 mL of phosphate buffer.

(NOTE: Glucose and glucose oxidase are used as the donor of hydrogen peroxide. With this procedure, no decrease in absorbance at 460 nm, the absorbance maxima of bilirubin, is observed after adding glucose and glucose oxidase (11). In usual clinical assays of

icteric sera, the oxidation of bilirubin takes place just after the addition of peroxidase (11). Other peroxidase test protocols call for the use of hydrogen peroxide or ethyl hydroperoxide (7). With these reagents, a decrease in absorbance at 460 nm is observed without the addition of peroxidase, thereby introducing error into the procedure (11).

*Peroxidase solution:* 4.0 mg of horseradish type 1 peroxidase (Sigma) was dissolved in 10 ml of PBS.

*Sodium benzoate stock solution:* 1 M solution of sodium benzoate was prepared using phosphate buffer.

*Bovine blood parameters:* Bovine whole blood was collected from Stones Meat Packing. 0.24 M EDTA and 1.0 M sodium bicarbonate were added to the blood to serve as an anticoagulant and pH buffer, respectively. Final blood concentrations of EDTA and sodium bicarbonate were approximately 8.4 mM and 0.5 M, respectively.

The blood was then centrifuged for 5 minutes at 2500 rpm on International Equipment Company PR 7000 centrifuge. The plasma and RBCs were separated and refrigerated at 5 °C.

The albumin concentration in the blood was measured using the Sigma company BCG test.

The exact parameters of the blood samples were:

	Sample #1	Sample #2
Volume collected	2800 mL	1800 mL
Amount of 0.24 M EDTA added	100 mL	63 mL
Amount of 1.0 M Sodium bicarbonate added	140 mL	90 mL
Hematocrit	30%	30%
[Albumin]	3.21 g% (483.7 uM)	3.17 g% (477.5 uM)

(NOTE: Sample #1 was collected and used on 7/6/89 in trials to determine the correlation between the Labo UB Analyzer and the manual peroxidase method. Sample #2 was collected on 7/19/89 and used on 7/20/89 in trials to study bilirubin-albumin binding at different physiological conditions.)

### PROCEDURES

*Bovine serum sample preparation:* Sample #1; 0.78 mL of bilirubin stock #1 (639.7 uM) were mixed with 2.07 mL of serum sample #1 ([albumin] = 483.7 uM) to give a sample solution with a bilirubin to albumin molar ration (B/A) of 0.5.

Sample #2; 5 mL of serum sample #2 were pipetted into 4 separate vials (total albumin = 2.39 umoles). 44.7 uL of bilirubin stock #2 (26.7 umoles/ml) were added to give a B/A = 0.5. Similarly, 89.4 uL and 134.1 uL of bilirubin stock #2 were added to 2 separate vials to give B/A = 1.0 and 1.5, respectively. The remaining vial had no bilirubin added and gave a B/A = 0.0.

Each of the above B/A plasma solutions were then used to prepare 16 samples with varying hematocrits (0, 20, 40, 60) and

sodium benzoate concentrations (0, 20, 50, 100 mM). All samples were prepared in 1 mL eppendorf tubes. The necessary amounts of B/A plasma solutions, red blood cells, 1.0 M sodium benzoate, and 0.1 M phosphate buffer were added to the eppendorf tubes to bring final sample volumes to 1.0 mL.

The samples were then gently rotated for 15 minutes to ensure proper mixing while trying to prevent hemolysis. Because the spectrophotometric methods involved in free bilirubin determination can only be carried out on serum (15), the sample tubes containing RBCs were spun down in an eppendorf centrifuge for 3 minutes. The supernatant of each tube was then collected and analyzed for unbound and total bilirubin with the Labo UB analyzer.

*Determination of rate constant for the peroxidase test:* Phosphate buffer (0.1 M) was used to bring all final cuvette volumes to 2100 uL. All cuvette samples contained 10 uL of bilirubin stock (cuvette concentration 3.05 uM), 10 uL of glucose oxidase, solution and 20 uL of glucose solution. 20.0-26.4 uL of peroxidase solution (prepared by dilutions of peroxidase stock solution) to give peroxidase concentrations ranging from 6.36 to 74.68 ug/L were also added to the cuvettes just before spectrophotometer readings were taken. All readings were done at 460 nm (the absorption maxima of free bilirubin), and 25 °C with a Beckman DU 65 spectrophotometer with a Peltier temperature control unit (11).

The rate of free bilirubin disappearance per minute was determined. Due to fluctuation in spectrophotometer readings in the

first 15 seconds of the reaction, the change in absorbance from 0 to 15 seconds was not used. Instead, the absorbance change from 15 to 60 seconds was utilized in order to give a stable rate of reaction.

It is important to note that when unknown free bilirubin concentrations are determined from experimental samples utilizing this method, the concentration of peroxidase that needs to be used varies. This is because the reaction of free bilirubin and peroxidase must run at a rate such that 20% of the reactants have disappeared between 2 and 10 minutes. Therefore, a range of peroxidase concentrations (6.36 -74.68 ug/L) was used in the experimental determination of the rate constant of the peroxidase reaction. The peroxidase concentrations and the associated rates of reaction were then plotted against each other and a smooth curve was generated (Figure 1). The curve that was generated allowed for the calculation of the rate constant (k) from the slope of the curve and the bilirubin concentration (3.05 uM). The first order bilirubin + peroxidase reaction equation is:

$$\Delta A_{460} = k \cdot [\text{POD}] \cdot [\text{Bil}],$$

$\Delta A_{460}$  represents the reaction velocity

Rearranging this equation to solve for k yields:

$$k = \Delta A_{460} / [\text{POD}] \cdot [\text{Bil}].$$

The slope of the curve represents  $\Delta A_{460} / [\text{POD}]$ . In order to determine the value of k associated with the initial reaction velocity, the slope of the curve at the origin was determined. Utilizing the above formula the rate constant was:  $k = 5.52 \times 10^{-4}$ .

*Labo UB Analyzer UA-1 specifications:* This instrument is designed to determine both total and unbound bilirubin concentrations in neonatal serum (11,15). Bilirubin and hemoglobin concentration in serum samples are photometrically determined at the wavelengths 460 nm and 575 nm. The Analyzer reads at 575 nm in order to eliminate the absorbance due to hemolysis in a given sample. The Analyzer then calculates the absorbtive difference mechanically and displays the total bilirubin concentration in the serum sample (15). The Analyzer calculates the unbound bilirubin concentration of a sample utilizing the peroxidase method principles set forth by Nakamura (11,15). In using this instrument, it is necessary to utilize the Analyzer reagent kits to make experimental runs. These kits include a buffer solution (containing sodium acid phosphate, potassium hydrophosphate and glucose), an enzyme pellet (containing glucose oxidase and peroxidase), and a diluent buffer without glucose, which is used to dissolve and dilute the enzyme (15). The exact specifications of these solutions were not listed in the literature from Labo Science.

The procedure for using the Analyzer involves pipetting 1.0 mL of buffer solution into the Analyzer cuvette along with a stirring bar and placing the cuvette into the optical unit of the Analyzer. Then 25 uL of a serum sample is pipetted into the cuvette and the total bilirubin is displayed. Next, 25 uL of the Analyzer enzyme solution is added to the cuvette and the Analyzer computes and displays the unbound bilirubin concentration. All samples are read at 30 °C. The ranges of the analyzer are: [total bilirubin], 0.3 - 30 mg%; [unbound

bilirubin], 0.1-2 ug%. At higher unbound bilirubin concentrations the reaction velocity will be rapid and the Analyzer is not capable of giving a reliable value. In this case, the enzyme solution is diluted with the diluent buffer. This will slow the reaction rate and give a reliable value which must then be multiplied by the dilution factor to give the true concentration of unbound bilirubin (15).

## OBSERVATIONS AND RESULTS

### Comparison of Manual and Automated Peroxidase Methods

Serum sample #1 was analyzed for unbound bilirubin by the manual and automated peroxidase method. The protocols used in the standardization of the rate constant were utilized in analyzing the serum by the manual method. Three experimental runs at two different peroxidase concentrations ([POD] = 45.6 and 67.7 ug/L) were carried out. The rate of reaction for each of these concentrations was between 2 and 10 minutes and the change in absorbance/minute at 460 nm was determined. The concentration of unbound bilirubin ([UB]) was then determined using the formula:  $[\text{unbound bil}] = (\Delta A_{460}/\text{min}) / ([\text{POD}] \cdot k)$ .

Labo Analyzer readings were done according to procedures described above. The enzyme had to be diluted 4.8 fold to give a reliable value for unbound bilirubin concentration.

The results of the comparison study were as follows:

	Manual Peroxidase Test	Labo Analyzer
[Unbound bil] umole/L	.355	.107
Standard Deviation (n=6)	.082	.009
Coefficient of Variation (CV)	23.1%	8.4%

It is obvious that the Analyzer results are far more accurate than the manual method. The undiluted Analyzer reagents used in this experiment gave accurate results (CV = 4.0%) when checked against a known bilirubin control solution provided by Labo Science.

This comparison study demonstrates the lack of precision involved in the manual peroxidase method. Because of this the Labo Analyzer was used in the experiments which examined bilirubin binding under different physiological conditions.

Note: A summary of the parameters and results of the samples used in the experimental trials performed on 7-20-89 is presented in the Appendix (Table 1).

Total Bilirubin Concentration ([TB]) with respect to Sodium Benzoate Concentration ([SB])

(Figures 3.1-3.3)

Figures 2.1; plots A, B, C, and D: 2.2; plot B: and 2.3; plots A, B, and D all showed approximately the same shape. There were marked inconsistencies in the remaining plots. From the plots that demonstrated relatively consistent shapes, however, some generalizations can be made. For each B/A ratio, an increase in hematocrit corresponded to a decrease in total bilirubin concentration, [TB]. The [TB] of each hematocrit at B/A = 1.5 (Figure 3.3) was much higher than the [TB] of the corresponding hematocrits at B/A = 0.5 (Figure 3.1). The [TB] of hematocrit = 20 at B/A = 1.0 (Figure 3.2, plot B) was higher than the [TB] of hematocrit = 20 at B/A = 0.5 (Figure 3.1, plot B), and slightly higher than the [TB] of hematocrit = 20 at B/A = 1.5 (Figure 3.3, plot B). Also, the [TB] in six of the plots remained relatively constant as the sodium benzoate concentration, [SB], increased from 50 to 100 mM. Additionally, at B/A = 0.5 (Figure 3.1), there was a substantial decrease in [TB] for

each hematocrit (plots A-D) as [SB] increased from 0 to 20 mM. The decrease became more pronounced at higher hematocrits. This trend was also observed at B/A = 1.0 (Figure 3.2) and B/A = 1.5 (Figure 3.3). Finally, two points on Figure 3.3, plot A corresponding to [SB] = 0 and 20 mM, were approximately 38.1 and 36.5 mg%, respectively. These values of [TB] exceeded the range specifications of the Labo Analyzer. However, they were proven to be fairly accurate with a manual caffeine test for [TB] (See below).

The Labo results generated apparent inconsistencies in Figure 3.2, plots A and C; and Figure 3.3, plot C. Because of this, a manual test for [TB] was performed on the samples corresponding to the graphs in question. The manual determination of [TB] was done utilizing a caffeine reagent which completely dissociates bilirubin from albumin. This method involved reading the absorbance of a sample containing 2.0 ml of caffeine reagent and 25 ul of serum sample. The effect of hemolysis on the absorbance at 460 nm (the absorbance maxima of bilirubin) was eliminated by employing the formula (11):

$$A_{460} - 0.8 \cdot A_{575}$$

575 nm is the absorbance maxima of heme (11).

By subtracting off a portion of the absorbance at 575 nm, the effect of hemolysis on bilirubin absorbance readings can be eliminated. The absorbance readings were then converted to bilirubin concentrations. The results used to generate the plot of B/A = 1.5, hematocrit = 0 (Figure 3.3, plot A) were also tested with the caffeine reagent. The [TB] results generated with the caffeine test showed close agreement with the [TB] generated by the Labo method for all the points composing the plots of B/A = 1.5 at hematocrit = 0 and 40

(Figure 3.3, plots A and C). However, the [TB] generated with the caffeine test for the points composing the plots of  $B/A = 1.0$  at hematocrit = 0 and 40 (Figure 3.3, plots A and C), differed markedly from the Labo results. The results of the caffeine test for the plots of  $B/A = 1.0$  at hematocrit = 0 and 40 (plots A.1 and C.1, respectively) are also shown on Figure 3.2. The  $B/A = 1.0$ , hematocrit = 0 plot of [TB] (Figure 3.2, plot A.1) based on the manual caffeine method had a general shape that resembled the shape of the majority of the graphs. However, the  $B/A = 1.0$ , hematocrit = 40 plot of [TB] (Figure 3.2, plot C.1) showed an unusual pattern which resembled that of the  $B/A = 1.5$ , hematocrit = 40 plot (Figure 3.3, plot C).

#### Unbound Bilirubin Concentration ([UB]) with respect to [SB].

(Figures 4.1-4.3)

The results showed conflicting data. However, some general observations could be made. As with the [TB], the general trend for [UB] was to decrease as hematocrit increased and an increase as the  $B/A$  ratio increased. The [UB] at all  $B/A$  ratios and hematocrits did not substantially increase or actually decreased as the [SB] increased from 50 to 100 mM [except for the plots of  $B/A = 0.5$ , hematocrit = 0 (Figure 4.1, plot A) and  $B/A = 1.5$ , hematocrit = 0 (Figure 4.3, plot A)].

For  $B/A = 0.5$ , hematocrit = 0 and 20 (Figure 4.1, plots A and B); and  $B/A = 1.5$ , hematocrit = 0 (Figure 4.3, plot A), an increase in [UB] of approximately 15, 16 and 30 ug%, respectively, occurred as the [SB] increased from 0 to 50 mM. Conversely, at  $B/A = 0.5$ , hematocrit = 60 (Figure 4.1, plot D; and  $B/A = 1.5$ ), hematocrit = 40

and 60 (Figure 4.3, plots C and D); an increase in [UB] ranging from 1 to 5 mg% occurred as the [SB] increased for 0 to 50 mM. For  $B/A = 1.0$ , hematocrit = 20 and 40 (Figure 4.2, plot B and C), an increase in [UB] of approximately 7 and 4 mg%, respectively, occurred as [SB] increased from 0 to 50 mM.

#### [UB] with respect to Hematocrit

(Figures 5.1-5.3)

Generally, for a given [SB] at hematocrit = 0, a higher B/A ratio resulted in a higher [UB]. Also, at a given B/A ratio and hematocrit = 0, higher [SB] resulted in higher [UB]. However, the point corresponding to hematocrit = 0 at  $B/A = 1.0$ , [SB] = 100 mM (Figure 5.2, plot D) does not support this observation. The results showed the [SB] plots of all three B/A ratio graphs at hematocrit = 60, gave a [UB] of 10 ug% or less. The trend for each [SB] plot was a substantial decrease in [UB] at each successive increase in hematocrit. However, a few points on the graphs did contradict this observation. Three graphic points corresponding to: hematocrit = 0 at  $B/A = 1.0$ , [SB] = 100 mM (Figure 5.2, plot D); and hematocrit = 20 at  $B/A = 1.5$ , [SB] = 20 and 100 mM (Figure 5.3, plots B and D); showed a [UB] that was low in comparison with the general trends of the other plots. Three of the plots:  $B/A = 0.5$ , [SB] = 50 mM (Figure 4.1, plot C);  $B/A = 1.0$ , [SB] = 0 and 20 mM (Figure 5.2, plots A and B); showed only a slight decrease in [UB] as the hematocrit increased from 0 to 20.

### [UB] with respect to B/A

(Figures 6.1-6.4)

The three points corresponding to  $B/A = 1.5$  at  $[SB] = 20$  mM, hematocrit = 20 (Figure 6.2, plot B);  $B/A = 1.5$  at  $[SB] = 100$  mM, hematocrit = 20 (Figure 6.4, plot B); and  $B/A = 1.0$  at  $[SB] = 100$  mM, hematocrit = 0 (Figure 6.4, plot A); all gave low [UB] readings that did not follow the general trend of the other respective plots of hematocrit. By neglecting these points, some general observations were made. First, for each separate graph (i.e. constant [SB]) decreasing hematocrits resulted in increased levels of [UB]. This effect was more pronounced at higher B/A ratios. Second, increasing [SB] gave increased [UB] at  $B/A = 1.5$  and hematocrit = 0. Third, as [SB] increased from 50 to 100 mM, little, if any, substantial increase in [UB] resulted at any B/A ratio when the hematocrit was greater than zero. Fourth, at B/A ratios = 1.0 or 1.5 and a constant hematocrit greater than zero, the addition of sodium benzoate at any concentration had little effect on [UB]. Finally, at  $B/A = 0.5$  and hematocrits of 0, 20, or 40; an increase in [SB] from 0 to 50 mM caused significant increases in [UB].

## DISCUSSION

The biggest problem encountered in carrying out these experiments was finding a reliable, accurate means of measuring unbound bilirubin concentrations. It took several weeks to develop a rate constant ( $k$ ) that could be utilized to determine unbound bilirubin concentrations with the manual peroxidase method. This method was time consuming and showed a substantial coefficient of variation (23%) for single sample analysis. The  $k$  finally determined differed greatly from the  $k$  reported by another investigator (Brian, unpublished data). Brian developed a  $k$  ranging from  $1.80 \times 10^{-4}$  to  $2.36 \times 10^{-4}$ . However, the  $k$  he reported was determined at high peroxidase concentrations, [POD], (i.e.  $>100 \mu\text{M}$ ). In the work presented an attempt was made to generate a rate constant that could be utilized over a lower range of [POD] because varying [POD] would have to be utilized for different samples so that the reaction rate would be such that 20% of the initial bilirubin had disappeared between two and ten minutes (8). In studying the rate constant of the peroxidase reaction, Jacobson has noted a coefficient of variation ranging from 2.3 to 19.2% for  $k$  determinations done within a 24 hour period. The reason for this was not clear but may be related to microaggregation in solutions (8).

In July, 1989, our lab received a Labo UB Analyzer UA-1. As stated previously, this clinical instrument is meant to analyze both [TB] and [UB]. The Labo was easy to use and greatly diminished the time involved in carrying out experimental runs. However, this

machine also had certain limitations because it is designed primarily for clinical use and not for research experimentation. For one thing, the operational ranges of the Labo may not always be sufficient for use in an experimental setting. For example, in Figure 3.3, plot A the two points corresponding to  $[SB] = 0$  and 20 mM show  $[TB]$  readings that exceed the Labo Analyzer range. Also, we were one of the first labs to use the Labo for purely experimental purposes. Additionally, due to our sample preparation protocols, the  $[UB]$  was such that the reaction rate was too rapid using the pure Labo enzyme solution provided. As a result the enzyme had to be diluted up to 25 fold in order to get stable  $[UB]$  readings. The Labo representative stated that the Labo should function accurately at these dilutions. However, a 4.8 fold dilution of the enzyme solution resulted in an increase of the coefficient of variation of  $[UB]$  from 4.0 to 8.4%. The variability of  $[UB]$  at higher dilutions was not tested. Also, diluting the serum resulted in false  $[UB]$  readings. A 40% dilution of a serum sample gave a  $[UB]$  that was 20% higher than the  $[UB]$  at no dilution. In an attempt to minimize this factor, only a 10% dilution consisting of a combination of sodium benzoate and PBS was used in preparing samples. This dilution was necessary in order to study the effect of different sodium benzoate concentrations on bilirubin/albumin binding.

## CONCLUSIONS

When the manual and Labo methods were used to analyze the same serum sample for [UB], the results differed greatly. The manual method gave a [UB] = 0.355 uM. This result was 3.3 times greater than the [UB] reading generated by the Labo ([UB] = 0.107 uM). Also, the manual readings showed a coefficient of variation (23.1%) that was much greater than the coefficient of variation of the Labo readings (8.4%). The Labo enzymes used in the experimental runs gave results that were in agreement with the Labo bilirubin control solution. Given these facts, the Labo was chosen to analyze the serum samples with varying B/A ratios, hematocrits, and sodium benzoate concentrations.

The single run per sample results generated with the Labo were inconsistent at times, as shown by the caffeine test for total bilirubin at B/A = 1.0 (see Figure 3.2). Also, there was evidence of some sample preparation error, or perhaps sampling errors in which samples became switched. An example of this was also shown by the caffeine test which confirmed the scattered points associated with the B/A = 1.0, hematocrit = 40 plot (Figure 3.1, plot C). Also, it was shown that the Labo [UB] values showed a coefficient of variation of at least 8.4%. Because of this, it did not seem prudent to report quantitative specifics of the results. However, several consistent trends were observed in the data. Therefore, the results were interpreted by looking at the plots that showed the most consistent shapes. From these interpretations, some qualitative generalizations

were presented. From the data generated, three areas merit discussion. First, red blood cell binding of unbound bilirubin was apparent in each of the sets of graphs presented (Figures 3, 4, 5, and 6). The data showed increases in hematocrit significantly lowered both total and unbound bilirubin levels. The binding of bilirubin to erythrocytes is increased markedly if there is a reduction in the capacity of albumin to bind bilirubin and can be greatly affected by the presence of any one of several antibiotics, other drugs, and hormones, (9,16). The addition of sodium benzoate in the presence of RBCs results in bilirubin leaving albumin and binding to RBCs. Also, Dourson has reported that the amount of RBC binding increases with both sodium benzoate concentration and hematocrit (4). For a given B/A ratio, a larger percentage of RBCs in sample solutions resulted in the uptake of greater quantities of unbound bilirubin. The decrease in [TB] at higher hematocrits (for a given B/A ratio) occurred because as the number of RBCs increased more unbound bilirubin could be taken up. This increase was accompanied by additional bilirubin-albumin unbinding to reestablish the equilibrium:



Figures 5.1-5.3 demonstrate the capacity of RBCs to take up bilirubin. For this set of data, the [UB] was less than 10 ug% at all points corresponding to a hematocrit of 60. For the plot of B/A =1.5 and [SB] = 50 mM (Figure 5.3, plot C), there was 60 ug% decrease in [UB] as the hematocrit increased from 0 to 60.

Second, sodium benzoate was shown to be an effective solutizer at

low B/A ratios and low to moderate hematocrit levels. The ability of sodium benzoate to unbind bilirubin from albumin has been demonstrated (3). For example, the plots of B/A = 1.5, hematocrit = 0, 20, and 40 ( Figure 4.1, plots A, B, and C) showed marked increases in [UB] as [SB] increased from 0 to 50 mM. However, it is possible that benzoate interferes with the readings of the Analyzer. The plot of B/A = 0.5, hematocrit = 0 (Figure 3.1, plot A) showed a significant drop in [TB] as [SB] increased from 0 to 20 mM. These points should show approximately the same [TB] because there were no RBCs present in the sample solutions to take up bilirubin. The drop in [TB] with the addition of sodium benzoate in the presence of RBCs would facilitate bilirubin-albumin unbinding, thereby making more unbound bilirubin available for uptake by RBCs.

In the presence of RBCs, it appeared that increasing [SB] from 50 to 100 mM did not significantly increase [UB] levels (Figures 3.1, 3.3, 4.1, 4.2, and 6.3 versus 6.4). In fact, for Figures 4.1 and 4.2, the curves corresponding to hematocrit = 20 and 40 (plots B and D, respectively) showed a decrease in [UB] as [SB] increased from 50 to 100 mM. Watson stated the presence of drugs greatly affects the capacity of RBCs to take up bilirubin (16). Perhaps [SB] in the 100 mM range facilitates an increase in RBC uptake ability.

Third, as the B/A ratio approached and exceeded 1.0, sodium benzoate in any concentration was not effective in increasing [UB] levels if the hematocrit was greater than zero. For example, in Figures 6.1-6.4, the points corresponding to hematocrit = 40 (plot B) at B/A = 1.0 show a narrow range of [UB] (7.6-11.6 ug%) as [SB]

increased from 0 to 100 mM.

The data presented on bilirubin-albumin binding demonstrated that different bilirubin/albumin molar ratios, hematocrits, and sodium benzoate concentrations all affect serum levels of total and unbound bilirubin. Preliminary experiments such as this are necessary when trying to develop new clinical intervention techniques. These results showed approximately how much unbound bilirubin can be expected under different conditions. This information can be used to evaluate the efficacy of the hemoperfusion column in experimental animal trials. Additionally, this type of information is necessary to aid in the development of protocols regarding column use.

## APPENDIX

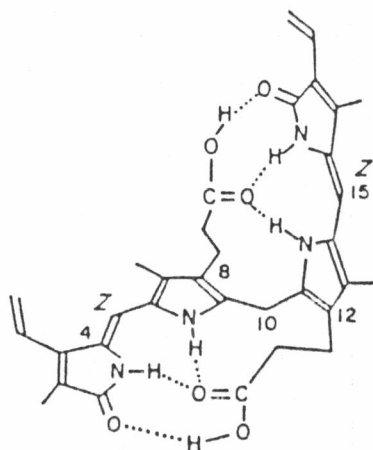
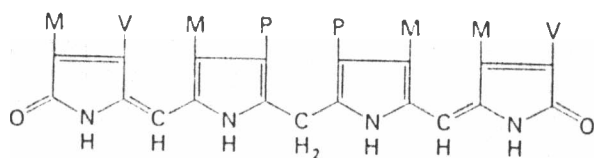


FIGURE 1. The bilirubin molecule: top, two dimensional diagram; middle, structural diagram of the preferred conformation; bottom bent paper clip analog (11,13).

Table 1

DETERMINATION OF VARIABILITY IN BILIRUBIN-  
ALBUMIN BINDING AT DIFFERENT B/A RATIOS,  
HEMATOCRITS, AND [SB] USING THE LABO  
ANALIZER.

B/A	Hct (%)	[S.B.] (mM)	[TB] (mg%)	[UB] raw (ug%)	ENZYME DILUTION	[UB] corrected (ug%)
0.0	00	000	00.1	0.00	01	00.00
0.0	00	020	00.1	0.00	01	00.00
0.0	00	050	00.1	0.00	01	00.00
0.0	00	100	00.1	0.00	01	00.00
0.0	20	000	00.3	0.00	01	00.00
0.0	20	020	00.3	0.00	01	00.00
0.0	20	050	00.3	0.00	01	00.00
0.0	20	100	00.3	0.00	01	00.00
0.0	40	000	00.2	0.00	01	00.00
0.0	40	020	00.2	0.00	01	00.00
0.0	40	050	00.2	0.00	01	00.00
0.0	40	100	00.2	0.00	01	00.00
0.0	60	000	00.3	0.00	01	00.00
0.0	60	020	00.2	0.00	01	00.00
0.0	60	050	00.2	0.00	01	00.00
0.0	60	100	00.2	0.00	01	00.00
0.5	00	000	14.9	1.32	06	07.92
0.5	00	020	13.5	1.37	14	19.18
0.5	00	050	13.2	1.65	14	23.10
0.5	00	100	12.5	1.55	20	31.00
0.5	20	000	13.1	1.33	04	05.32
0.5	20	020	10.9	1.71	06	10.26
0.5	20	050	09.5	1.54	14	21.56
0.5	20	100	06.6	0.84	20	16.80
0.5	40	000	10.2	1.08	04	04.32
0.5	40	020	05.3	0.26	06	01.56
0.5	40	050	04.8	0.53	20	10.60
0.5	40	100	04.6	0.47	20	09.40
0.5	60	000	06.2	0.43	04	01.72
0.5	60	020	02.8	0.13	14	01.82
0.5	60	050	02.2	0.15	20	03.00
0.5	60	100	02.1	0.27	20	05.40
1.0	00	000	28.0	1.57	14	21.98
1.0	00	020	12.3	1.36	20	27.20
1.0	00	050	24.9	1.99	20	39.80
1.0	00	100	27.0	0.22	20	04.40
1.0	20	000	24.3	1.57	14	21.98
1.0	20	020	19.1	1.32	20	26.40

B/A	Hct (%)	[SB] (mM)	[TB] (mg%)	[UB] raw (ug%)	Enzyme Dilution	[UB] corrected (ug%)
1.0	20	050	17.2	1.44	20	28.80
1.0	20	100	15.8	1.22	20	24.40
1.0	40	000	13.0	0.54	14	07.56
1.0	40	020	17.2	0.44	20	08.80
1.0	40	050	09.8	0.58	20	11.60
1.0	40	100	16.8	0.43	20	08.60
1.0	60	000	10.4	0.15	20	03.00
1.0	60	020	11.1	0.08	20	01.60
1.5	00	000	38.0	2.59	20	51.80
1.5	00	020	36.4	3.28	20	65.60
1.5	00	050	29.0	3.43	20	68.60
1.5	00	100	28.1	3.24	25	81.00
1.5	20	000	22.5	2.05	14	28.70
1.5	20	020	16.8	0.14	20	02.80
1.5	20	050	14.9	1.35	20	27.00
1.5	20	100	14.7	0.15	20	03.00
1.5	40	000	20.4	1.25	14	17.50
1.5	40	020	13.5	0.88	20	17.60
1.5	40	050	24.1	0.83	20	16.60
1.5	40	100	11.3	0.73	25	18.25
1.5	60	000	13.0	0.42	14	05.88
1.5	60	020	06.4	0.37	20	07.40
1.5	60	050	05.5	0.39	20	07.80
1.5	60	100	05.2	0.49	20	09.80

Figure 2

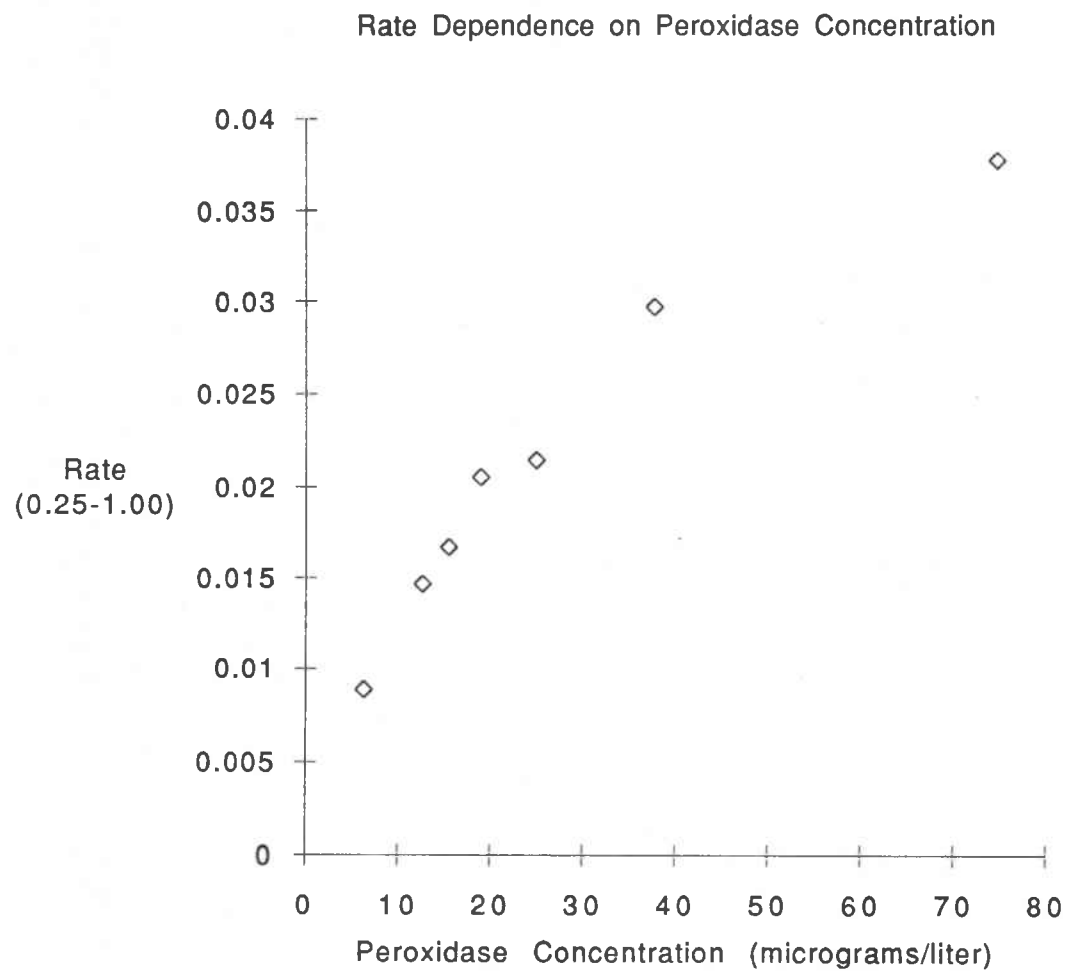


Figure 3.1

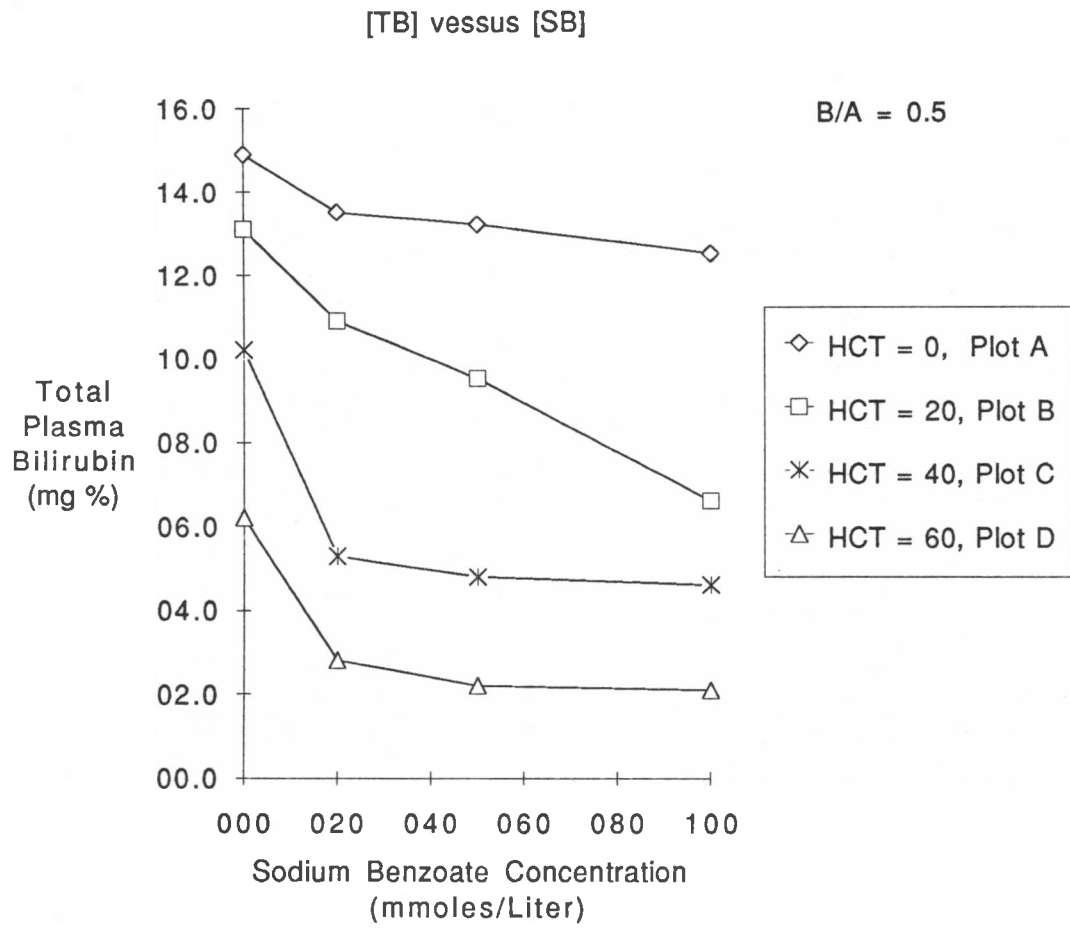


Figure 3.2

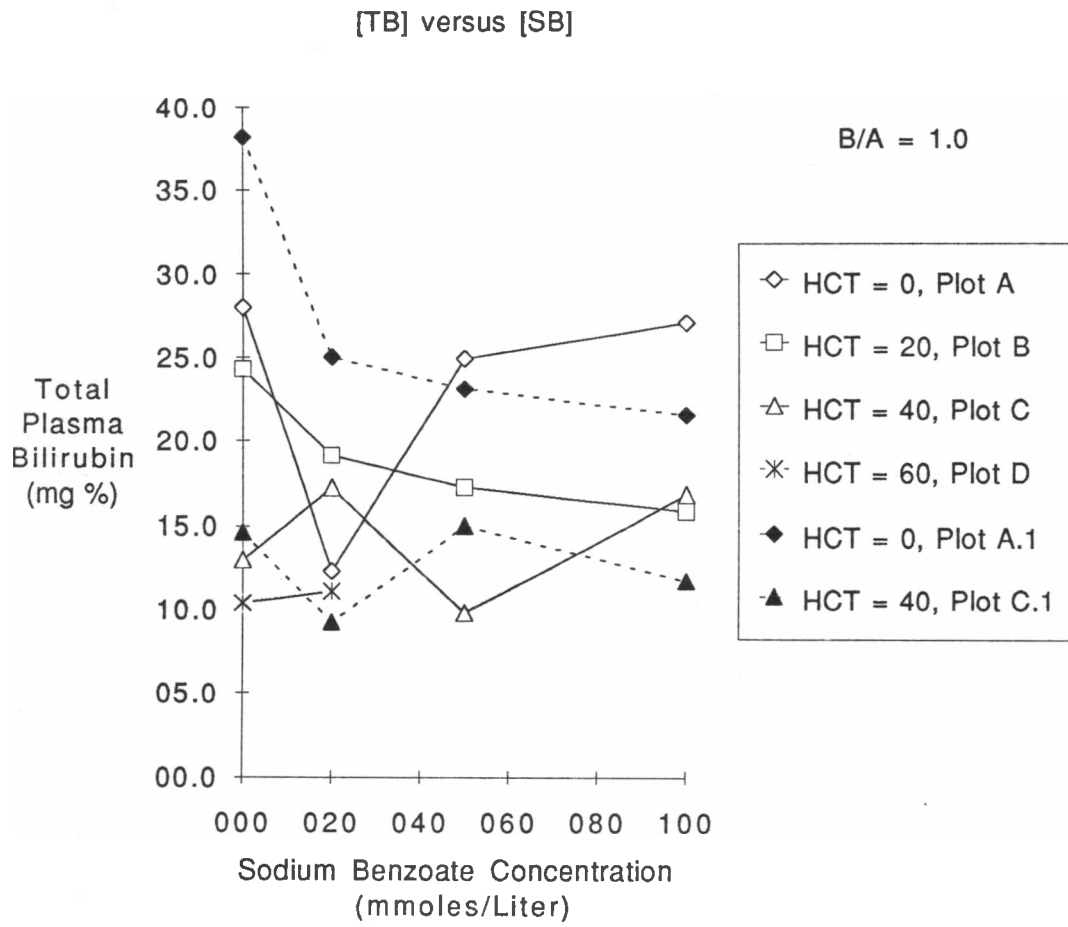


Figure 3.3

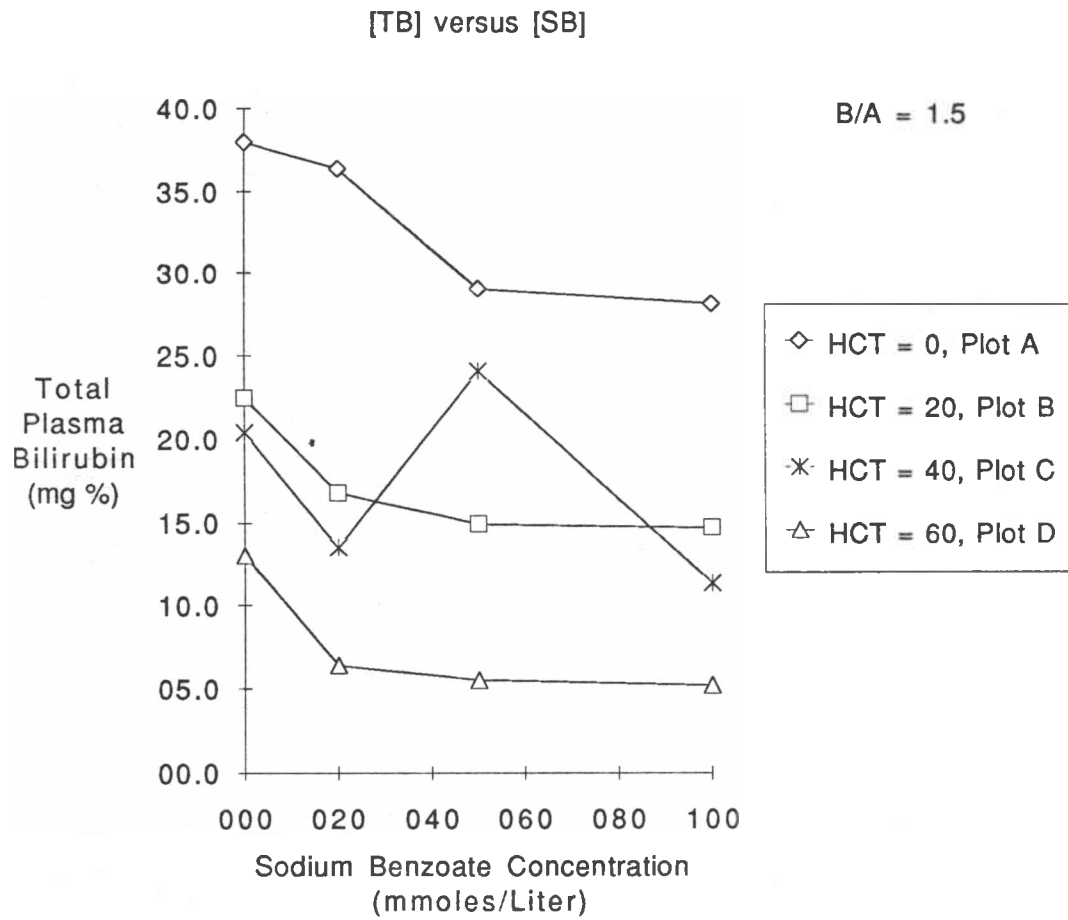


Figure 4.1

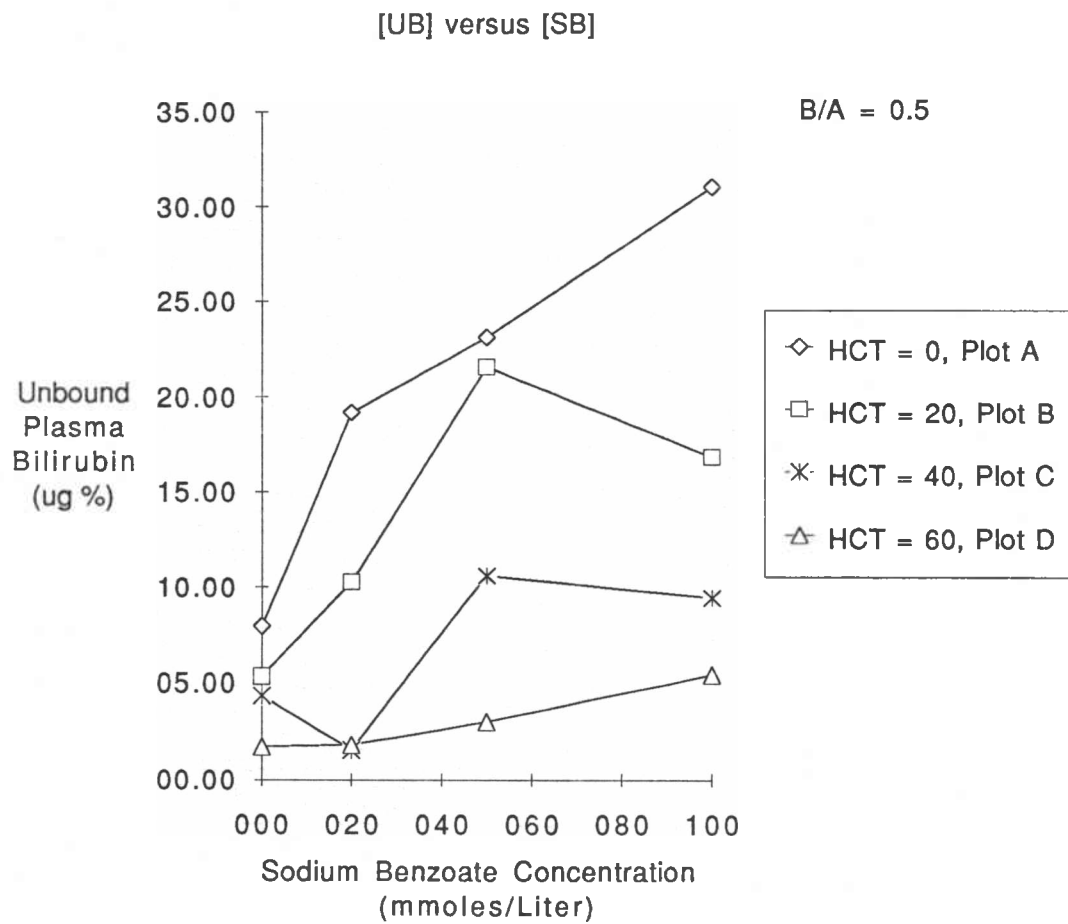


Figure 4.2

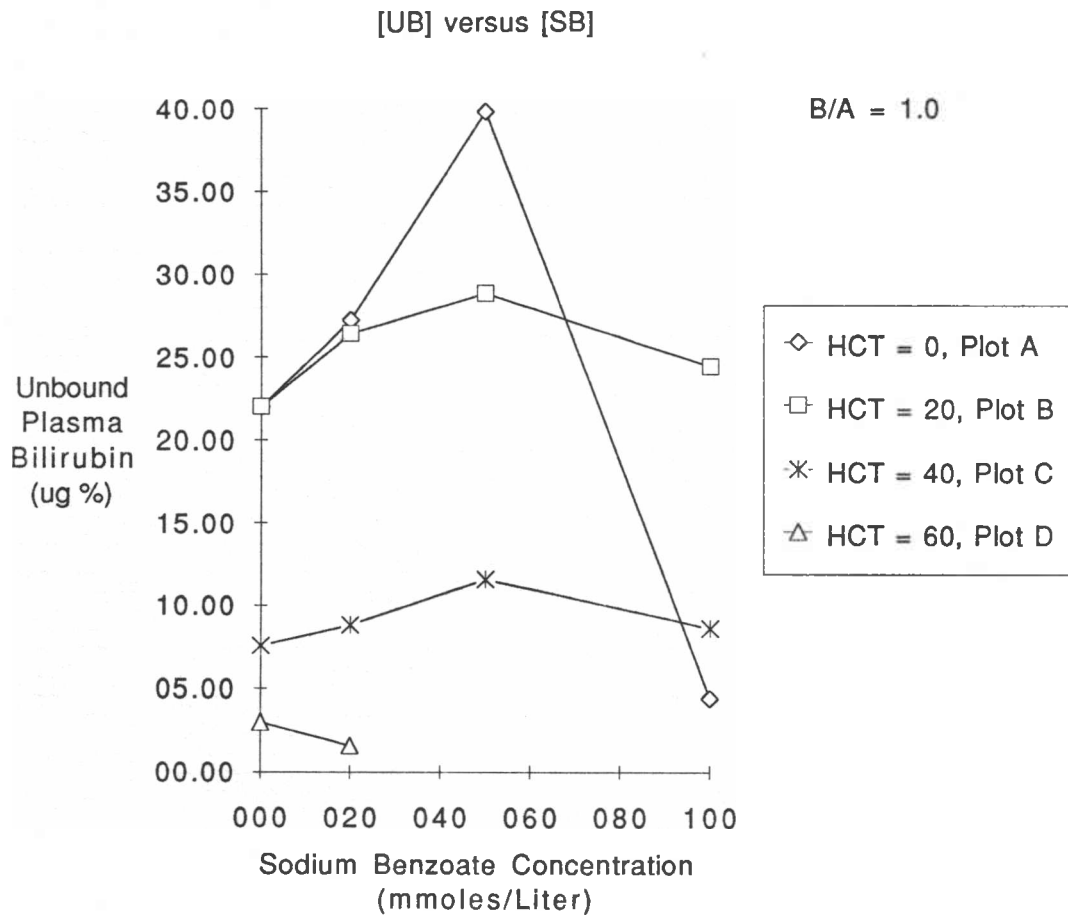


Figure 4.3

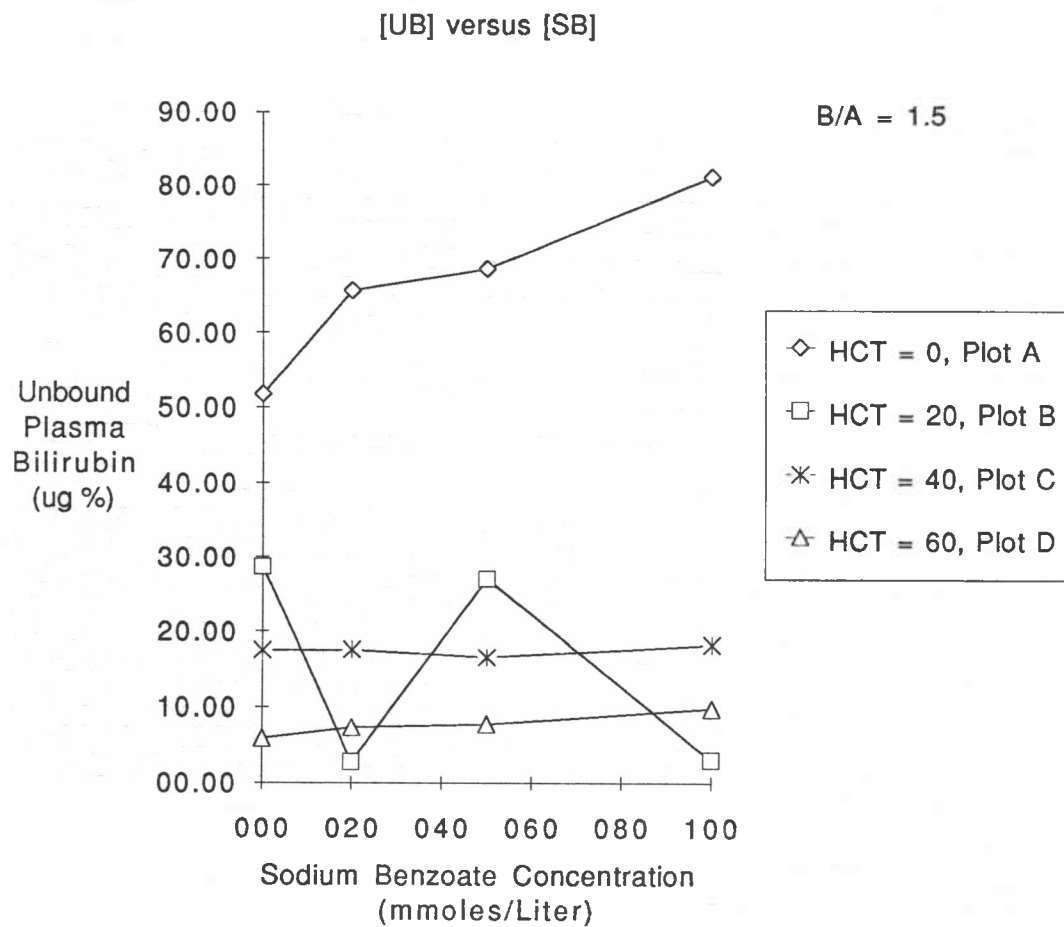


Figure 5.1

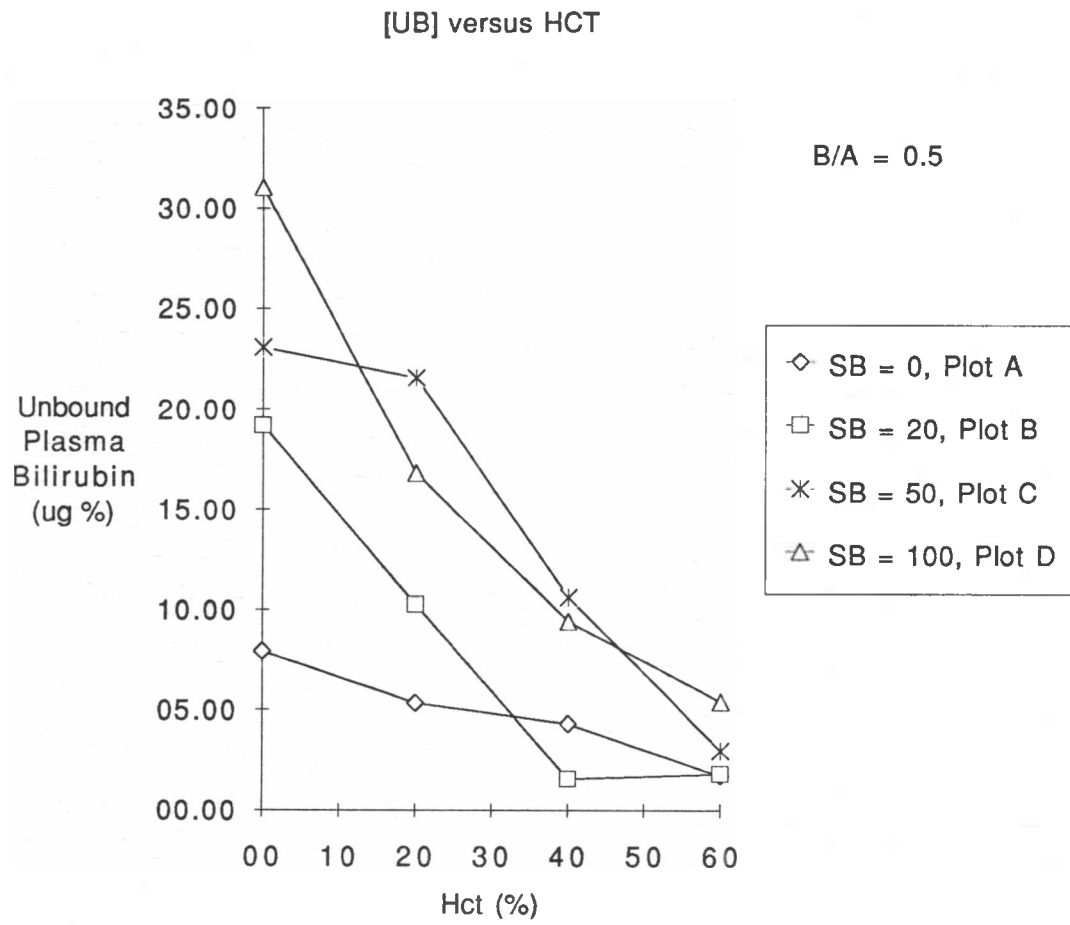


Figure 5.2

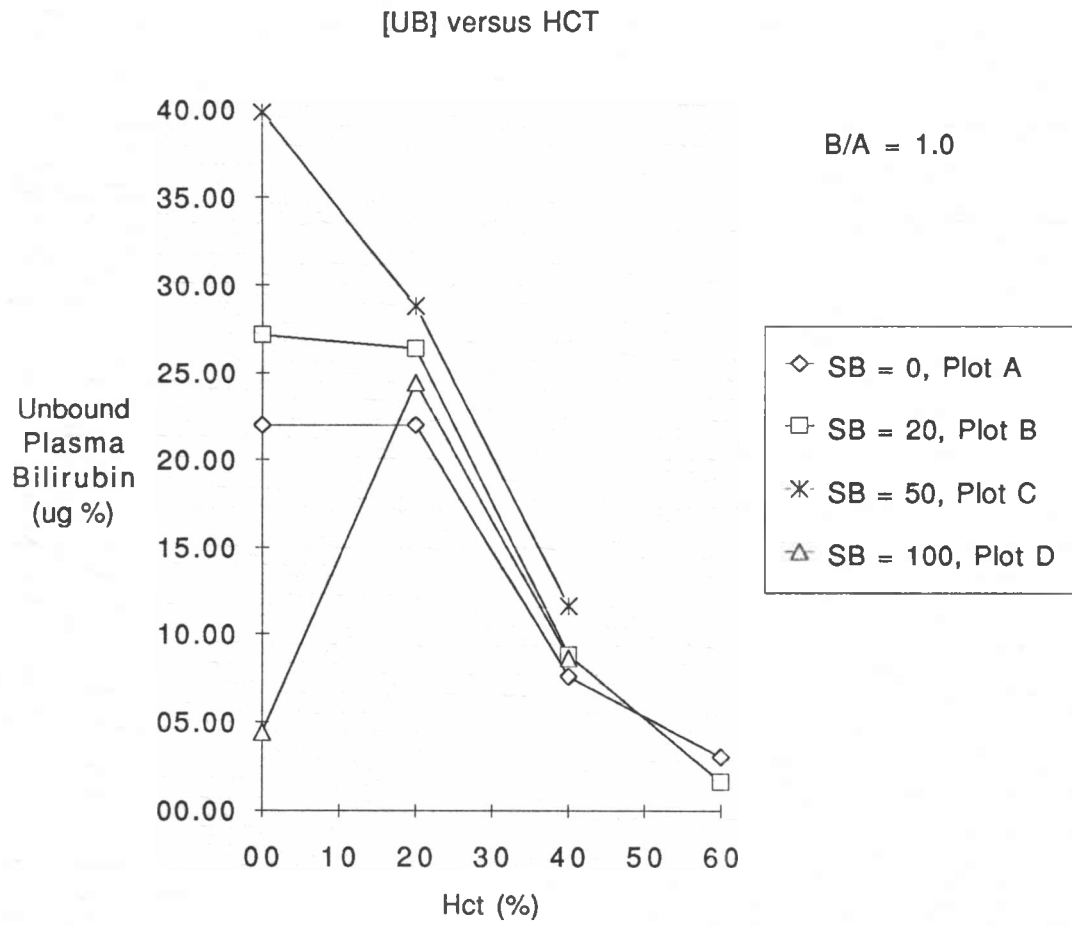


Figure 5.3

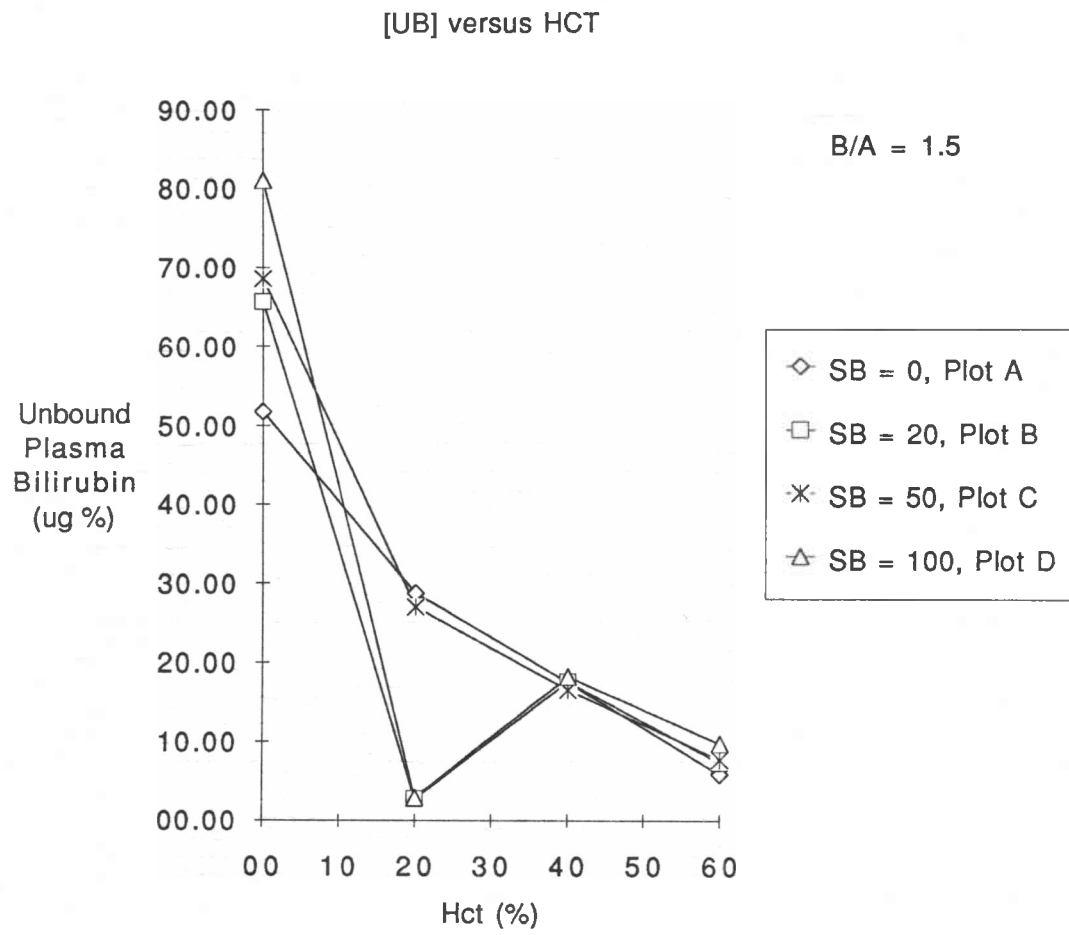


Figure 6.1

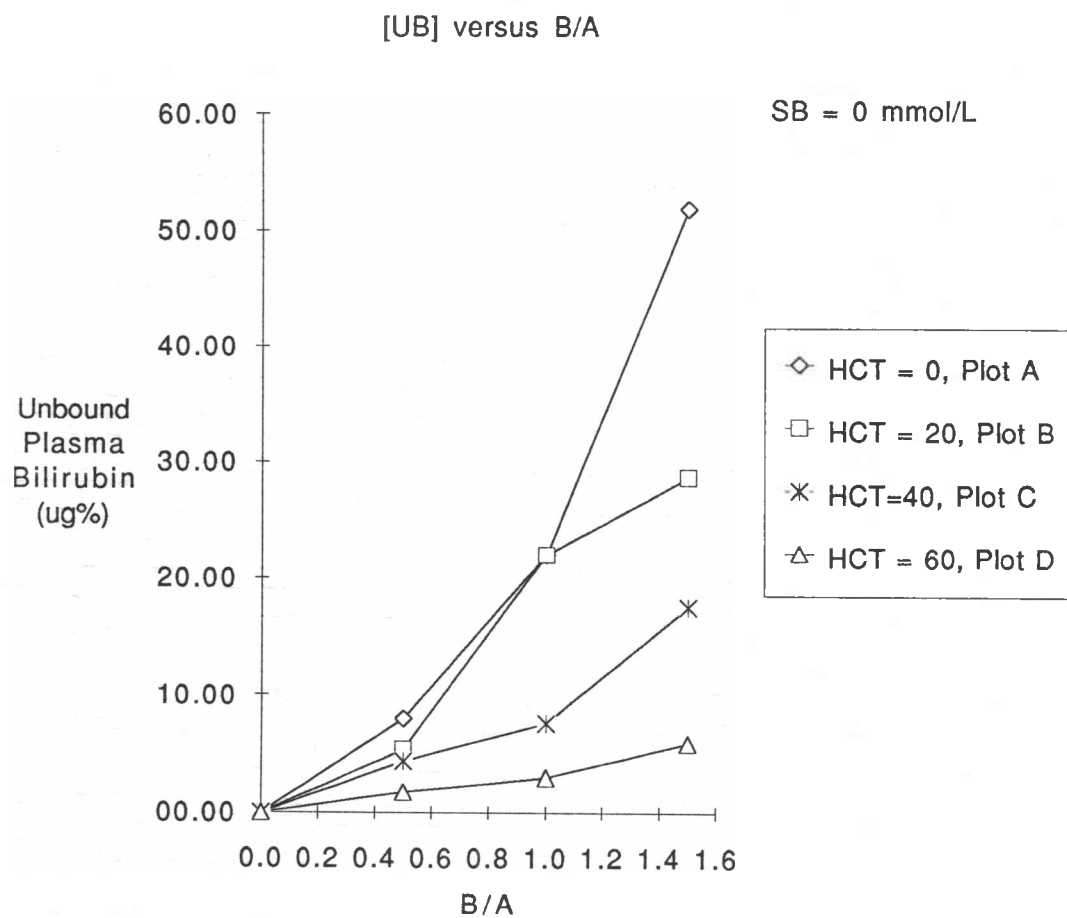


Figure 6.2

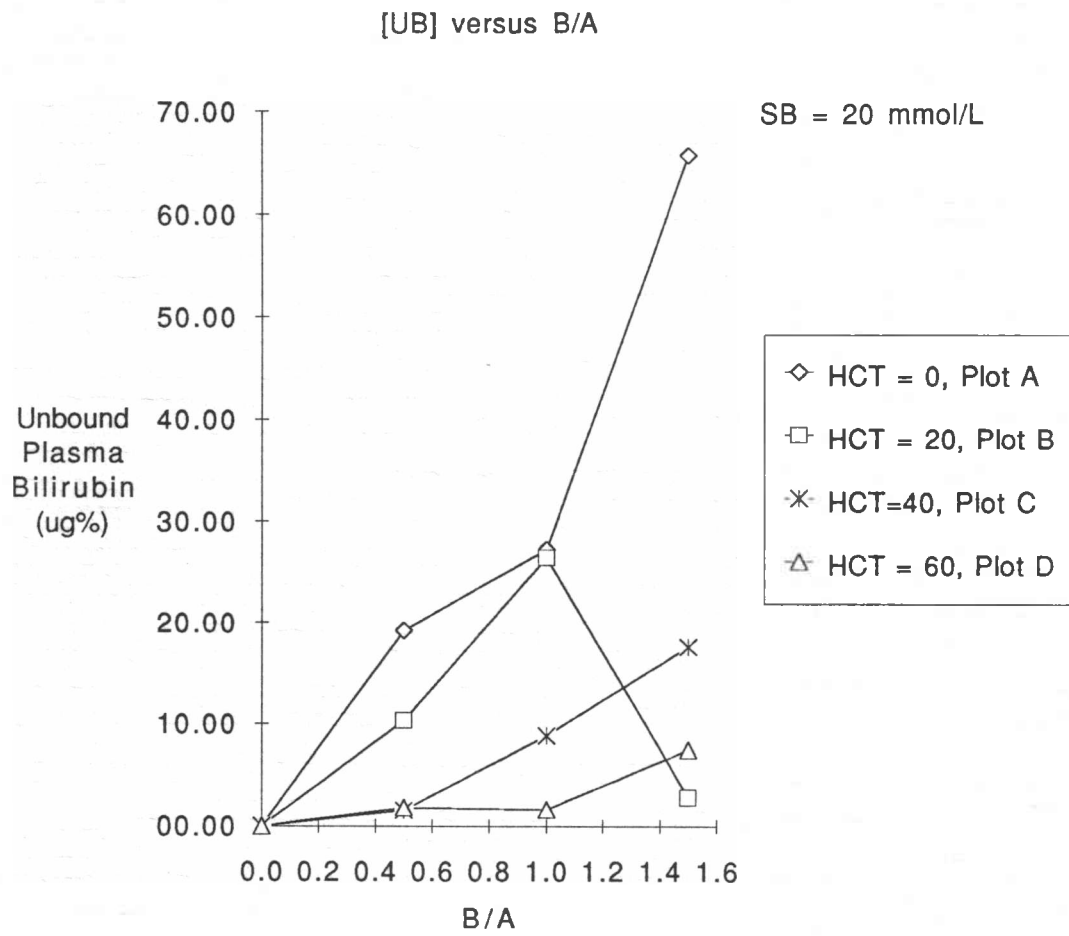


Figure 6.3

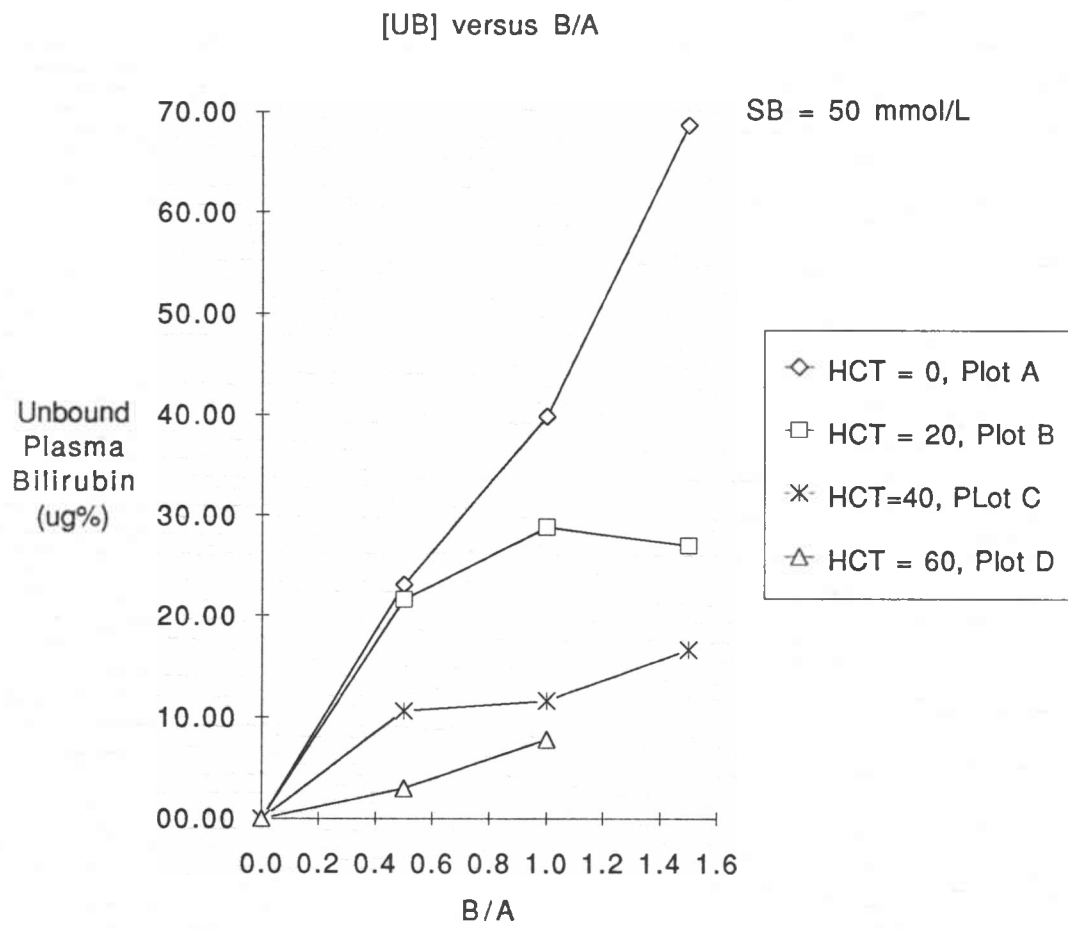
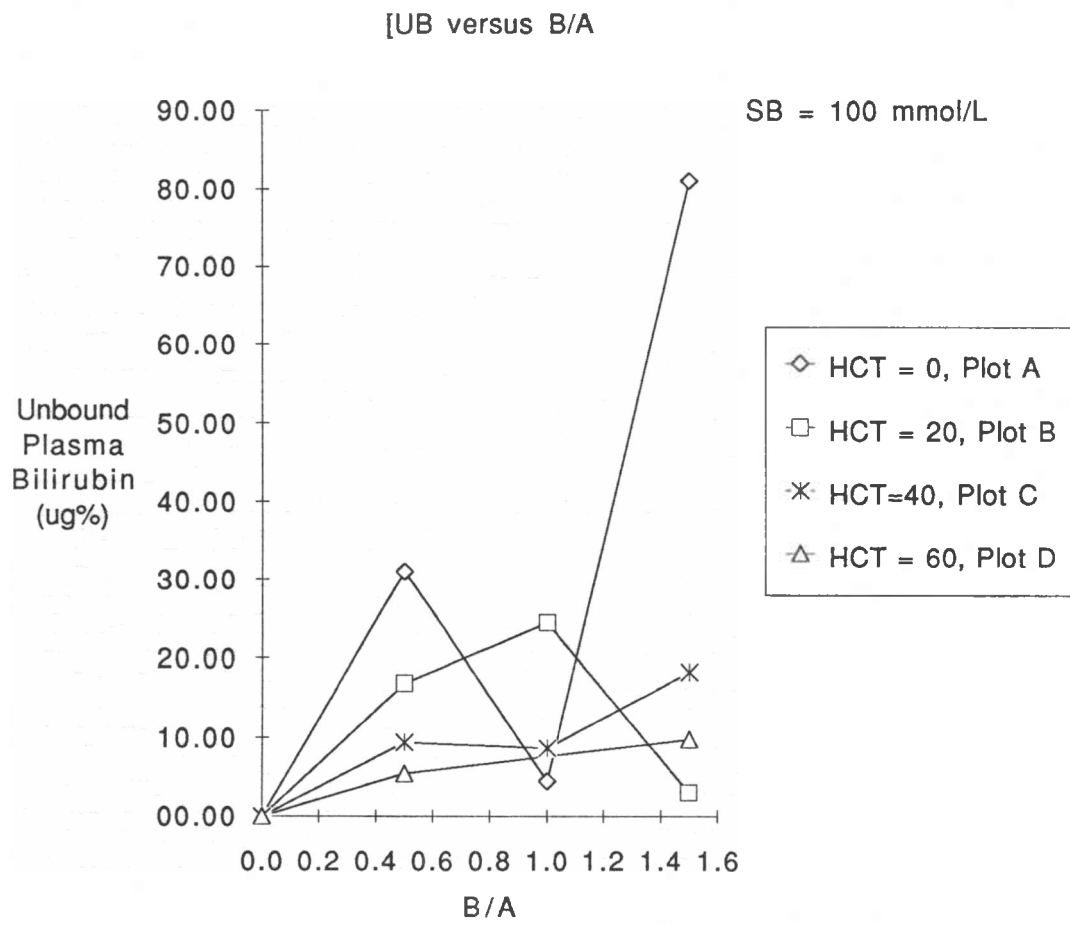


Figure 6.4



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