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TITLE STUDIES OF THE SEPARATION AND PURIFICATION OF BILIRUBIN CONJUGATES FROM HUMAN BILE

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ABSTRACT

STUDIES OF THE SEPARATION AND PURIFICATION OF BILIRUBIN CONJUGATES FROM HUMAN BILE

Frank J. Schenck

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The present work is concerned with the separation and purification of bilirubin monoglucuronide and diglucuronide from human bile, in a pure form so that they can be used as reference standards for the various clinical determinations of bilirubin conjugates in serum. The first step was to partially remove the lipid components of bile (ie. cholesterol and bile salts) by chromatography on a column packed with Amberlite XAD-2 resin, followed by ultrafiltration using a 500 molecular weight cut-off membrane filter. Then further separation was achieved on glass columns packed with silica gel or silicic acid. The behavior of bilirubin and its conjugates on Eastman Chromagram silica gel G thin layer chromatography sheets was studied at various stages of purification.

The Amberlite XAD-2 removed from 23 to 48% of the bile salts present in samples of centrifuged bile. It also removed 10% of the conjugated bilirubin.

Diafiltration was carried out at 45-65 psi., and approximately 250 ml. of diafiltrate was collected over a 24 hour period. Diafiltration carried out over 4 to 8 day periods resulted in losses of 50% of the total bilirubin and 75% of the direct reacting bilirubin present.

When the bile was subjected to ultrafiltration, concentrating it to one fifth of its original volume, 71% of the cholesterol and 73% of the bile salts were passed out into the ultrafiltrate. During the ultrafiltration 18% of the total and 36% of the direct reacting bilirubin were hydrolyzed or oxidized, even though the process was carried out under red light and a nitrogen atmosphere. The five fold concentration gave a bile sample containing 96 mg.% direct reacting and 114 mg.% total bilirubin.

The removal of the bile salts also had an effect on the thin layer chromatography of bilirubin and its conjugates. As the bile salt concentration was lowered the two conjugated pigments migrated considerably slower than they did in corresponding samples of centrifuged bile.

By eluting the partially purified bile in a column packed with silica gel or silicic acid, using a solvent system of acetone, butanol, propionic acid and water (7:4:3:1), it was possible to attain a complete separation of the two conjugates from free bilirubin. With slower flow rates the silica gel or silicic acid columns produced a 75% reduction in bile salts and 25% reduction in cholesterol in the eluates as compared to the bile introduced onto the column. At faster flow rates the cholesterol and bilirubin conjugates were eluted at the same time.

The product was a solution of approximately 2.0 mg.% bilirubin conjugates in the acetone, butanol, propionic acid and water mixture. The solution also contained about 10 mg.% cholesterol and approximately 0.0012 g. per 100 ml. bile salt. When the solution was stored in the dark at -15°C., there was no loss in direct reacting bilirubin, even after three weeks.

Attempts to evaporate the solvent and dissolve the solid residue in phosphate buffer or deionized water resulted in hydrolysis of the glucuronic acid from the bilirubin or oxidation to biliverdin.

In the mixed solvent system the bilirubin conjugates had an $A_{max.}$ at 420 nm., and in phosphate buffer the A_{max} was at 410 nm. The Malloy-Evelyn bilirubin assay on pure conjugated bilirubin gave a higher value for the direct reacting than for the total bilirubin.

Recycling of the column eluates a second time through a silicic acid column did not result in separation of the monoglucuronide from the diglucuronide.

ACKNOWLEDGEMENTS

I would like to dedicate this thesis to my research adviser, Dr. Leonard B. Spiegel, without whose constant guidance and help this research would have not been possible.

I would also like to extend my appreciation to Dr. John Van Norman and Dr. Marvin Lukin, for reading the manuscripts and for their many helpful suggestions.

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Finally, I would like to extend my thanks to my wife for her help and encouragement.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
r.p.m.	Revolutions per minute
g's	Relative Centrifugal Force
mg.%	Milligrams Solute per 100 ml. Solution
R_f	$\frac{\text{Migration Distance of Compound}}{\text{Migration Distance of Solvent Front}}$
T.L.C.	Thin Layer Chromatography
$^{o}C.$	Degrees Centigrade
ACD	Anticoagulant Citrate Dextrose
CPD	Citrate Phosphate Dextrose
psi.	Pounds per Square Inch
nm.	Nanometers (10^{-9} m.)
$\lambda_{max.}$	Wavelength in nm. of Maximum Absorbance
$\varepsilon_{max.}$	Molar Absorptivity at Wavelength of Maximum Absorbance
$A_{max.}$	Maximum Absorbance

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CHAPTER I

INTRODUCTION

A. General

Bilirubin is an orange, lipid soluble, cytotoxic compound belonging to a class of compounds found in the human body called bile pigments. It is produced in the reticuloendothelial system of the liver, spleen and bone marrow. It is a product of the degradation of hemoglobin, released during the destruction of senescent red blood cells. Red blodd cells have an average life of 110 to 130 days and the hemoglobin cannot be reused. Being insoluble in blood at physiologic pH, free bilirubin can only be transported to the liver from extra-hepatic sources by binding to serum albumin.[1,2,3] (Figure 1)

In the liver bilirubin reacts with uridine diphosphate glucuronate, utilizing the enzyme uridine diphosphate glucuronyl transferase, to form the water soluble compounds bilirubin monoglucuronide and bilirubin diglucuronide. The glucuronides are excreted into the bile canaliculi and then into the bile. They are then excreted through the bile duct into the small intestine. Being lipid insoluble the glucuronides are unable to diffuse across cell membranes of the intestinal mucosa back into the blood. In the large intestine the bilirubin is converted into urobilin through bacterial action. [3,4,5] (Figure 1)

Jaundice or icterus, is a condition characterized by yellow discoloration of the skin, mucuous membranes, and numerous organs. It is caused by the retention of bile pigments in the blood, and it is

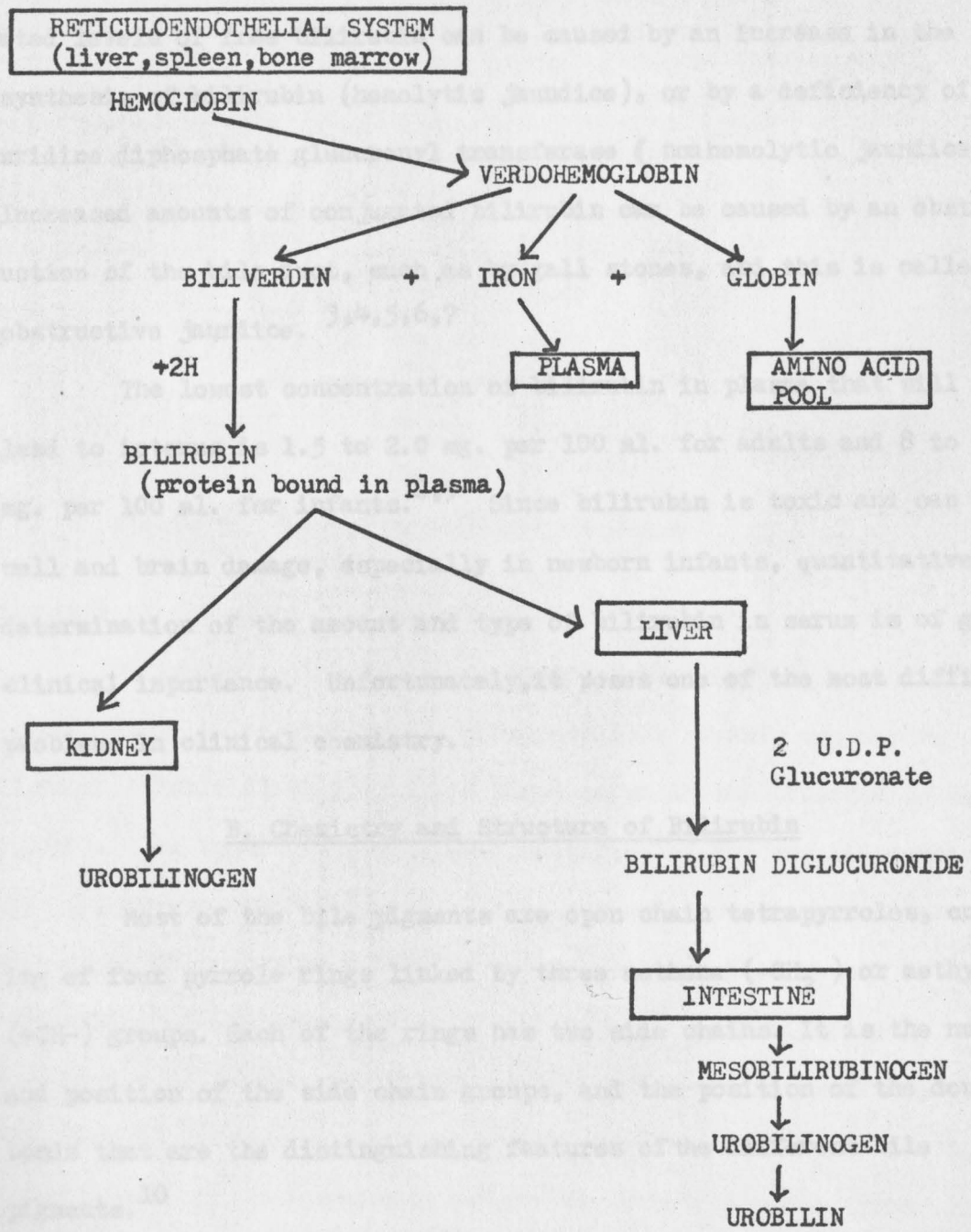


Fig. 1. Hemoglobin Degradation and Formation of the Bile Pigments[4]

always accompanied by elevated levels of bilirubin in the serum. Elevated levels of free bilirubin can be caused by an increase in the synthesis of bilirubin (hemolytic jaundice), or by a deficiency of uridine diphosphate glucuronyl transferase (nonhemolytic jaundice). Increased amounts of conjugated bilirubin can be caused by an obstruction of the bile duct, such as by gall stones, and this is called obstructive jaundice. [3,4,5,6,7]

The lowest concentration of bilirubin in plasma that will lead to icterus is 1.5 to 2.0 mg. per 100 ml. for adults and 8 to 9 mg. per 100 ml. for infants.[8,9] Since bilirubin is toxic and can cause cell and brain damage, especially in newborn infants, quantitative determination of the amount and type of bilirubin in serum is of great clinical importance. Unfortunately,it poses one of the most difficult problems in clinical chemistry.

B. Chemistry and Structure of Bilirubin

Most of the bile pigments are open chain tetrapyrroles, consisting of four pyrrole rings linked by three methene ($-CH_2-$) or methyne ($=CH-$) groups. Each of the rings has two side chains. It is the number and position of the side chain groups, and the position of the double bonds that are the distinguishing features of the different bile pigments.[10]

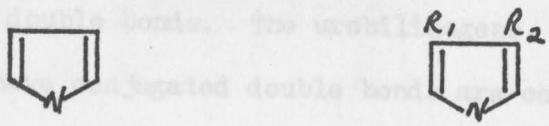


Fig. 2. Pyrrole Ring and Pyrrole Side Chains

The bile pigments are derivatives of the porphyrins, specifically protoporphyrin IX. (See fig. 4). They are formed by oxidative

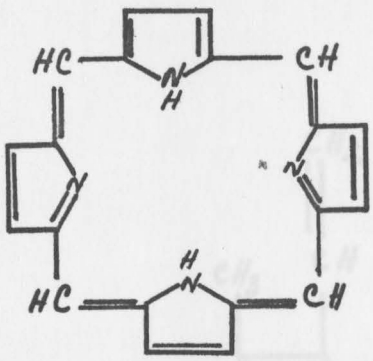


Figure 3. Porphyrin Skeleton Structure

cleavage of the alpha methyne bridge.[11]

Bilirubin is insoluble in water at physiologic pH, but it is quite soluble in chloroform and other nonpolar organic compounds. It is water soluble at pH 11.3 or in blood serum in the presence of serum albumin.[12] Bilirubin is a weak acid by virtue of the two side chain carboxyl groups. (See figure 6) It is commonly accepted that the insolubility of bilirubin in neutral aqueous solutions is a result of strong intramolecular hydrogen bonding of the type shown in figure 9. This has given rise to the possibility of two tautomeric forms of bilirubin. (See fig. 9) Arguments favoring both tautomeric forms have been presented, based on optical, infrared and nuclear magnetic resonance spectroscopy.[12,13,14]

The red color of the bile pigments is produced by conjugated carbon-carbon double bonds. The urobilinogens, derivatives of bilirubin which do not have conjugated double bonds are colorless. When they are oxidized to urobilin, the central bridge becomes unsaturated and

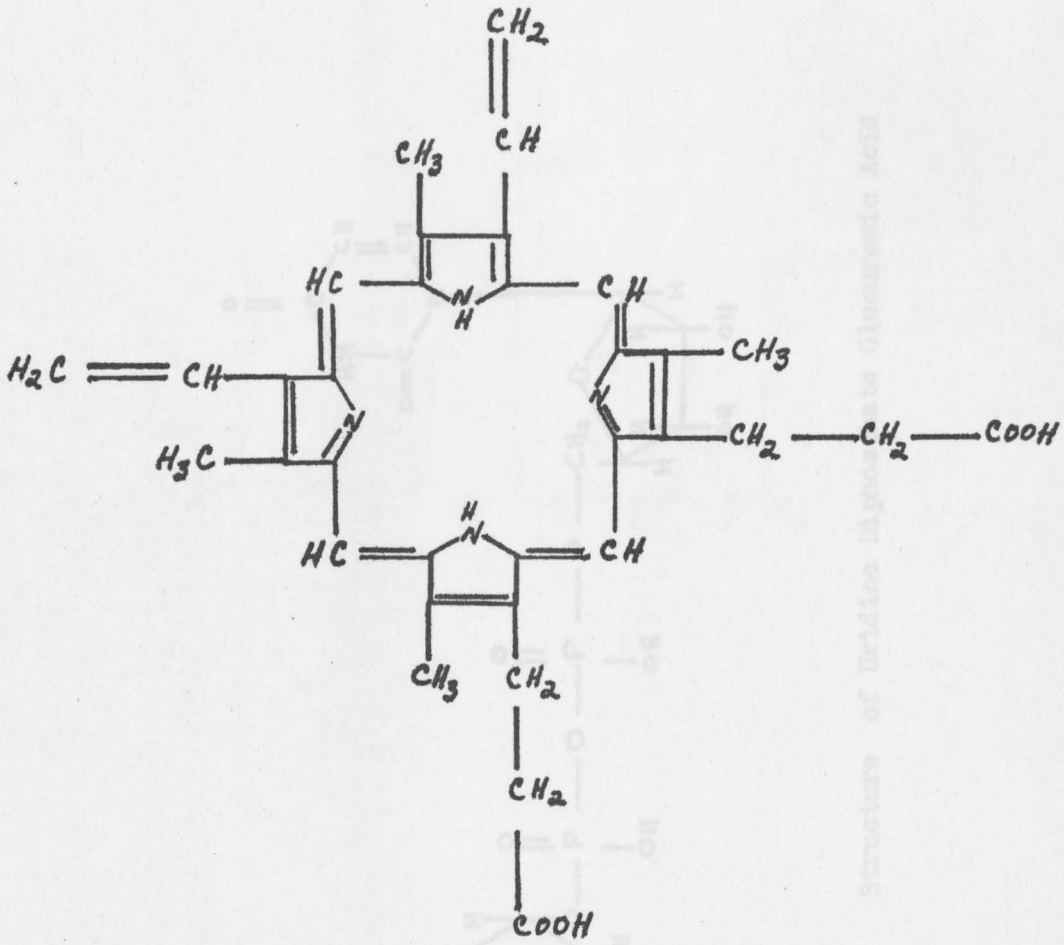


Figure 4. Structure of Protoporphyrin IX.

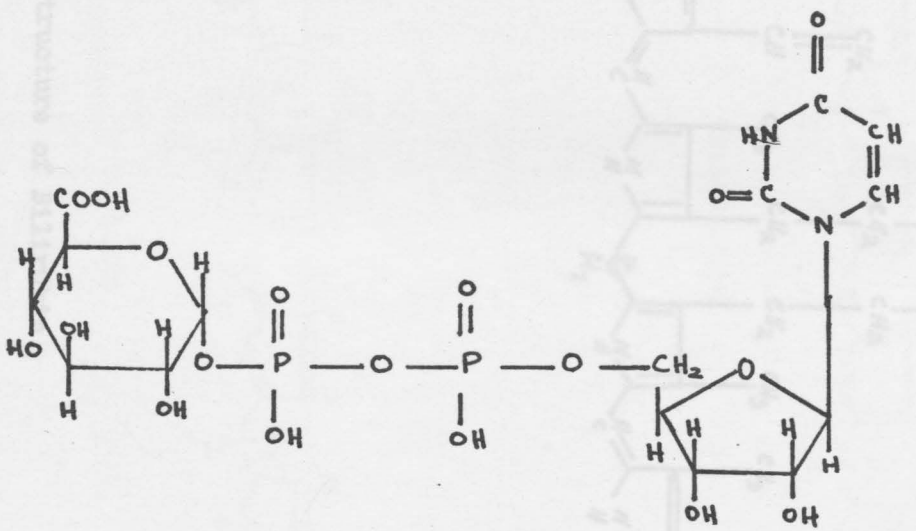


Figure 5. Structure of Uridine Diphosphate Glucuronic Acid

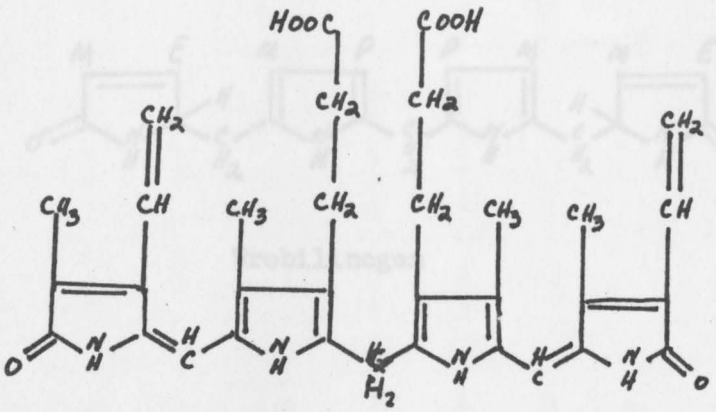
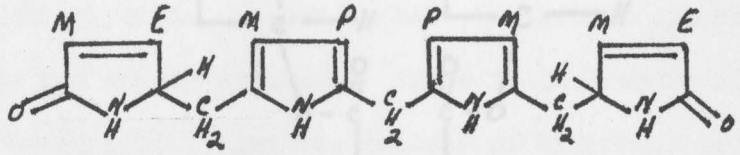
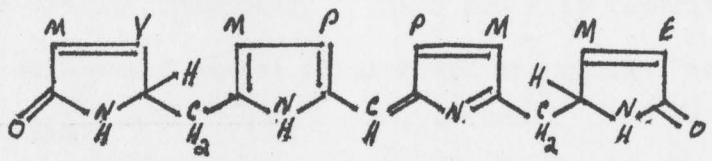


Figure 6. Structure of Bilirubin



Urobilinogen



Urobilin

M = CH_3

V = $-CH=CH_2$

P = $-CH_2-CH_2-COOH$

E = $-CH_2-CH_3$

Fig. 7. Structure of Urobilinogen and Urobilin

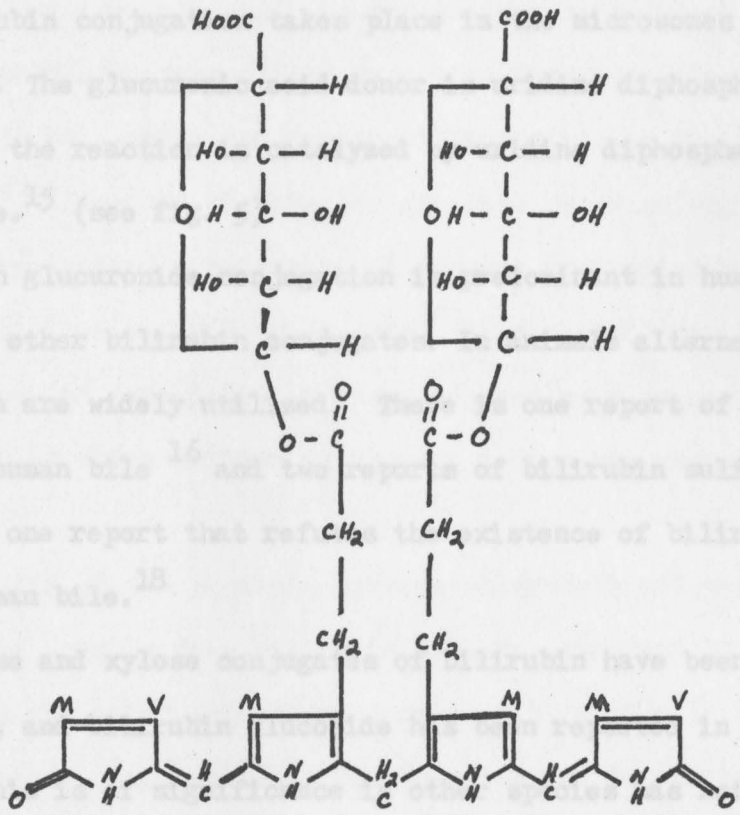


Figure 8. Structure of Bilirubin Diglucuronide

electron resonance over the conjugated double bonds results in a very strong color.[10] (see Fig. 7)

Bilirubin conjugation takes place in the microsomes of the hepatic cells. The glucuronic acid donor is uridine diphosphate glucuronic acid and the reaction is catalyzed by uridine diphosphate glucuronyl transferase.[15] (see fig. 5)

Though glucuronide conjugation is predominant in humans, there are traces of other bilirubin conjugates. In animals alternative methods of conjugation are widely utilized. There is one report of bilirubin phosphate in human bile [16] and two reports of bilirubin sulfate.[16,17] There is also one report that refutes the existence of bilirubin sulfate in human bile.[18]

Glucose and xylose conjugates of bilirubin have been reported in dog bile[19], and bilirubin glucoside has been reported in rat bile,[20] but whether this is of significance in other species has not been established.[21] Taurine conjugates have been reported in chick and to a much lesser extent human bile.[19] There are also reports of disacharridic conjugates identified as: a hexuronyl hexuronide, and a mixture of aldobiuronides.[21,22]

C. The Clinical Determination of Bilirubin and Its Conjugates

What is of importance in the clinical laboratory is not only the determination of the total serum bilirubin level, but also the amounts of free and conjugated bilirubin, commonly referred to as the indirect reacting and direct reacting bilirubin respectively. The methods used for serum bilirubin determination involve direct spectrophotometry, solvent partition, and the reaction of bilirubin and its

conjugates with a diazo salt of an aromatic acid.

The direct spectrophotometric method measures the absorbance of a substance at the wavelength of its maximum extinction. The methods used take advantage of the fact that free bilirubin has an E_{max} around 454 nm., while conjugated bilirubin has an E_{max} around 420 nm.[23,24,25] From the absorbances at these two wavelengths, Fogg and Baaken have derived a mathematical formula from which the direct and indirect bilirubin can be calculated.[25] This method may be affected by the presence of hemoglobin in cases of hemolysis in newborn infants.[24] The wavelength at which maximum extinction occurs may vary with pH and solvent.[26,27]

Partition of the pigments between chloroform and water phases where the lipid soluble free bilirubin will be extracted by the chloroform phase and the water soluble conjugated bilirubin will be extracted by the water phase, has been used for quantitative determination of free and conjugated bilirubin. The concentration of free and conjugated bilirubin can then be determined by direct spectrophotometry[24,25] or by the diazo method.[24] One problem is that the partition is very pH dependent. Also bilirubin monoglucuronide seems to have properties intermediate between free bilirubin and bilirubin diglucuronide, and it is extracted to some extent into the chloroform giving a falsely high indirect bilirubin.[24] There is also no pH value where free bilirubin can be extracted quantitatively into chloroform.[24,28]

The diazo methods are based on the assumption that at strong acid pH values the bilirubin conjugates will react with diazotized sulfanilic acid quickly, while the unconjugated bilirubin will react slowly and only after the addition of an accelerator such as, methanol,

ethanol, diphylline, sodium benzoate, sodium acetate or urea. Actually free bilirubin may react slowly in the absence of an accelerator, and this reaction may be hastened by the presence of impurities in the serum. Even after one minute with no accelerator present, measurable amounts of unconjugated bilirubin may react. [29] Conversely, bilirubin monoglucuronide reacts sluggishly with the diazo reagent. Billing, Cole and Lathe,[30] using trace amounts of conjugated bilirubin obtained chromatographically have shown that after 30 minutes fairly large percentages of bilirubin monoglucuronide failed to react with diazo reagent.

D. The Diazo Reaction

The diazo reaction, discovered in 1883 by P. Ehrlich, is the most widely used method for bilirubin determination. He added 0.1 % diazobenzene-sulfonic acid in HCl-alcohol to bilirubin in chloroform to yield an intense blue color,[31]

In 1916, Hijmans van den Bergh and Muller discovered that icteric sera from persons with obstructive jaundice reacted with aqueous diazo solution, while sera from persons with hemolytic jaundice did not react with the diazo solution to give a color until ethanol was added. The two reactions were named direct reacting and indirect reacting bilirubin respectively. It was postulated that there were two forms of bilirubin involved but not much progress was made in proving it until 1951 when Yamaoko and Kosaka postulated that the direct reacting bilirubin is an ester of the indirect reacting bilirubin, which is an acid.[32] Cole and Lathe[33] in 1953 separated the two fractions by reverse phase partition chromatography, and other workers have shown that the direct reacting pigment is the glucuronic acid ester of the indirect reacting pigment, (ie. free bilirubin) [33,34,3

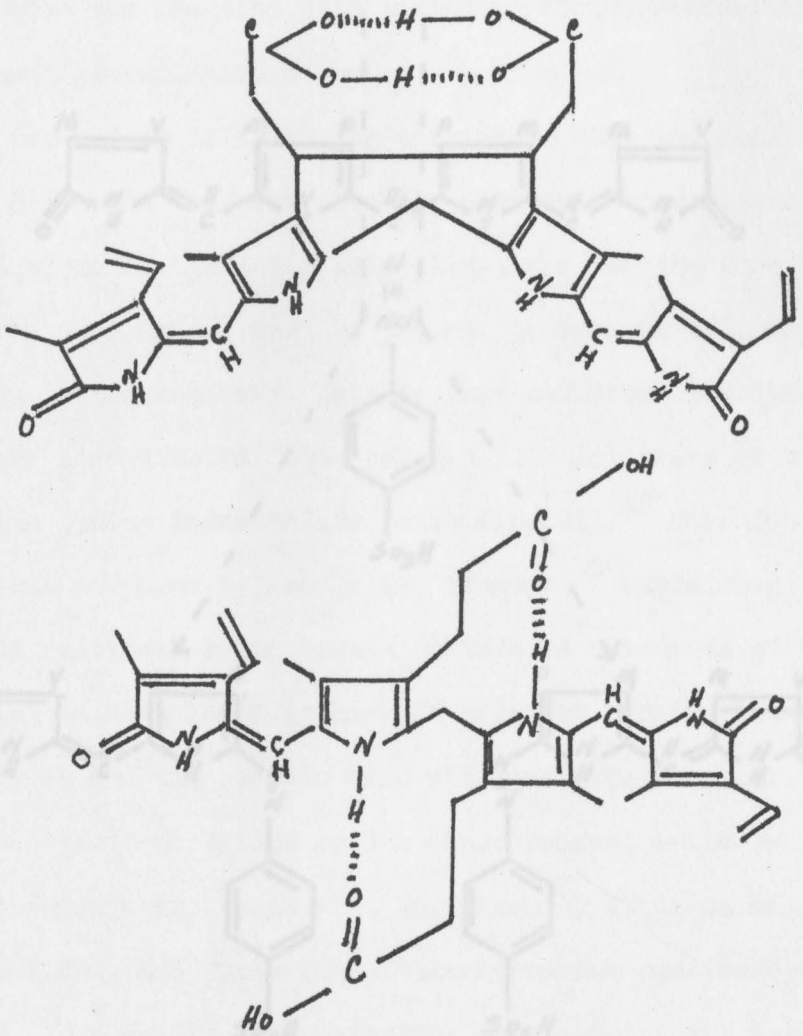


Figure 9. Intramolecular Hydrogen Bonding in the Tautomeric Forms of Bilirubin

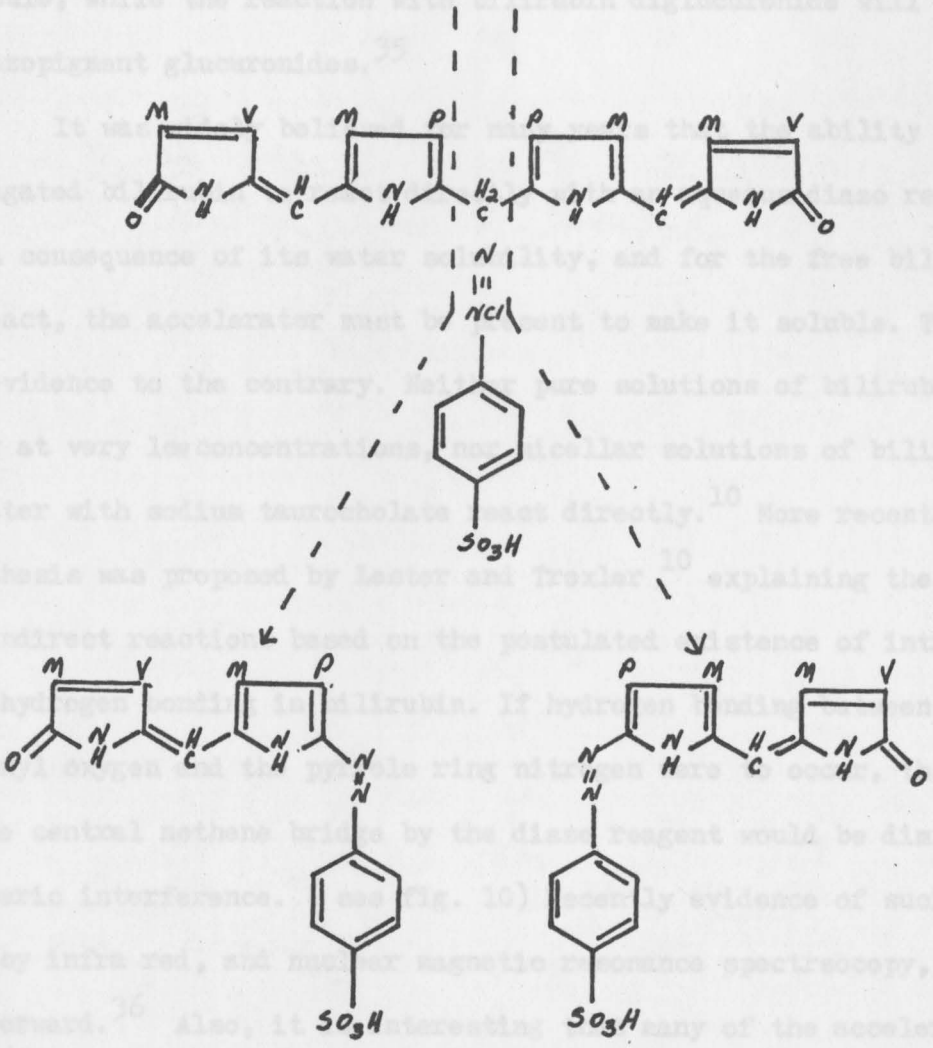
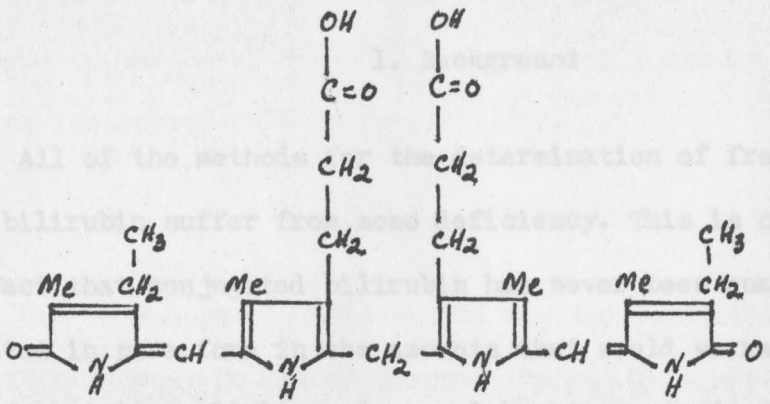


Figure 10. Reaction of Bilirubin Diglucuronide with Diazo Reagent.[2]

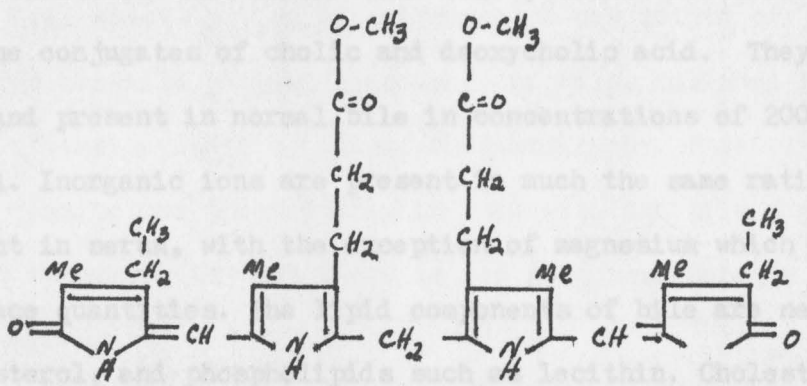
The reaction of bilirubin with diazo reagent proceeds with the cleavage of the central methene bridge to form two dipyrrolic isomeric azopigments. (see fig.10) The cleavage of bilirubin monoglucuronide will produce one azopigment molecule and one azopigment glucuronide molecule, while the reaction with bilirubin diglucuronide will produce two azopigment glucuronides.[35]

It was widely believed for many years that the ability of conjugated bilirubin to react directly with an aqueous diazo reagent was a consequence of its water solubility, and for the free bilirubin to react, the accelerator must be present to make it soluble. There is now evidence to the contrary. Neither pure solutions of bilirubin in water at very low concentrations, nor micellar solutions of bilirubin in water with sodium taurocholate react directly.[10] More recently, a hypothesis was proposed by Lester and Troxler [10] explaining the direct and indirect reactions based on the postulated existence of intramolecular hydrogen bonding in bilirubin. If hydrogen bonding between the carbonyl oxygen and the pyrrole ring nitrogen were to occur, the access to the central methene bridge by the diazo reagent would be diminished by steric interference. (see fig. 10) Recently evidence of such bonding, by infra red, and nuclear magnetic resonance spectrsocopy, has been put forward.[36] Also, it is interesting that many of the accelerators of the diazo reaction, such as methanol and 6 M urea, are compounds that are known to disrupt hydrogen bonds.

Lester and Troxler,[10] have found that dimethyl mesobilirubin, which is similar to conjugated bilirubin in that it has its carboxylic acid groups tied up in an ester linkage, reacts directly. But, it is insoluble in water. Conversely, mesobilirubin, which is soluble in water, but has its carboxyl groups free to engage in hydrogen bonding, reacts



Mesobilirubin



Dimethyl Mesobilirubin

Fig. 11. Mesobilirubin and Dimethyl Mesobilirubin

indirectly. (See fig. 11)

E. Statement of the Problem

1. Background

All of the methods for the determination of free and conjugated bilirubin suffer from some deficiency. This is complicated by the fact that conjugated bilirubin has never been quantitatively isolated in pure form in the amounts that would warrant its manufacture so that it could be used as a laboratory standard. Only free bilirubin is available in sufficient quantity and purity that it can be used as a standard for clinical determinations.[10]

One of the reasons it is so difficult to separate conjugated bilirubin from bile, is the complexity of bile. One of the major constituents of bile is the bile salts, sodium salts of the glycine and taurine conjugates of cholic and deoxycholic acid. They are water soluble and present in normal bile in concentrations of 200 to 800 mg. per 100 ml. Inorganic ions are present in much the same ratio as they are present in serum, with the exception of magnesium which is found only in trace quantities. The lipid components of bile are neutral fats, cholesterol, and phospholipids such as lecithin. Cholesterol is very important, considering the role it plays in the formation of gallstones. It may be present in concentrations of 200 to 800 mg. per 100 ml. Also present are mucoproteins, ammonia, urea, purine derivatives and amino acids. The bile pigments are present almost totally in the form of bilirubin, mainly in the conjugated form. The bilirubin content of normal bile may be 2.3 to 18 mg. per 100 ml., although during and after cases of obstructive jaundice the level may be higher.[37]

Another problem is the extreme instability of the bilirubin conjugates. Ostrow and Murphy[38] have isolated small amounts of conjugated bilirubin which was dissolved in phosphate buffer. The solution was stored in the dark, at -5°C., but it still slowly oxidized to biliverdin or isomerized to dihydrobiliverdin.

2. Proposed Method

One very commonly used method for the separation of different chemical components is column chromatography. This method has been tried by some workers,[23,30,39] but because of the immense complexity of bile, quantitative recovery has never been achieved. Some workers have tried solvent extraction methods to separate the conjugated bilirubin from the other components of bile,[38,40,41] but these methods are very involved and tedious.

This investigation has attempted a new method of partial removal of non-bile pigment components of bile, followed by further separation of the pigments by column chromatography. Possibly one of the reasons that column chromatographic work with the bile pigments has never been too successful is that there is too much interference from the other components.

The preliminary purification was by ultrafiltration, using a 500 molecular weight exclusion value membrane filter. The bile pigments (m.w. free bilirubin=540) will be retained by the filter, while the lower molecular weight bile salts and cholesterol and lecithin will pass through into the ultrafiltrate.

CHAPTER II

REVIEW OF THE LITERATURE

A. Solvent Partition Methods

One of the first solvent partition methods for the separation of the conjugated bile pigments was developed by J. Lucassen[40] in 1961. It is based on the extreme solubility of the sodium salt of conjugated bilirubin in water. Lucassen started with fresh human T-tube bile from patients with obstructive jaundice, which was put through two pH changes followed in each case by centrifugation. The first pH change precipitated the bile salts as bile acids, and the second precipitated the conjugated bilirubin. The conjugates were subsequently dissolved in acetone, the pH was again readjusted, and by using alcoholic .1 N NaOH the sodium salts of conjugated bilirubin were precipitated. Lucassen claimed a 90% purity for his preparation.

He found the di-sodium salt of bilirubin diglucuronide to be an extremely hygroscopic, amorphous, yellow powder that is very soluble in water. Upon exposure to sunlight or standing in moist air it turned green, but if it was kept in a vacuum dessicator, in the dark, it was stable for one year.[40,42]

A more extensive solvent partition method was developed by Talafant and Appelt.[41] Their starting material was dog gallbladder bile, which was readily available. It also contained many taurine conjugated, rather than glycine conjugated, bile salts. The taurine conjugated bile salts are easier to separate by solvent partition. They also noted

that gallbladder bile is more stable than hepatic bile.

A total of 2.5 volumes of acetone was added to one volume of bile. A precipitate formed and was removed, and an additional 7 volumes of acetone were added to the clear supernatant. A precipitate formed which was dissolved in water and re-precipitated by addition of acetone. This acetone precipitate contained 4-7% bilirubin, mainly in the conjugated form, bile salts, and some ninhydrin positive material. The precipitate was removed, dried, and subsequently dissolved in water and then acidified with 1 N acetic acid. Two volumes of acetone were added, and the precipitate was removed by centrifugation. Seven volumes of acetone were then added to the supernatant and the precipitate was collected, washed with acetone and dried. The product was a powder which contained more than 10% bilirubin.

To obtain a more concentrated preparation, this powder was dissolved in water, precipitated with 0.1 M cinchonidine acetate, suspended in aqueous tris (hydroxymethyl) aminomethane and extracted with chloroform. The aqueous layer of the preparation was acidified and the bile pigments were precipitated with 1% lead acetate. They were suspended in water and shaken with a Dowex 50-X slurry. The mixture was centrifuged, the clear layer was evaporated to dryness, dissolved in methanol and evaporated to dryness again. A solid powder was produced which the author said contained 91.4% bilirubin diglucuronide. The properties were similar to those described by Lucassen.

The most extensive solvent partition method was that of Ostrow and Murphy.[38] Their starting materials were human T-tube postoperative bile and rat bile. They first separated the lipids from the bile pigments and the bile salts by extraction of bile first with chloroform

and then with petroleum ether. The aqueous phase from these extractions was then acidified to pH 3.2 and extracted with ethyl acetate. The bile pigments were extracted into the ethyl acetate phase, as they are insoluble in water at low pH values. The bile salts remained in the aqueous phase. The ethyl acetate phase was changed to pH 7.4 with phosphate buffer, at which point the bilirubin conjugates now dissolved in the phosphate buffer phase. The steps of lowering the pH, ethyl acetate extraction and extraction back into phosphate buffer were then repeated. This was followed by another chloroform extraction. The resulting substance was a mixture of bilirubin conjugates in 50 millimolar phosphate buffer, which the authors referred to as C.B.M. preparation.

The yield of this process according to the diazo method and radioactive labelling was 42-60% for rat bile and 15-22% for human bile. The yield with human bile was lower because of the extra difficulty involved in the extraction of glycine conjugated bile salts. The concentration of bilirubin in the human C.B.M. preparation was 60 to 100 mg. per 100 ml. Chemical analysis of six C.B M. preparations and two crystalline samples yielded no detectable bile salts, cholesterol, phospholipids or free bilirubin.

Thin layer chromatography of the C.B.M. preparation gave only two spots. When these spots were hydrolyzed and then developed in another direction, they were both shown to be glucuronides. Thin layer chromatography with a solvent system of chloroform, methanol, acetic acid (85:15:3) showed the monoglucuronide to be more mobile than the diglucuronide.

All C.B.M. or crystalline conjugated bilirubin samples had

absorption maxima at 450 and 420 nm. The former peak was more intense in aqueous and methanolic solution, and the latter was more intense in ethyl acetate. The millimolar extinction coefficient of the bilirubin conjugates at 450 nm. (their concentration being determined by radioactive labelling techniques) was 59.8 ± 0.8 $cm.^{-1}$ The wavelengths of the maxima and the extinction coefficients were independent of the proportions of bilirubin monoglucuronide and diglucuronide in the preparations.

The direct diazo reaction of the C.B.M. preparation had an absorption maximum of 560 nm., with the extinction averaging 12 ± 2 % higher than that for the total diazo reaction of the same sample in methanol. These values were independent of the ratio of monoglucuronide and diglucuronide. The direct but not the total absorbance was supressed when the C B.M. preparation was added to human or rat serum. No reason for this was given or suggested by the authors.

When stored in the dark under air at -15°C., C B M. preparations oxidized at a rate of less than 1% per day. When incubated in the dark under air at 37° C. at pH 7.0, the rate of oxidation was less than 1% per hour. Thin layer chromatography of the oxidized pigment showed that biliverdin monoglucuronide and diglucuronide were formed. Under neither set of conditions was free bilirubin found.

B. Chromatographic Methods of Separation

Cole and Lathe,[33] in 1953 first separated the direct and indirect reacting bilirubin of icteric sera on silicone treated columns. A solvent system of chloroform, methanol, carbon tetrachloride, pH 6 phosphate buffer (25:25:18:18) was used. The direct reacting pigment moved faster than the indirect. In some cases the direct broke up into

two bands, but they were always eluted together.

Cole, Iathe and Billing,[23] in 1953, used reverse phase column chromatography on siliconized kieselguhr with bile, serum and urine of patients with obstructive jaundice. The solvent system was chloroform,' methanol, pH 6 phosphate buffer, carbon tetrachloride (25:25:18:18). The polar direct pigment broke up into two bands; the less polar designated pigment I and the more polar pigment II. They also used a solvent system of n-butanol and 0.005 M pH 6 phosphate buffer which resolved the direct reacting pigment into two pigments. With the first solvent system both of the polar direct reacting pigments had absorption maxima at 419 nm., while the indirect reacting pigment had its maximum at 454 nm. With the second solvent system the indirect pigment had an A_{max} at 450 nm., pigment I at 452 nm. and pigment II at 448 nm. It was also noted that urine contained greater proportions of pigment I while serum contained proportionately more pigment II.

Billing, Cole and Iathe,[30] in 1956, found that if the polar direct reacting pigments are diazotized, there will be two azopigments formed, azopigments A and B. These two pigments were separated by paper chromatography, using a mobile phase of isopropyl ether and n-heptane (3:2) and a stationary phase of 70 % acetic acid. Pigment A moved with an R_f value of 0.8, while pigment B had an R_f value of 0.15. The spots were tested for amino acids with ninhydrin and for sugars with napthoresorcinol. Pigment B showed a blue color with napthoresorcinol, which is indicative of the presence of a pentose or uronic acid. Pigment B was hydrolyzed, and the product of the hydrolysis moved with the same R_f value and stained with the same color as a uronic acid spot which was also applied to the paper. Pigment B was thus shown to be the glucuronide of pigment A.

Tenhunen[16], in 1965, applied human bile which had been centrifiged at 300 g's for ten minutes to thin layer chromatography plates of kieselgel G. The plates were dried 30 minutes at 105-110° C. and stored in a dessicator prior to use. The plates were developed in a solvent system of acetone, n-butanol, propionic acid and water (7:4:3:3) for 35 to 40 minutes during which time the solvent front travelled 12-14 cm. The fractions were removed with a steel splinter, and they were eluted from the kieselgel with a mixture of 0.001 M pH 6 phosphate buffer and methanol (1:1). Half of each eluate was assayed for bilirubin by the diazo method, while the other half was assayed for glucuronic acid.

Tenhunen was able to separate five fractions on his thin layer plates as shown in table 1.

TABLE 1

BILE FRACTIONS SEPARATED BY TENHUNEN BY THIN LAYER CHROMATOGRAPHY

Fraction	R_f value	Identification
I	0.70	Free bilirubin
II	0.50	Bilirubin monoglucuronide
III	0.35	Bilirubin diglucuronide
IV	0.25-0.30	Bilirubin sulfate
V	0.04-0.06	Bilirubin phosphate

Tenhunen also separated diazotized bile pigments by thin layer chromatography. Centrifuged bile was diazotized with sulfanilic acid, and was then allowed to stand for thirty minutes. The azopigments were then extracted three times with a three fold volume of heptane, n-butanol

(7:3) and three times with a three fold volume of chloroform, to remove the bile salts. Twenty five to fifty microliters of diazotized solution thus extracted were applied to plates which were prepared as described above. The solvent system was methyl ethyl ketone, propionic acid and water (20:5:5). Five fractions were again separated as enumerated in Table 2.

TABLE 2

DIAZOTIZED BILE FRACTIONS SEPARATED BY TENHUNEN

Fraction	R_f value	Identification
I	0.70	Diazo compound of free bilirubin
II	0.30-0.35	Taurine conjugates of bilirubin
III	0.18-0.24	Azobilirubin glucuronides
IV	0.15	Sulfate derivative of diazotized bilirubin
V	0.02-0.06	Phosphate containing fraction

Tenhunen also used radioactive labelled sulfate, phosphate and taurine to help in the identification of these spots.

Jacobsen,[39] in 1969, separated bilirubin monoglucuronide and diglucuronide by gel filtration. A sample of bile prepared by Lucassen's method was applied to a column of Sephadex LH-20 (Pharmacia), an alkylated cross linked dextran. An ethanol-water solvent system (1:1) was used with a flow rate of 15 ml. per hour. The concentration of the eluate was 2 mg. bilirubin per 100 ml. solvent, but the results were variable depending on the age of the starting materials.

Bilirubin diglucuronide was the faster of the two fractions. They both reacted directly with the diazo reagent, even when the alcohol

was evaporated from the solvent. They both showed an A_{max} at 454 nm. The eluate consisted of 10% azo negative impurities which were thought to be ethanol soluble substances leached out of the Sephadex LH-20

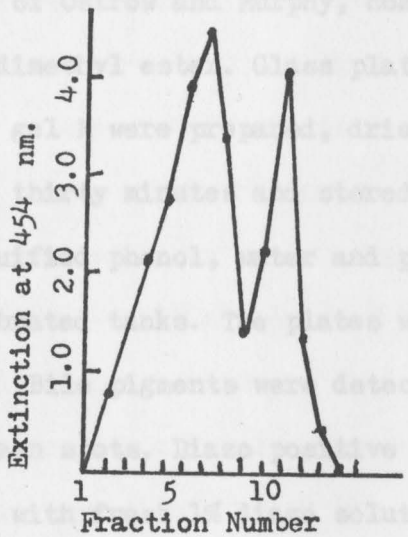


Fig. 12 Elution Curve of Sephadex LH-20 Elution of Bilirubin Conjugates by J. Jacobsen

Kuenzele,[22] in 1970, was able to separate 8 different azopigments from diazotized bile bilirubin. The three bilirubin fractions of human hepatic bile were separated by reverse phase partition chromatography on silicone treated celite with a solvent system of 1-butanol and pH 6 phosphate buffer (1:1). The three fractions were treated with diazotized aniline, and the resulting azopigments were re-chromatographed on a silicone treated celite column. The fractions thus obtained were further separated by adsorption chromatography on anhydrous sodium sulfate with the use of chloroform followed by a gradient of ethyl acetate in chloroform. Finally the azopigments were eluted again through

a silicone treated celite column. This time a solvent system of 1-octanol, di-isopropyl ether, ethyl acetate, methanol and acetic acid (1:2:2:3:4) was used.

Thompson and Hoffman,[42] in 1971, used thin layer chromatography to study concentrated bilirubin diglucuronide solutions prepared by the method of Ostrow and Murphy, commercial bilirubin and synthesized bilirubin dimethyl ester. Glass plates coated with 0.5 mm. thick layers of silica gel H were prepared, dried at room temperature, activated at 110° C. for thirty minutes and stored over calcium sulfate. A solvent system of liquified phenol, water and pH 4.6 buffer (36:5:9) was used in pre-equilibrated tanks. The plates were developed in the dark at 22°C.

Bile pigments were detected by examining the plates for yellow and green spots. Diazo positive pigments were detected by spraying the plates with fresh 1% diazo solution. Carbohydrates were identified by spraying with concentrated sulfuric acid, water (1:1) and heating.

TABLE 3

RESULTS OF THIN LAYER CHROMATOGRAPHY OF BILE
BY THOMPSON AND HOFFMAN

R_f	Identity	Comments
L.0	cholesterol lecithin	detected by charring with H_2SO_4
1.0	bilirubin dimethyl ester	----
.70-.85	bilirubin	----
.60	biliverdin	blue green spot
.48-.50	glycine and taurine conjugates of deoxycholic acid	detected by charring with H_2SO_4
.40-.44	glycine and taurine conjugates of cholic acid	detected by charring with H_2SO_4
.38	bilirubin monoconjugates	----
.34	bilirubin diconjugates	----

Hayes[43] attempted to separate conjugated bilirubin from hog gall bladder bile by the method of Cole, Iathe and Billing.[23] His starting material had oxidized in the gall bladder to a green fluid, probably biliverdin and biliverdin glucuronides. The separation procedure yielded no diazo-positive pigments. Reduction of the bilirubin with sodium borohydride was attempted, and even though there was a slight lessening of the deep green color, the pigments still gave a negative diazo test.

A second bile sample from slaughtered hogs,which had been immediately frozen after collection was subjected to $NaBH_4$ reduction. The sample was sealed in an air tight flask and stood 24 hours at room temperature. Small aliquots of the sample gave an immediate diazo positive red color. He reported that the golden yellow fluid thus obtained after reduction of whole bile evidently contained reduced bilirubin conjugates in a form which was stable for a long time and gave an immediate diazo test. The pigments were not extracted from the bile sample.

Pavlicko[44] used the method of Cole, Iathe and Billing[23] to obtain bilirubin diglucuronide from hog bile. The bile was first purified and then chromatographed on a siliconized kieselguhr column. It was eluted from the column with .005 M phosphate buffer (pH 6) saturated with butanol. Fractions of 2 to 5 ml. were collected, and out of 160 fractions collected, 149 showed visible color with an A_{max} at 430 nm. The fractions contained 12.6 mg. per 100 ml. bilirubin compared to the original bile which contained 32 mg. per 100 ml.

Next an attempt was made to separate bilirubin diglucuronide from the fractions. An adapatation of Lucassen's[40] was used. The fractions were made to contain 90% acetone. To this a 0.1 N NaOH solution in 96% ethanol was added to bring about a pH change from 5 to 7. At this

point a flocculent precipitate of bilirubin diglucuronide was to form.[40] However, upon the addition of acetone to the eluate (pH 5), a sticky tarry material formed. This material was thought to be some lipid material or bile acid that had not been completely removed.

The kieselguhr eluate was then further purified by chromatography on a column of Sephadex G-10 (Pharmacia). The Sephadex eluate was again subjected to the Lucassen method of precipitation of bilirubin diglucuronide. The eluate was made to contain 90 % acetone with no problem. The 0.1 N NaOH was added to change the pH from 5 to 7, but no precipitate or any other material formed.

Killmeyer[45] tried to separate the bilirubin conjugates from human t-tube bile purified by the method of Lucassen.[40] He used a 15 x 240 mm. glass column packed with Sephadex G-10 (Pharmacia). The elution was run under a nitrogen atmosphere and the eluting solvent was pH 7.4 phosphate buffer.

A liter of bile with 56 mg.% direct reacting and 77 mg.% total bilirubin was purified by Lucassen's method to yield 65 mg. of solid residue, which contained 14.5% direct reacting and 22.5% total bilirubin by weight. Five mg. of this residue was dissolved in 1.0 ml. of pH 7.4 phosphate buffer, introduced onto the Sephadex column and eluted at a flow rate of 0.46 ml. per minute. The Lucassen residue introduced onto the column contained 0.725 mg. direct reacting and 1.125 total bilirubin. The total volume of eluate contained only 0.09 mg. of direct reacting and 0.5 mg. total bilirubin. He attributed the failure of the greater portion of the bilirubin to be eluted from the column to the possibility that the bilirubin became permanently adsorbed on the surface of the gel, due to the aromaticity of bilirubin.

Killmeyer also did some thin layer chromatography work which was the basis of a part of the present paper. He used samples of centrifuged bile (4,300 g's for 25 minutes), bile acidified to pH 6 with oxalic acid, bile purified by Lucassen's procedure and commercial bilirubin dissolved in phosphate buffer. The work was done on pre-heated glass plates coated with 0.25 mm. silica gel G, pre-heated Eastman Chromagram sheets and non pre-heated Eastman Chromagram sheets. He used two solvent systems. Solvent system A was a mixture of acetone, butanol, propionic acid and water (7:4:3:3), while solvent system B contained butanol, acetone, propionic acid and water (7:4:3:3). This was the first demonstration of separation of bile pigments using Eastman Silica Gel G Chromagram sheets. The results of Killmeyer's thin layer chromatography work are tabulated in Table 4.

TABLE 4

BILE FRACTIONS SEPARATED BY KILLMEYER BY THIN LAYER CHROMATOGRAPHY

Sample utilized	Plate	R_f values of fractions			Solvent system
Cent. bile	glass	.45	.66	1.00	A
Commercial bilirubin	glass	1.00			B
Lucassen residue	glass	.49	.59	1.00	B
Lucassen residue	glass	.59			B
pH 6 bile	glass	.71	1.00		A
Lucassen residue	glass	.81			A
pH 6 bile	pre-heat Chromagram	.58	.72	1.00	A
Lucassen residue	pre-heat Chromagram	.58	.72		A
Commercial Bilirubin	pre-heat Chromagram	1.00			A
Cent. bile	Chromagram	.55	.74	1.00	A
Cent. bile	Chromagram	.62	.80	1.00	A
Commercial bilirubin	Chromagram	1.00			A
Lucassen residue	Chromagram	.62	.80		A
pH 6 bile	Chromagram	.60	.77	1.00	A

CHAPTER III

MATERIALS AND APPARATUS

A. Materials

Absolute alcohol was U.S.I. pure ethyl alcohol, U.S.P., obtained from U.S. Industrial Chemicals, New York. Bilirubin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. It was certified by the American College of Pathologists, and it had a molar absorptivity in chloroform of 60,000 ± 600. The cholesterol used was Fisher Certified Reagent. The sodium taurocholate was obtained from Nutritional Biochemicals Corp. The manufacturer stated that it was obtained from the bile of carnivores and that it contained small amounts of sodium glycocholate. The glucuronic acid was crystalline, grade 1, 99% pure, from Sigma Chemical Co., St. Louis. The Carbazole was Fisher Certified Reagent, purchased from Fisher Scientific, Fair Lawn, New Jersey.

All water used for the preparation of reagents, and the rinsing of glassware, was distilled and deionized.

The bile was obtained from local hospitals from patients who had T-tube drainage. The bile was placed in a brown bottle and refrigerated until it could be brought back to the laboratory.

ACD and CPD plasma was obtained from the blood banks of local hospitals. It was stored at 4° C. and centrifuged 15 minutes at 3,200 r.p.m. prior to use.

Silica gel, .2-.5 mm., was purchased from Brinckmann Instruments, Westbury, N.Y. The 100 mesh silicic acid was purchased from Mallinkrodt.

For thin layer chromatography work, Eastman Chromagram Sheets, #6061 silica gel G without fluorescent indicator, were used. They were obtained from Eastman Kodak Co.

The Amberlite XAD-2, polystyrene column packing resin, was a gift from Rohm and Haas, Philadelphia, Pa.

All other chemicals used were reagent grade.

B. Apparatus

All fluorometric work was done on a Turner Model 111 Fluorometer (G.K. Turner Associates), with Turner filters. Spectral scans and the determination of the extinction coefficient of bilirubin in chloroform were carried out in a Beckmann Model DB Spectrophotometer. A Sargent Recorder, model SRL, was used for recording the spectral scans and the progress of the elutions. Quantitative assays for bilirubin and glucuronic acid were carried out with a Spectronic 20 spectrophotometer (Bausch and Lomb). A Thomas Flow Cell (Arthur H. Thomas Co.) was used in conjunction with the Spectronic 20 for column elutions.

The Buchi Rotary Evaporator was purchased from Buchi, Switzerland. The Amicon Stirred Ultrafiltration Cell, Reservoir and UM-05 membrane filters were purchased from The Amicon Corp., Lexington Mass.

Centrifugation was carried out using either an MSE HS-25 refrigerated centrifuge (MSE Ltd.) or a Sorvall Model GLC-1 (Ivan Sorvall, Norwich Conn.).

The thin layer chromatography Chromagram Sheets were spotted with a Ziptrol 0-5 microliter capillary pipette and were developed in an Eastman Chromagram Developing Apparatus.

CHAPTER IV

EXPERIMENTAL

A. Assay Methods

1. Bilirubin

a. Reagents

The procedure for the determination of bilirubin was the Malloy-Evelyn method,[46,47] as modified by Kingsley et al.[12,48] For solution A, 15 ml. of concentrated HCl was added to 985 ml. of deionized water. To this, one gram of sulfanilic acid was added, and it was stirred until it dissolved. For solution B 0.5 g. of sodium nitrite ($NaNO_2$) was added to 100 ml. of deionized water. Solution B remains stable for several months if stored at 4° C.

A diazo blank was prepared by adding 15 ml. of concentrated HCl to 985 ml. of deionized water. To prepare the diazo reagent for use, 0.3 ml. of solution B was added to 10 ml. of solution A. This reagent is unstable and a fresh mixture was prepared for each set of determinations. The mixed reagent was always stored on ice.

b. Procedure

To each of two standard 19 x 105 mm. cuvettes were added 4.0 ml. of water and 0.5 ml. of serum. To one tube 1.0 ml. of diazo blank was added, and to the other 1.0 ml. of fresh diazo reagent. The Spectronic 20 was set with the serum diazo blank at zero absorbance.

The absorbance of the serum was read after exactly one minute, at 540 nm. for the direct reacting bilirubin.

To both tubes 5.5 ml. of absolute methanol was added, and mixed by inversion. These were read after exactly 20 minutes to give the total bilirubin.

The absorbanees were compared to those on a standard curve. The absorbance of the direct reacting bilirubin was divided by two to compensate for the fact that the direct readings were based on a volume of 5.5 ml., while the totals were based on 11.0 ml.

When using bile, which had a greater concentration of pigments than serum, high absorbances (ie. greater than 1.5) were encountered and they could not be read accurately. In these cases the procedure was modified by adding 0.1 ml. of bile to 4.4 ml. of water. The absorbances were then read from the standard curve, and the values were corrected for dilution.

c. Preparation of a Standard Curve

A 0.1 M phosphate buffer, pH 7.4, was made by dissolving 11.92 grams of anhydrous dibasic sodium phosphate (Na_2HPO_4) and 2.8 grams of anhydrous monobasic potassium phosphate (KH_2PO_4) in deionized water and diluting it to one liter. A 5 N NaOH solution was prepared by dissolving NaOH in an appropriate amount of CO_2 free deionized water. Alkaline solvent was made by adding 1.0 ml. of 5 N NaOH to 100 ml. volumetric flask and diluting it to 100 ml. with pH 7.4 phosphate buffer. The pH of this 20 mg.% solution should be 7.4 ± 0.1.

Another solution was prepared in a like manner, but the bilirubin was omitted. This served as a blank. Both standard and blank

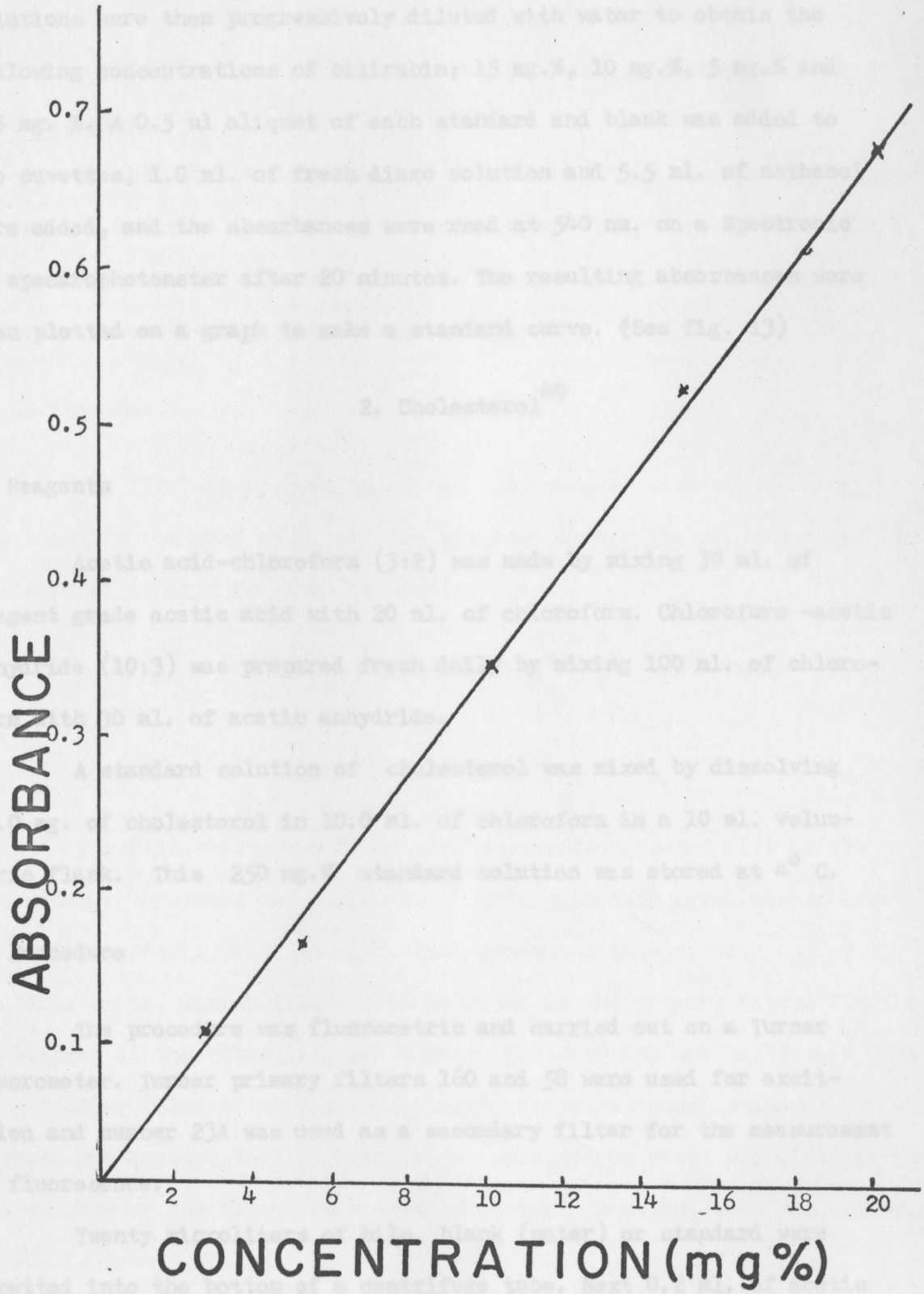


Figure 13. Absorbance of Diazotized Bilirubin in ACD Plasma-Phosphate Buffer vs. Concentration at 540 nm.

solutions were then progressively diluted with water to obtain the following concentrations of bilirubin; 15 mg.%, 10 mg.%, 5 mg.% and 2.5 mg. %. A 0.5 ml aliquot of each standard and blank was added to two cuvettes, 1.0 ml. of fresh diazo solution and 5.5 ml. of methanol were added, and the absorbances were read at 540 nm. on a Spectronic 20 spectrophotometer after 20 minutes. The resulting absorbances were then plotted on a graph to make a standard curve. (See fig. 13)

2. Cholesterol[49]

a. Reagents

Acetic acid-chloroform (3:2) was made by mixing 30 ml. of reagent grade acetic acid with 20 ml. of chloroform. Chloroform -acetic anhydride (10:3) was prepared fresh daily by mixing 100 ml. of chloroform with 30 ml. of acetic anhydride.

A standard solution of cholesterol was mixed by dissolving 25.0 mg. of cholesterol in 10.0 ml. of chloroform in a 10 ml. volumetric flask. This 250 mg.% standard solution was stored at 4° C.

b. Procedure

The procedure was fluorometric and carried out on a Turner Fluorometer. Turner primary filters 160 and 58 were used for excitation and number 23A was used as a secondary filter for the measurement of fluoresence.

Twenty microliters of bile, blank (water) or standard were pipetted into the bottom of a centrifuge tube. Next 0.2 ml. of acetic acid-chloroform reagent was added. A momentary precipitate may form, but it will dissolve upon mixing. Then 5.0 ml. of chloroform-acetic

anhydride was added, followed by a brief mixing. Next 0.2 ml. of concentrated sulfuric acid was added. This was followed by thorough mixing in a vortex mixer.

If the mixture was turbid, it was centrifuged and the clear supernatant was decanted into the fluorometer tube. Forty minutes after the addition of the sulfuric acid the tube was placed in the fluorometer, which had been blanked with a dummy cuvette.

c. Calculations

The fluorescent reading of the bile sample is recorded as R_u, the blank is recorded as R_b and the standard as R_s. The concentration of cholesterol in mg.% is given by the formula:

$$\text{mg.\% cholesterol} = \frac{R_u - R_b}{R_s - R_b} \times 250 \text{ mg.\%}$$

3. Bile Salts

The bile salts were determined spectrofluorometrically by the procedure of Mroszcak and Riegelman.[50] This procedure gives almost identical fluorescent intensities for equal concentrations of each one of the bile salts. Turner filter # 48 was used as the primary filter for the excitation of the molecules, and Turner filters# 65A and 2A-12 were used as secondary filters for the measurement of fluorescence. Evaporation to dryness was accomplished by using a jet of nitrogen which was directed onto the surface of the liquid in a test tube, while the test tube was immersed in 40° C. water.

a. Reagents

A barium hydroxide solution was prepared by adding 6.0 g. of $Ba(OH)_2 \cdot 8H_2O$ to 100 ml. of freshly boiled water. The mixture was then quickly filtered through glass wool and stored in a tightly stoppered polyethylene bottle. A zinc sulphate solution was prepared by adding 10.0 g. of $ZnSO_4$ to 100 ml. of deionized water and storing in a glass stoppered bottle.

"Pure solvents upper phase", was a solvent mixture used by Folch et. al.[51] for lipid extraction. It consisted of chloroform, methanol and water (3:48:47). The 0.29% pure solvents upper phase sodium chloride solution was made by substituting 0.58% Na Cl solution for water in the above mixture.

b. Procedure

To 0.1 ml. of bile was added 1.75 ml. of absolute ethanol, 0.1 ml. of barium hydroxide solution and 0.5 ml. of zinc sulphate solution. This precipitates the protein and bilirubin (the latter causes high fluorescent readings). After mixing, the samples were centrifuged at 2,400 rpm. for 10 minutes. One tenth volume (0.2 ml.) of the supernatant fluid was transferred to another test tube and evaporated to dryness. To the residue was added 0.1 ml. of deionized water and 3.9 ml. of chloroform-methanol (2:1). This was then mixed well with an aqueous NaCl solution (7.0 g. per liter). The phases separate, the bile salts are partitioned into the more polar upper phase (methanol-water), which is drawn off with a Pasteur pipette. Phospholipids, fatty acids and cholesterol migrate into the less polar (chloroform) lower phase. To ensure quantitative removal of the bile salts, the lower phase was

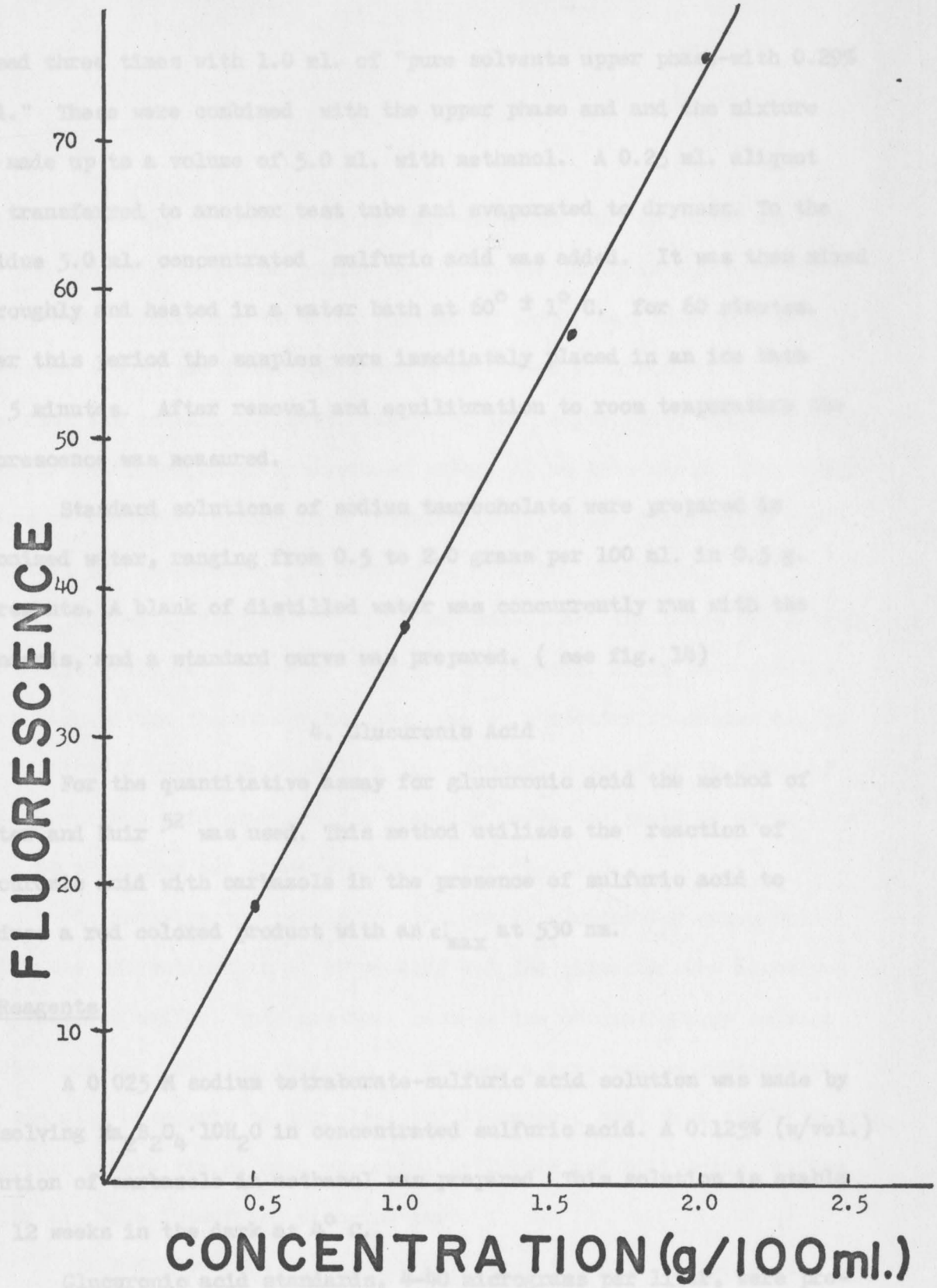


Figure 14. Fluorescence of Sodium Taurocholate vs. Concentration

rinsed three times with 1.0 ml. of "pure solvents upper phase-with 0.29% NaCl." These were combined with the upper phase and and the mixture was made up to a volume of 5.0 ml. with methanol. A 0.25 ml. aliquot was transferred to another test tube and evaporated to dryness. To the residue 5.0 ml. concentrated sulfuric acid was added. It was then mixed thoroughly and heated in a water bath at $60^{\circ} \pm 1^{\circ}$ C. for 60 minutes. After this period the samples were immediately placed in an ice bath for 5 minutes. After removal and equilibration to room temperature the fluorescence was measured.

Standard solutions of sodium taurocholate were prepared in deionized water, ranging from 0.5 to 2.0 grams per 100 ml. in 0.5 g. increments. A blank of distilled water was concurrently run with the standards, and a standard curve was prepared. (see fig. 14)

4. Glucuronic Acid

For the quantitative assay for glucuronic acid the method of Bitter and Muir [52] was used. This method utilizes the reaction of glucuronic acid with carbazole in the presence of sulfuric acid to produce a red colored product with an ε_{max} at 530 nm.

a. Reagents

A 0.025 M sodium tetraborate-sulfuric acid solution was made by dissolving $Na_2B_2O_4 \cdot 10H_2O$ in concentrated sulfuric acid. A 0.125% (w/vol.) solution of carbazole in methanol was prepared. This solution is stable for 12 weeks in the dark at 4° C.

Glucuronic acid standards, 4-40 micrograms per liter, were prepared from a standard stock solution. The stock solution was glucuronic acid (40 micrograms per liter) in deionized water saturated with benzoic

acid.

b. Procedure

Five ml. of sodium tetraborate-sulfuric acid solution was placed in test tubes with ground glass or teflon stoppers, in a rack. The tubes were then cooled to 4° C. One ml. of sample was then layered carefully on top of the acid. The tubes were closed with stoppers, and the rack was shaken, at first slowly and then vigorously, while being constantly cooled under a stream of water. At no time should the temperature of the tubes exceed room temperature. If these precautions are omitted, the temperature can rise to 135° in the interface.

Carbazole reagent, 0.1 ml., was added, and the tubes are heated in a vigorously boiling water bath for 25 minutes. After they cool to room temperature the absorbance was read on a spectrophotometer at 530 nm. against a blank prepared in a like manner, with water in place of the glucuronic acid standard. A standard curve was prepared using the 4 to 40 microgram per liter standards (see fig. 15).

In assaying the column chromatography eluates for glucuronic acid, the solvent had to be evaporated and the pigments were dissolved in phosphate buffer. This was done because the chromatography solvent system reacted with the reagents to produce a dark opaque substance. It was also necessary to hydrolyze the glucuronic acid from the bilirubin, and then remove the colored pigments, bilirubin and biliverdin, as the color may have interfered with the test.

To hydrolyze the glucuronic acid from the bile pigments, a phosphate buffer solution of bile pigments eluted from the chromatography column was mixed with an equal volume of 2 N HCl. This mixture was heated

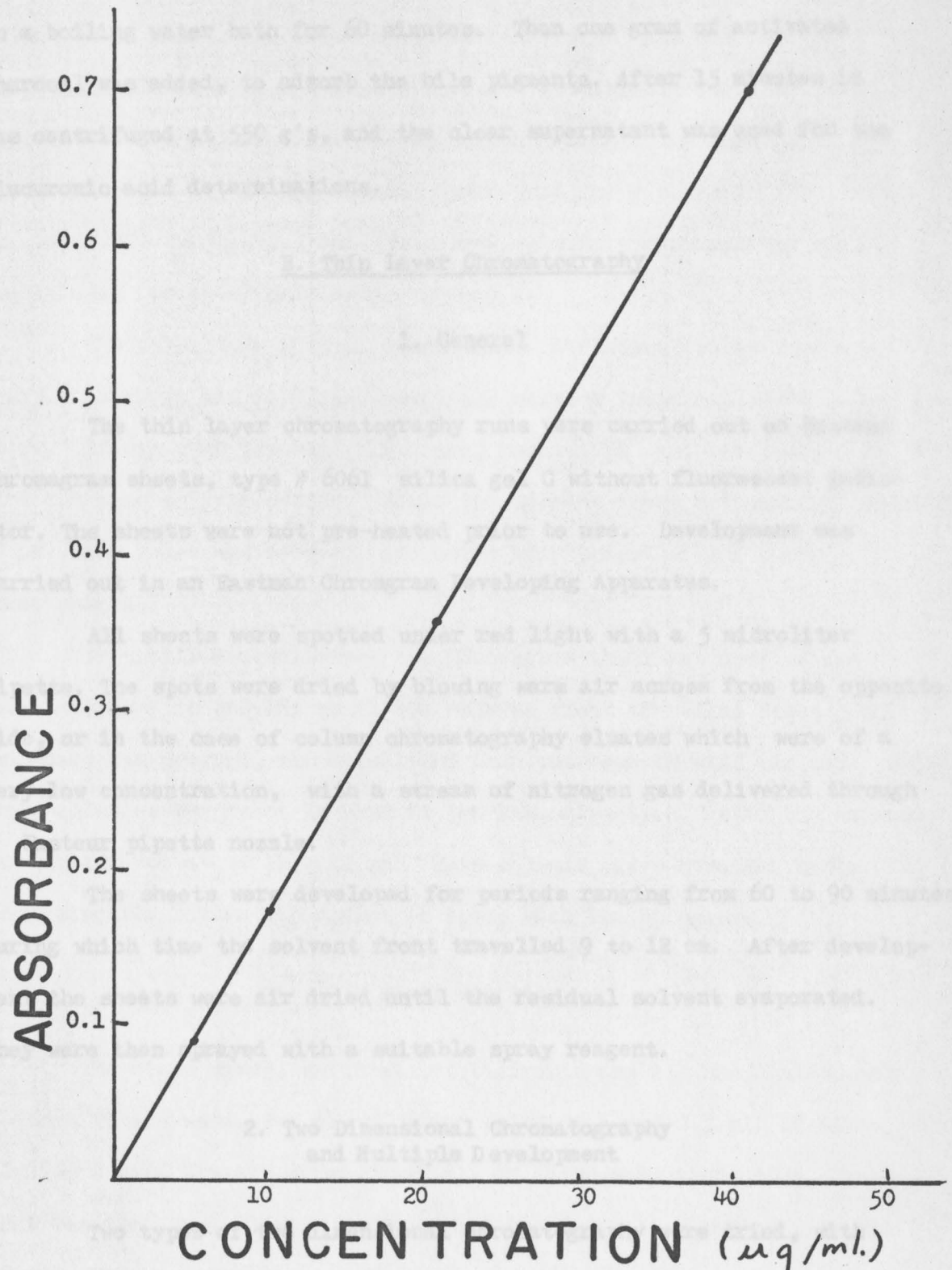


Figure 15. Absorbance of Glucuronic Acid-Carbazole Complex vs. Concentration at 530 nm.

in a boiling water bath for 60 minutes. Then one gram of activated charcoal was added, to adsorb the bile pigments. After 15 minutes it was centrifuged at 550 g's, and the clear supernatant was used for the glucuronic acid determinations.

B. Thin Layer Chromatography

1. General

The thin layer chromatography runs were carried out on Eastman Chromagram sheets, type # 6061 silica gel G without fluorescent indicator. The sheets were not pre-heated prior to use. Development was carried out in an Eastman Chromgram Developing Apparatus.

All sheets were spotted under red light with a 5 microliter pipette. The spots were dried by blowing warm air across from the opposite side, or in the case of column chromatography eluates which were of a very low concentration, with a stream of nitrogen gas delivered through a Pasteur pipette nozzle.

The sheets were developed for periods ranging from 60 to 90 minutes, during which time the solvent front travelled 9 to 12 cm. After development the sheets were air dried until the residual solvent evaporated. They were then sprayed with a suitable spray reagent.

2. Two Dimensional Chromatography and Multiple Development

Two types of two dimensional chromatography were tried, with and without hydrolysis. In the two dimensional chromatography with hydrolysis the sheet is developed until the solvent front moves about 10 cm. It was then removed, air dried and put into a tank saturated with NH_3

vapor for 24 hours. The sheet was then rotated 90° and put into a different solvent system. After the second development resulted in an appoximately 10 cm. migration of the solvent front, the sheet was removed and air dried. The ammonia hydrolyzed the glucuronic acid from the bilirubin conjugates, and the second solvent system was one specifically used for glucuronic acid.

The other type was two dimensional development without hydrolysis. The sheet was developed until the solvent front had moved about 10 cm. It was then removed, air dried, rotated 90° and developed again with the same solvent system. After the solvent front had again migrated 10 cm., the sheet was removed, air dried and sprayed with a suitable spray reagent.

In multiple development the Chromagram sheet was spotted and then developed in solvent until the solvent front travelled about 5 cm. The sheet was removed, air dried, and then put back in with the same solvent, allowing the front to move in the same direction, until the solvent front had moved about 10 - 12 cm. This methods gives stronger spots and a slightly better separation of spots with low R_f values.

3. Spray Reagents

The diazo spray, which stains bilirubin and its conjugates, was the most widely used. Ten ml. of diazo solution A and 0.3 ml. of diazo solution B were freshly mixed. Five ml. of water were then added. The sheet was then sprayed with methanol and the indirect reacting pigments showed up after a few minutes.[16]

Zinc acetate spray[53] was also used to identify the bile pigments. The sheet was sprayed with a saturated solution of zinc acetate in

methanol followed by a 0.006% solution of I_2 in methanol. This resulted in the formation of zinc complexes with bilirubin and biliverdin, which fluoresced under ultra-violet light.

Rhodamine 6-G, phosphomolybdic acid, 2'7' dichlorofluorescein, and bromphenol blue sprays were used for the detection of lipids. Some sheets were placed in a tank saturated with I_2 vapors for lipid detection. A solution of 3% p-anisidine HCl was used for the identification of glucuronic acid.

4. Solvent Systems

The most commonly used solvent system for the separation of the bile pigments was; acetone, butanol, propionic acid and water (7:4:3:3). This solvent system was used by Tenhunen[16] and subsequently by Killmeyer[45] in their thin layer chromatography work with the bile pigments. From here on it it will be referred to as solvent system A.

The thin layer separation of lipid components from bile was accomplished using solvent system B, chloroform-methanol (2:1). Solvent system C contained butanol, acetic acid and water (12:3:5). It was used for the separation of glucuronic acid from the bile pigments after hydrolysis.

C. Membrane Ultrafiltration

1. Introduction

Membrane filtration is a process for separating dissolved or particulate substances from a fluid using a selectively permeable barrier to retain desired constituents. The fluid may be driven through the

membrane by gravity or external pressure. Membrane ultrafilters with molecular weight exclusion values of 500 to 30,000 are available, but it should be noted that these values are approximate indicators. Actual rejection depends on molecular size, shape and charge.[54] For this work a membrane with a 500 molecular weight exclusion value was used. This would allow cholesterol (m.w. 384) and bile salts (m.w. approx. 400) to pass through, while the bile pigments (m.w. 573,bilirubin) would be retained.

2. The Membrane

An Amicon UM-05 Diaflo membrane was used with an Amicon ultrafiltration apparatus as shown in fig. 16. With careful handling, and where cross contamination is not a problem, these membranes have been re-used 25 times.[54] When not in use the membrane was removed from the cell and stored in deionized water at 4° C. The membrane was occasionally cleaned by soaking in a 10 % ethanol in water solution for at least 24 hours.

Membrane polarization is a concentration gradient in the fluid, with solute or suspensoid content highest at the membrane surface and diminishing to the general concentration level of the liquid some distance from the membrane.[54] This situation exists even in well mixed solutions to a certain extent. If the solution were left undistrubed, some of the material that should pass through the membrane will consolidate into a second membrane which will reduce or cut trans-membrane flow. This can lead to an increased rejection of the membrane permeating species. To counter this effect the solution was stirred and kept at dilute concentration levels, by allowing the cell to be filled with either deionized

water or 1% sodium thiosulphate solution during the ultrafiltration process.

3. Diafiltration

The samples introduced into the ultrafiltration cell were either centrifuged bile, or bile samples which had been partially purified by passing it through a glass column packed with Amberlite XAD-2 (Rohm and Haas). All filtrations were carried out at room temperature, in the dark or in the presence of two 25 watt light bulbs shielded with a red filter and at a distance of two feet.

The solvent reservoir (see fig. 16) was filled with either 1 % aqueous $Na_2S_2O_3$ (to prevent oxidation) or deionized water. The cell was kept under pressures ranging from 45-75 psi., by nitrogen gas. During the filtration nitrogen was directed through the solvent reservoir so that for each ml. of liquid diafiltrate that passed through the membrane, one ml. of water or sodium thiosulphate solution was put back into the cell. This kept the cell liquid volume constant so as to prevent polarization of the membrane. The solution in the cell was also stirred constantly by means of a magnetic stirrer. The filtrate was tested quantitatively for bile pigments and qualitatively for cholesterol and bile salts periodically. Occasionally the pressure was cut, and the cell was opened so that the bilirubin content of the retentate in the cell could be measured.

4. Concentration or Ultrafiltration

The nitrogen gas could be directed directly into the cell, causing a decrease in volume of the bile and a greater concentration of bilirubin per unit volume. The manufacturer of the apparatus referred to this

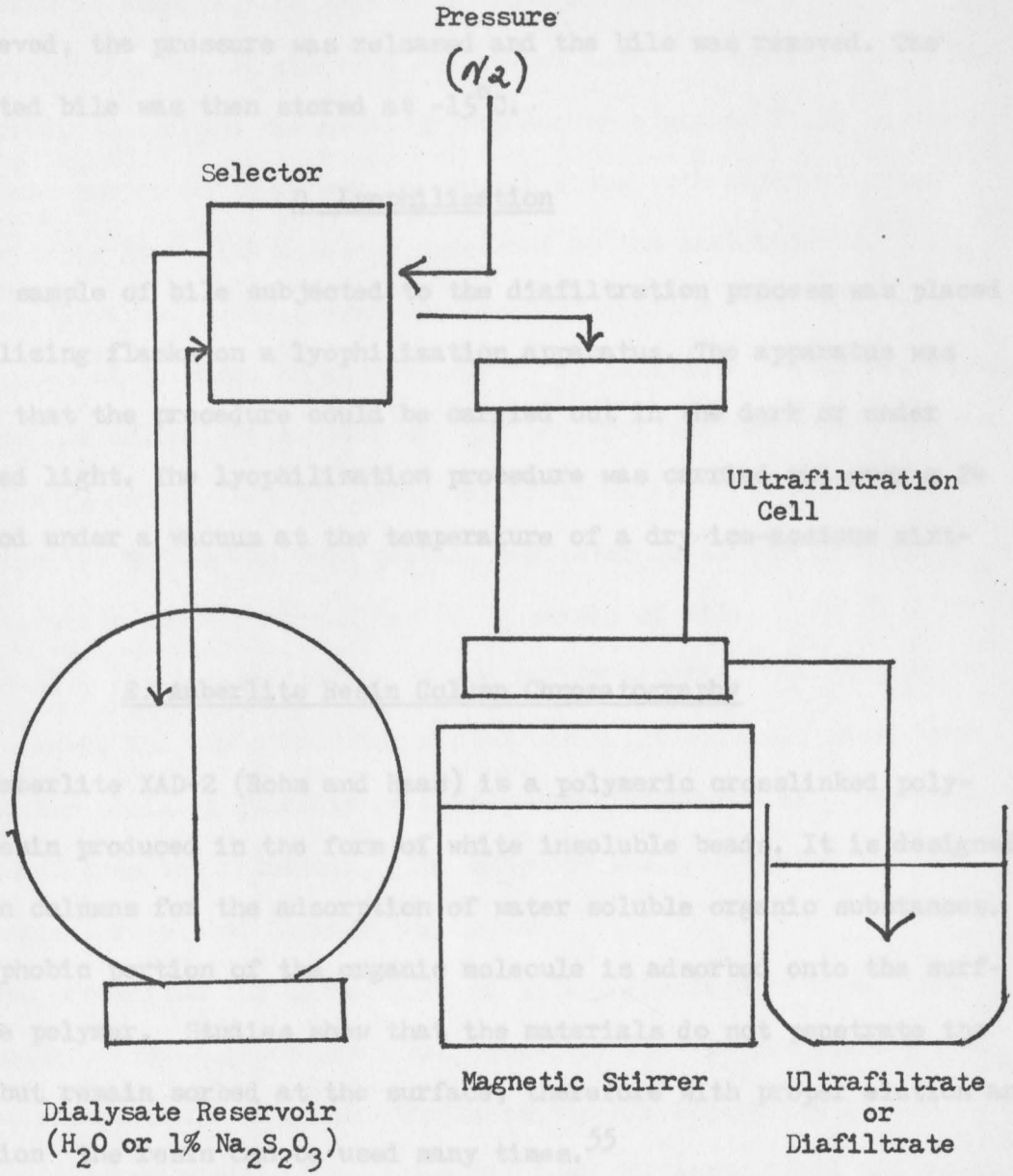


Fig. 16. Amicon Ultrafiltration Apparatus

process as ultrafiltration. When a two or three fold concentration had been achieved, the pressure was released and the bile was removed. The concentrated bile was then stored at -15°C.

D. Lyophilization

A sample of bile subjected to the diafiltration process was placed in lyophilizing flasks on a lyophilization apparatus. The apparatus was set up so that the procedure could be carried out in the dark or under subdued red light. The lyophilization procedure was carried out over a 24 hour period under a vacuum at the temperature of a dry ice-acetone mixture.

E. Amberlite Resin Column Chromatography

Amberlite XAD-2 (Rohm and Haas) is a polymeric crosslinked polystyrene resin produced in the form of white insoluble beads. It is designed for use in columns for the adsorption of water soluble organic substances. The hydrophobic portion of the organic molecule is adsorbed onto the surface of the polymer. Studies show that the materials do not penetrate the surface, but remain sorbed at the surface; therefore with proper elution and regeneration the resin can be used many times.[55]

One of the manufacturer's tests with this resin showed that it was able to remove 80% of the bile salt sodium cholate present from a solution containing 2.0 g. sodium cholate and 2.0 g. sodium chloride in 200 ml. of water.[55] Since the bile salts are present in bile at a concentration of approximately 1.0 g. per 100 ml., the Amberlite XAD-2 was used to effect a partial removal of the bile salts from the bile.

Prior to packing, the Amberlite XAD-2 had been rinsed with a solution containing sodium chloride and sodium carbonate by the manufacturer, to control mold and bacterial growth during storage. Prior to use the resin was subjected to a four bed volume rinse with water at a two gallon per cubic foot flow rate, as suggested by the manufacturer.[55]

An 11x300 mm. glass column was prepared, with a glass wool plug and a stopcock at the base. An aqueous slurry of Amberlite resin was slowly poured into the column while water was drained from the base. Care was taken to always assure that the resin was under water.

When the column had been packed, the water level was lowered to about 2 mm. above the top of the resin. A sample of bile, usually 50, 100 or 150 ml., was layered on the column with a pipette. The stopcock was then opened, and the column was eluted until the bile was level with the top of the resin. A quantity of water, usually 300-500 ml., was then carefully added to the reservoir. The stopcock was then opened and the bile pigments were eluted through the column.

Two procedures for the elution of bile pigments were used. These will be referred to as procedure I and procedure II. In procedure I the column was eluted until there was the first visible indication of the bile pigments being eluted. The eluate was then collected until the eluate appeared to lose its strong pigmentation. If 50 ml. of bile were placed on the column, then 50 ml. of strongly pigmented eluate was collected.

In procedure II, after the bile had been placed on the column, and the water had been added to the reservoir, all the eluate was collected, from the first aliquot of water being eluted to the last noticeable evidence of pigment in the eluate. This usually involved collecting 150 ml. of eluate for a 50 ml. sample of bile.

The aqueous bile pigment eluates were quantitatively assayed for bilirubin, cholesterol and bile salts. The eluates were also chromatographed on Eastman Chromagram sheets. The eluates were stored in the dark at -15° C. Most of these eluates were further purified by ultrafiltration.

After use the Amberlite resin had taken on a definite green coloration. It was washed with 200 ml. portions of acetone, methanol and pH 7.4 phosphate buffer. Between runs the column was routinely washed with 1,000-1,200 ml. of deionized water.

F. Silica Gel Column Chromatography

All column chromatography work was done in subdued red light. A slight modification of thin layer chromatography solvent system A was used: acetone, butanol, propionic acid, water (7:4:3:1) . This mixture will be referred to as mixed solvent . The quantity of water was reduced so as to reduce the polarity of the solvent system, to achieve a better separation of the bilirubin monoglucuronide and diglucuronide. Three columns using two different support media were used.

The eluates of all the columns passed through a polypropylene stopcock, into glass and polyethylene tubing. A careful choice of tubing was made because of the possibility of plasticizers being leached out of the tubing by the solvent.

The eluate was passed through a Thomas Flow Cell in a transistorized Spectronic 20. The Spectronic 20 was connected to a Sargent SRL chart recorder, set at slow speed (0.2 inch per minute). The spectrophotometer wavelength was set at 450 nm. initially, but after the third run it was set at 420 nm., as this was consistently shown to be the wavelength of

maximum extinction of bile pigment in this particular solvent system. The eluates were stored in the dark at -15° C. The eluate solution did not freeze at this temperature.

Column number 1 was a 15 x 250 mm. glass column with a built in porous filter at the base. Silica gel, 0.2-0.5 mm., was stirred thoroughly in water and allowed to settle, after which the fines were decanted. This procedure was repeated two more times. The silica gel was pre-equilibrated by putting it into the mixed solvent system and letting it stand for one hour. The solvent slurry of silica gel was then poured into the column, care being taken to assure that the level of the solvent was always above the silica gel. If at any time the solvent level went below the silica gel, the column was re-packed.

Samples were layered onto the column by the following procedure. The solvent level was allowed to drop to within one mm. of the top of the silica gel. The bile sample was then added with a pipette. The stopcock was then opened and the bile was allowed to move into the silica gel. It was closed when the bile was one mm. below the top of the silica gel. The solvent was added very carefully and slowly, so that there would be no mixing with the bile. The flow rate of the elution was controlled by a stopcock and a pinch clamp attached to the polyethylene outlet tube.

Column number 2 was a 25 x 600 mm glass column with a 1,000 ml. solvent reservoir. It was packed with silica gel, 0.2-0.5 mm., which had been prepared in the same manner as column number one above. The column was kept under nitrogen during the entire elution. This was accomplished by feeding in nitrogen from a tank at approximately 1 psi. through a fitting above the solvent reservoir. A flow rate of 2.7 ml. per minute was used with a 3.0 ml. sample.

Column number 3 was exactly like column number 2 except that the column was packed with 100 mesh silicic acid. The silicic acid was added to water and stirred, after which it was allowed to sit for 15 minutes. The fines were decanted. This procedure had to be repeated 8 times because of the tremendous quantity of extremely fine silicic acid grains that were present. If these were not removed, they would have slowed the flow rate to a prohibitively slow speed. Sample volumes of 1.5, 3.0, 4.5, and 6.0 ml. were used. Flow rates between 1.0 amd 3.0 ml. per minute were used.

Since many of the eluates showed an absorbance greater than 2.0, they were quantitatively diluted with"mixed solvent", and then the absorbances were read on the spectrophotometer. A graph was drawn with absorbances from 0 to 5.0 plotted, which were calculated by using a dilution factor.

G. Treatment of the Column Chromatography Eluates

The silica gel and silicic acid column eluates, mainly conjugated bilirubin dissolved in "mixed solvent", were stored at - 15° C. in the dark. The "mixed solvent" remained a liquid at this temperature. Small aliquots were occasionally withdrawn for thin layer chromatography work and quantitative bile salt, cholesterol and bilirubin determinations. The diazo test was run over a period of days to determine the stability of the conjugated bilirubin under these conditions.

To separate the solid bilirubin conjugates from the solvent a Buchi Rotary Evaporator was used. The solvent was rotated in a round bottom flask in a 37° C. water bath, while being subjected to a vacuum from a vacuum pump. A trap immersed in dry ice-acetone, was placed between the evaporator and the pump. This is where all the vaporized solvent

accumulated. When the evaporation was completed nitrogen gas was always bled into the system,so that the solid bilirubin conjugates were not exposed to oxygen.

A solid red powder, thus obtained was then dissolved in either deionized water or 0.1 M pH 7.4 phosphate buffer. The water and buffer were de-oxygenated by boiling for five minutes and bubbling nitrogen gas through while they cooled. These solutions were stored in the dark, in a flask flushed out with nitrogen gas, at -15° C. Samples were then taken over the next few days to determine the stability of the bilirubin conjugates in these solvents.

The phosphate buffer samples were used for the glucuronic acid determinations of the eluates. The actual eluates themselves could not be used because the "mixed solvent" reacted with the reagents to give a dense dark cloudy precipitate.

Spectral scans were taken on all"mixed solvent" eluates and all phosphate buffer and deionized water preparations. This work was done with a Beckmann DB spectrophotometer connected to a Sargent Model SRL Recorder. The spectral scans were run between 800 and 320 nm. When the absorbance of the eluate was too high for a spectral scan, it was quantitatively diluted with"mixed solvent"to bring it within the range of the instrument.

CHAPTER V.

RESULTS AND DISCUSSION

A. Thin Layer Chromatography

1. Bile

Bile sample I was human T-tube post-operative bile collected in 1970 and stored at -15° C. for two years prior to use. Sample volumes ranging from 5 to 15 microliters were spotted on Eastman Chromagram silica gel G sheets, which were developed in solvent system A. One half of the sheet was sprayed with diazo spray followed by methanol, while the other half was sprayed with zinc acetate spray. All of the spots showed some streaking, though there were three definitely indentifiable spots. The two spots of lower R_f values reacted directly with the diazo spray, while the spot that travelled with the solvent front reacted only after the sheet was sprayed with methanol. The two direct reacting spots were probably bilirubin monoglucuronide and diglucuronide while the indirect reacting spot was probably free bilirubin. The results of this run are shown in table 5.

Bile sample II was human T-tube post operative bile, collected July 1972, and containing 1% $Na_2S_2O_3$. It was stored at -15° C. until use. The Malloy Evelyn test showed 37 mg.% direct reacting and 42 mg.% total bilirubin. Two Chromagram sheets were spotted, developed in solvent system A, and sprayed with diazo or zinc acetate spray. There was some streaking with some of the spots, but it was worse with larger samples. From these

runs it was concluded that smaller samples were desirable if streaking was to be kept to a minimum. The results are shown in table 5.

Bile sample III was human, T-tube post-operative bile collected July, 1973, containing 1% $Na_2S_2O_3$. It was centrifuged at 20,000 g's (11,000 rpm.) at 4° C. in an MSE high speed centrifuge. The Malloy Evelyn assay showed 35 mg.% direct reacting and 38 mg.% total bilirubin. The bile was stored at -15° C. and thawed in warm water when needed.

Two sheets were spotted with portions of bile sample III, developed in solvent system A and sprayed with diazo spray. The results of these runs are shown in table 5.

Tenhunen[16] used the same solvent system with bile samples on glass plates coated with silica gel G, but his resulting R_f values were quite different from those reported in this paper. He found the indirect reacting free bilirubin at R_f 0.70, while the monoglucuronide and diglucuronide conjugates had R_f values of 0.50 and 0.35. Killmeyer,[45] used solvent system A with glass plates coated with silica gel G and Eastman Chromagram sheets. On the coated glass plates the two direct reacting pigments moved with R_f values of 0.45 and 0.66, while the indirect reacting spot moved with the solvent front. Using Eastman Chromagram sheets, Killmeyer found that the first spot moved with R_f values ranging from 0.55-0.62, while the second spot moved with R_f values ranging from 0.74 to 0.80. The indirect spot travelled with the solvent front. Both of the direct reacting spots and the indirect reacting spot travelled at approximately the same rate as Killmeyer's in this work.

The slow moving yellow, diazo negative spots are similar to the polar oxidation products reported by Thompson and Hoffman.[42]

TABLE 5

RESULTS OF T.L.C. OF BILE

Bile sample number	Sample volume (microliters)	Spray reagent	R_f values
I	5	diazo	.55 .70 1.00
I	10	diazo	.55 .70 1.00
I	10	diazo	.55 .70 1.00
I	12	diazo	.55 .70 1.00
I	15	diazo	.55 .70 1.00
I	5	zinc acetate	.50 .65 1.00
I	10	zinc acetate	.50 .65 1.00
I	10	zinc acetate	.50 .65 1.00
I	12	zinc acetate	.50 .65 1.00
I	15	zinc acetate	.50 .65 1.00
II	10	diazo	.51 .68 streaked
II	25	diazo	long streak, no resolution
II	10	zinc acetate	.51 .68
II	25	zinc acetate	nothing visible
II	2	diazo	.50 .70
II	4	diazo	badly streaked
II	7	diazo	badly streaked
II	10	diazo	badly streaked
II	2	zinc acetate	.53 .73
II	4	zinc acetate	badly streaked
II	7	zinc acetate	badly streaked
II	10	zinc acetate	badly streaked
III	5	diazo	.15(yellow) .52 .72
III	5	diazo	.15(yellow) .52 .72
III	5	diazo	.57 .82
III	5	diazo	.56 .74
III	5	diazo	.55 .72

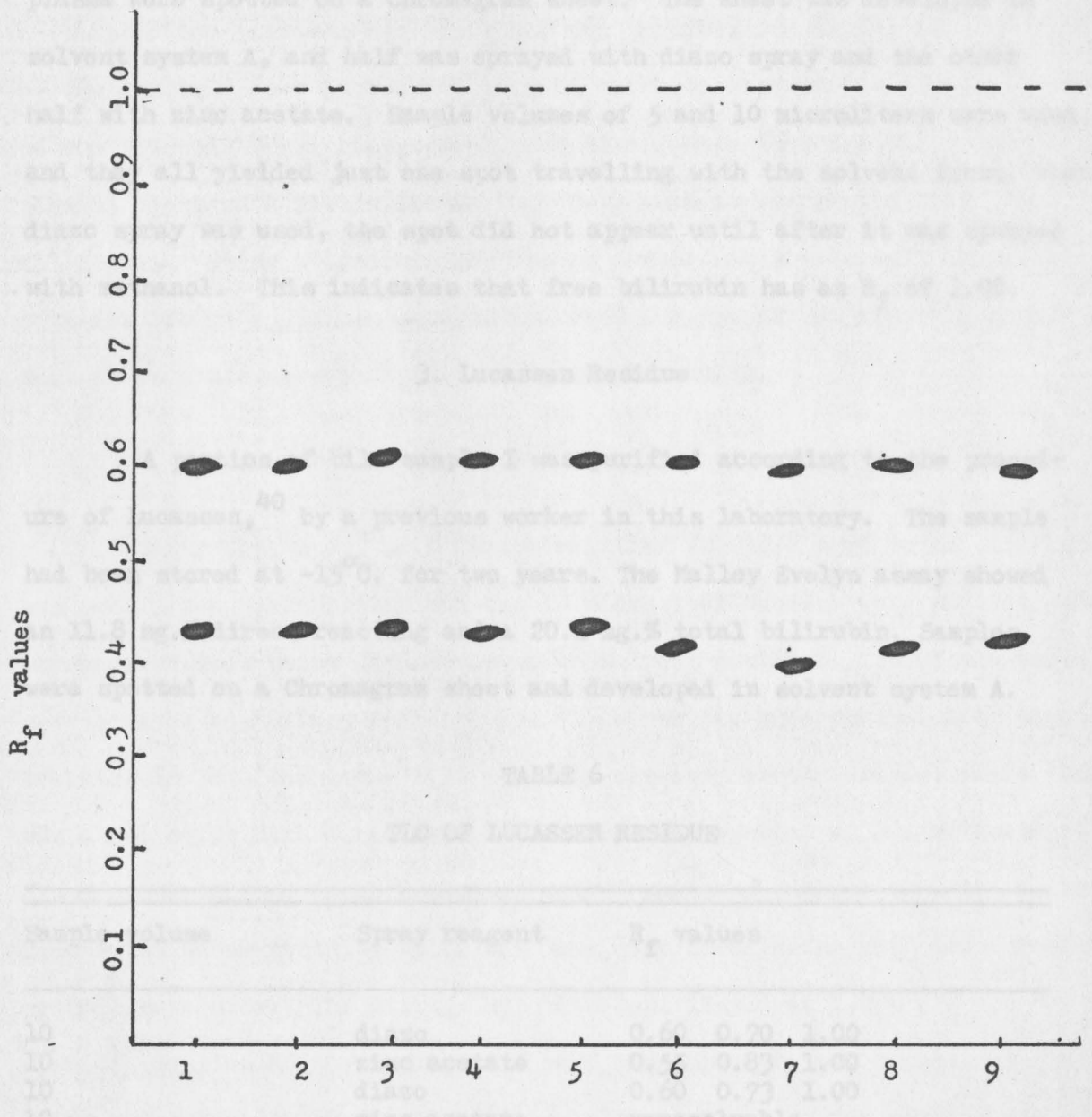


Fig. 17. Thin Layer Chromatography of Ultrafiltered Bile Spots # 1-5 sprayed with Diazo; # 6-9 sprayed with Zinc Acetate

2. Bilirubin in ACD Plasma

Portions of a 20 mg.% solution of commercial bilirubin in ACD plasma were spotted on a Chromagram sheet. The sheet was developed in solvent system A, and half was sprayed with diazo spray and the other half with zinc acetate. Sample volumes of 5 and 10 microliters were used, and they all yielded just one spot travelling with the solvent front. When diazo spray was used, the spot did not appear until after it was sprayed with methanol. This indicates that free bilirubin has an R_f of 1.00.

3. Lucassen Residue

A portion of bile sample I was purified according to the procedure of Lucassen,[40] by a previous worker in this laboratory. The sample had been stored at -15°C. for two years. The Malloy Evelyn assay showed an 11.8 mg.% direct reacting and a 20.2 mg.% total bilirubin. Samples were spotted on a Chromagram sheet and developed in solvent system A.

TABLE 6

TLC OF LUCASSEN RESIDUE

Sample volume	Spray reagent	R_f values
10	diazo	0.60 0.70 1.00
10	zinc acetate	0.55 0.83 1.00
10	diazo	0.60 0.73 1.00
10	zinc acetate	unresolvable
10	diazo	0.60 0.73 1.00
10	diazo	unresolvable

Generally the Lucassen residue spots that were resolved streaked less and were clearer than those from samples of centrifuged bile. The unresolvable spots were probabably the result of improper drying. The better resolution of the Lucassen residue spots was probably due to the greater purity of the sample, since Lucassen's solvent extraction procedure removes much of the bile salts and cholesterol from bile. Note that the slower conjugated pigment migrated a little faster (R_f= 0.6) than it did in the bile (R_f=0.5). Killmeyer,[45] using a Lucassen residue on Chromagram sheets with solvent system A found R_f values of 0.62 and 0.80 for the direct reacting pigments. This is in close agreement with the present results.

4. Sera From Patients With Hyperbilirubinemia

Serum with a 7.3 mg.% direct reacting and 10.4 mg.% total bilirubin was used. Two 5 microliter and two 10 microliter samples were spotted on a Chromagram sheet which was developed in solvent system A. Two of the spots were sprayed with diazo reagent, and the other two were sprayed with zinc acetate. In both cases the bile pigments remained at the origin, where there was a red color with diazo spray and a red fluorescence with zinc acetate.

One part of serum was mixed with 9 parts of ethanol to denature and remove the serum proteins which may have been interfering with the chromatographic separation. The mixture was then centrifuged at 1,200 g's for 5 minutes. Samples of the supernatant were then spotted on a Chromagram sheet which was subsequently developed with solvent system A. The results are shown in table 7.

A 60 microliter sample of serum with 24 mg.% total bilirubin was spotted on a Chromagram sheet and developed in solvent system A. The result is shown in table 7.

TABLE 7

TLC OF SERUM

Sample	Sample volume (microliters)	Spray reagent	R_f values
Deproteinized serum	10	diazo	.86
Deproteinized serum	10	zinc acetate	.84
Deproteinized serum	25	diazo	.63 .80
Deproteinized serum	25	zinc acetate	.61 .82
Serum	60	diazo	.54 .74

The pigments moved at the same rate in the serum as they did in bile, but when the serum proteins are denatured and removed, both of the pigments migrate at a faster speed. The lack of free bilirubin at R_f 1.00 was probably due to its low concentration in this particular serum sample. All diazo sprays were followed by methanol.

5. Bile Subjected to Ultrafiltration and Diafiltration

A portion of bile sample II was subjected to 4 days of diafiltration at 20°C. under 65 psi. pressure with nitrogen gas. Samples of the diafiltered were spotted side by side with samples of centrifuged bile sample II. The diafiltered bile generally gave sharper spots, less streaking, better resolution and they dried faster after spotting. The latter may have been due to the removal of lipid materials during the diafiltration. The only changes in the rate of migration seemed to be more related to the size of the sample rather than the purity. The Malloy Evelyn reaction showed a 14 mg.% direct reacting and 22 mg. % total bilirubin for the diafiltered bile. The results are shown in

table 8.

The diafiltered bile was then subjected to four more days of diafiltration under the same conditions. Samples were again spotted on a Chromagram sheet and developed in solvent system A. The results are shown in table 9. The Malloy Evelyn assay showed a 6 mg.% direct reacting and 22 mg.% total bilirubin.

TABLE 8

TLC OF BILE AFTER 4 DAYS DIAFILTRATION

Sample	Sample volume (microliters)	R_f Values
Diafiltered bile	3	.50 .71 1.00
Cent. bile	3	.50 .69 1.00
Diafiltered bile	8	.41 .63 1.00
Cent. bile	8	.44 .65 1.00
Diafiltered bile	15	.41 .63 1.00
Cent. bile	15	unresolvable

TABLE 9

TLC OF BILE AFTER 8 DAYS DIAFILTRATION

Sample volume (microliters)	Spray reagent	R_f values
2	diazo	.15 .47 .71 1.00
4	diazo	.15 .50 .73 1.00
6	diazo	.15 .47 .75 1.00
8	diazo	.15 .45 .75 1.00
10	diazo	.15 .45 .75 1.00
2	zinc acetate	----------
4	zinc acetate	.50 .74
6	zinc acetate	.50 .74
8	zinc acetate	.50 .72
10	zinc acetate	.54 .76

A portion of bile sample III was brought to pH 6.0 with .1N HCl, put into the ultrafiltration cell, and concentrated from a starting volume of 200 ml. to a final volume of 42 ml. Two 10 microliter samples were spotted on a Chromagram sheet which was then developed in solvent system A. The results are shown in table 10. The Malloy Evelyn bilirubin assay showed a 92 mg.% direct reacting and 114 mg.% total bilirubin.

TABLE 10

TLC OF pH 6.0 ULTRAFILTERED BILE

Sample volume (microliters)	Spray reagent	R_f values
10	diazo	.15 .55 .75
10	diazo	.15 .55 .75
10	diazo	.15 .55 .75

No free bilirubin spot at the solvent front was observed. This may have been due to the insolubility of free bilirubin in water at pH 6.0. Values for the direct reacting pigments were in the same range as centrifuged bile and diafiltered bile.

6. Two Dimensional Development Without Hydrolysis

A 5 microliter sample of bile sample II was subjected to two dimensional development without hydrolysis as described in chapter IV. A full Chromagram sheet was used. It was developed with solvent system A and sprayed with diazo followed by methanol. It yielded yellow spots at the origin and the solvent front. There were two direct reacting spots, the slower one having a purple color and the faster one having a red

color. (See fig. 18) The fact that they had different colors was a pH effect, since the purple spot turned red when exposed to NH_3 vapors, and they both turned purple when exposed to HCl vapors.

A 10 microliter sample of bile sample II was subjected to two dimensional development, and the sheet was sprayed with diazo spray and methanol after development. Again there were two strongly pigmented spots, the one with the lower R_f value being purple and the one with the higher R_f value being red. There were also yellow areas at the origin and at the solvent front.

A 15 microliter sample of bile sample II was subjected to two dimensional development and sprayed with zinc acetate spray. There were two spots between the origin and the intersection of the solvent fronts, and one spot at the intersection of the solvent fronts. All the spots were yellow and fluoresced red under ultra violet light. (See fig. 19)

7. Two Dimensional Development with Hydrolysis

Chromagram sheets were spotted, developed in solvent system A, and subjected to 24 hours of hydrolysis in a tank of NH_3 vapors. It was then rotated 90° and developed in solvent system C, which consists of butanol, acetic acid and water (12:3:5). After development it was sprayed with a 3% para-anisidine hydrochloride in butanol (water saturated) solution, and heated in a 100° C. oven for 2 to 3 minutes.

Sample volumes of 3 and 15 microliters were used in two separate runs, and both yielded two definite dark brown spots, indicative of glucuronic acid.

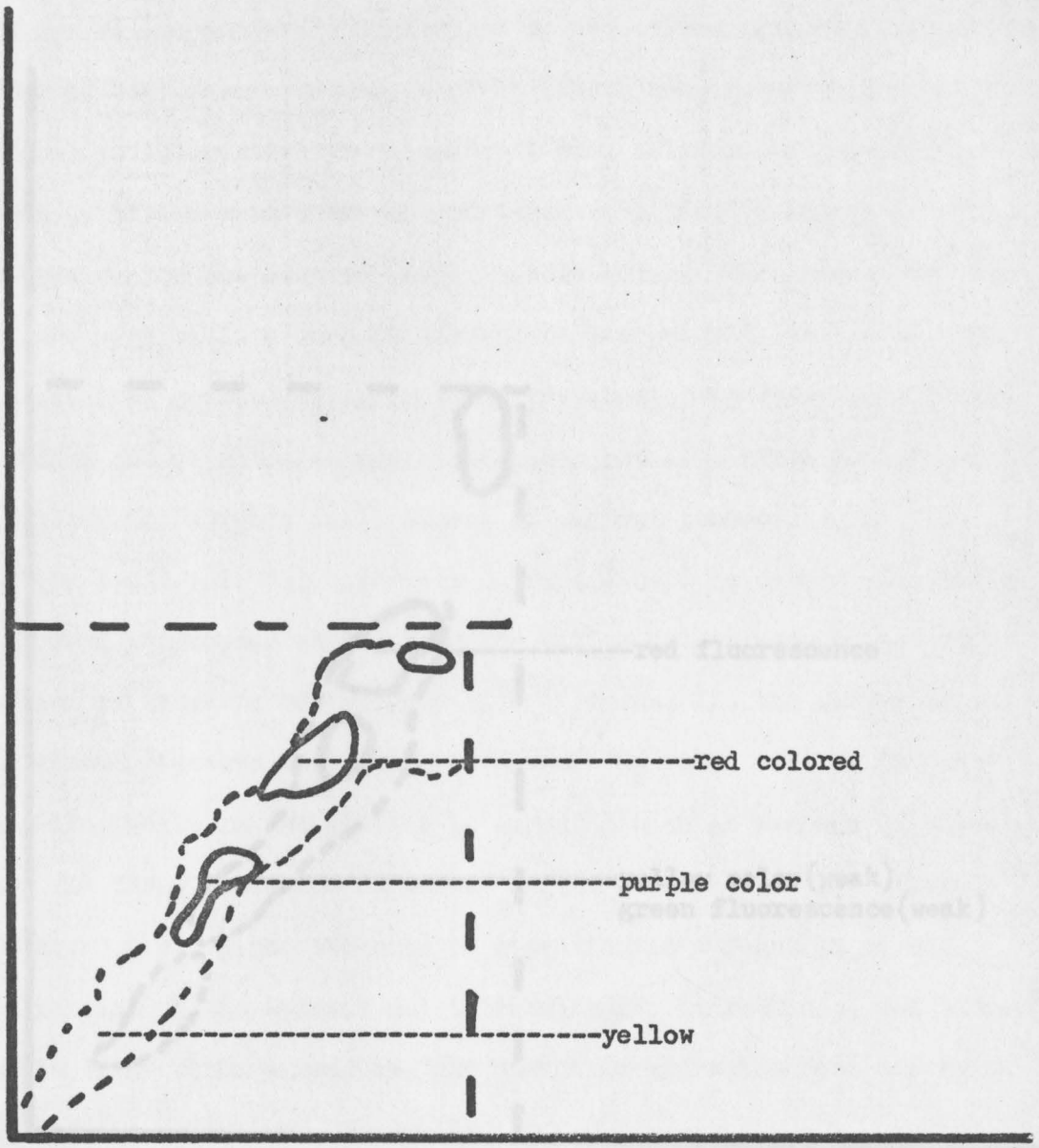


Fig. 18 Two Dimensional Chromatography without Hydrolysis of 15 microliters of Bile Sample II; Sprayed with Diazo Spray

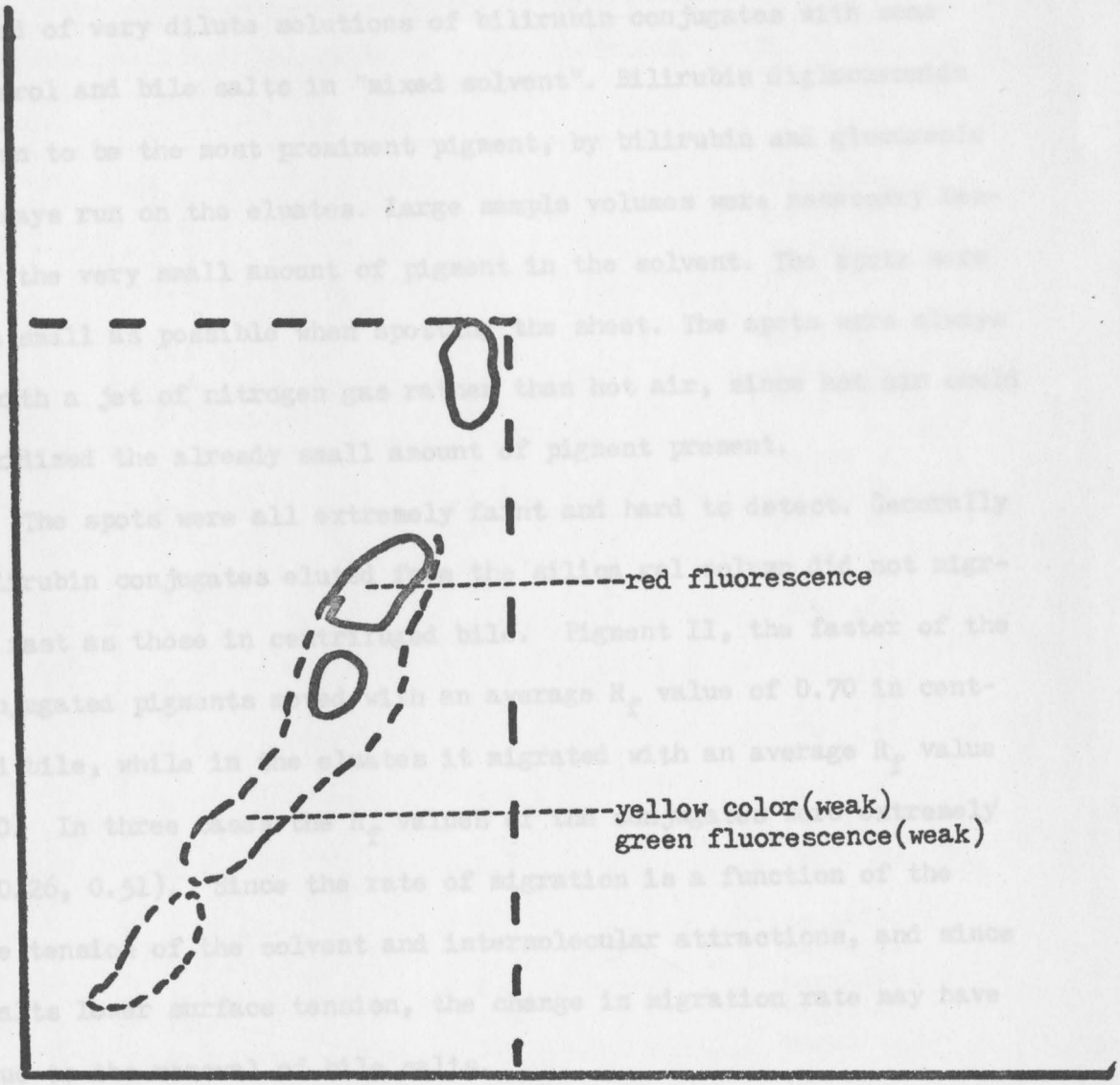


Fig. 19. Two Dimensional Chromatography Without Hydrolysis of 15 microliters of Bile Sample II; Sprayed with Zinc Acetate Spray.

8. Column Chromatography Eluates

The silica gel and silicic acid column chromatography eluates consisted of very dilute solutions of bilirubin conjugates with some cholesterol and bile salts in "mixed solvent". Bilirubin diglucuronide was shown to be the most prominent pigment, by bilirubin and glucuronic acid assays run on the eluates. Large sample volumes were necessary because of the very small amount of pigment in the solvent. The spots were kept as small as possible when spotting the sheet. The spots were always dried with a jet of nitrogen gas rather than hot air, since hot air could have oxidized the already small amount of pigment present.

The spots were all extremely faint and hard to detect. Generally the bilirubin conjugates eluted from the silica gel column did not migrate as fast as those in centrifuged bile. Pigment II, the faster of the two conjugated pigments moved with an average R_f value of 0.70 in centrifuged bile, while in the eluates it migrated with an average R_f value of 0.60. In three cases the R_f values of the conjugates were extremely low (0.26, 0.51). Since the rate of migration is a function of the surface tension of the solvent and intermolecular attractions, and since bile salts lower surface tension, the change in migration rate may have been due to the removal of bile salts.

The results of this work are shown in table 11. The samples in table 11 are grouped, so that all the samples from the same elution run are together. It can be seen that there is a great amount of internal consistency when three samples of the same eluate were run together. The three samples that had very slow migration rates (R_f values 0.26,0.51) were from an eluate that contained only .01 g. bile salt per 100 ml. This represented a large reduction in the bile salt concentration, when

TABLE 11

TLC OF COLUMN CHROMATOGRAPHY ELUATES

Sample volume (microliters)	Direct bilirubin mg.%	Total bilirubin mg.%	R_f values and comments
15	1.6	1.6	negative
15	1.6	1.6	negative
15	1.6	1.8	negative
15	1.0	1.0	negative
5	1.1	1.1	negative
10	1.1	1.1	negative
15	1.1	1.1	negative
10	1.2	1.2	negative
30	1.2	1.2	.51 .60
40	1.2	1.2	.51 .60
15	1.0	1.0	.45 .66
30	1.0	1.0	.45 .66
20	2.0	2.0	.26 .51
20	2.6	2.6	.29 .48
20	2.6	2.6	.34 .53
20	2.0	2.3	.44 .62
20	2.0	2.3	.50 .65
20	2.0	2.3	.46 .60
20	1.6	1.8	negative
20	1.6	1.8	negative
20	1.6	1.8	negative
50	1.6	1.8	negative
50	1.6	1.8	negative
50	1.6	1.8	.51
10 [1]	1.8	2.6	.54 .70
50 [2]	0.5	0.8	.65

1- eluate evaporated to dryness, dissolved in water, extracted with chloroform, water layer spotted

2- eluate evaporated to dryness and dissolved in phosphate buffer

compared to the original centrifuged bile which had bile salt concentrations of between 1.0 and 2.0 grams per 100 ml.

No free bilirubin was observed at the solvent front, which was to be expected, since the eluates contained only minute amounts of free bilirubin or none at all.

B. Purification of Bile and Concentration of Pigments

1. Diafiltration and Lyophilization

Bile sample number 1 consisted of 386 ml. of post-operative human t-tube bile which was centrifuged at 3,441 g's at 4°C. for 15 minutes. The Malloy Evelyn bilirubin assay showed a 37 mg.% direct reacting and 42 mg.% total bilirubin, both before and after centrifugation. The whole sample was placed in the ultrafiltration cell with a 30 psi. nitrogen pressure being used to wash a 1% solution of sodium thiosulfate through. After 24 hours 200 ml. of diafiltrate had passed through. It contained less than 0.1 mg.% total bilirubin. After four days a total of 400 ml. of diafiltrate was collected.

A sample was taken from the cell and the Malloy Evelyn test showed a 15 mg.% direct reacting and 28 mg.% total bilirubin. Despite the precautions taken there was a loss of pigment due to hydrolysis and oxidation during the diafiltration process.

The pressure was now set at 65 psi., with deionized water being washed through the cell. After four days an additional 1250 ml. of diafiltrate was collected. The cell was opened and a layer of dark material covered the membrane while dark particles were found floating in the bile. The bile was centrifuged at 3,000 g's for five minutes, yielding a yellow precipitate that was insoluble in chloroform, slightly soluble

in ether, powdery in acetone and soluble in methanol. The Malloy Evelyn assay showed 6 mg.% direct reacting and 22 mg.% total bilirubin before and after centrifugation. The precipitate was never identified, but it may have been phospholipids which are both abundant in bile and soluble in slightly polar solvents.

The filter was washed with a 10% ethanol-water solution, and the pressure was set at 65 psi., with an additional 250 ml. of deionized water being washed through. The bile was removed from the cell, a small amount was retained for testing while the rest of it was put into a lyophilizing flask and lyophilized in the dark. The lyophilization took 24 hours after which the residue was weighed and transferred to a vacuum dessicator in the dark.

When the bile was suctioned from the cell the membrane was coated with a very dark material that was insoluble in water. A concentrated water suspension of this membrane coating material gave a 0.0 mg.% direct reacting and a 30 mg.% total bilirubin. It was free bilirubin which was no longer soluble in the bile because of the removal of some of the bile salts during the diafiltration process. The results of the diafiltration are shown in table 12.

TABLE 12

RESULTS OF DIAFILTRATION OF BILE

Sample	Direct reacting bilirubin	Total Bilirubin
Centrifuged bile	37 mg.%	42 mg.%
Bile after 4 days diafiltration	15 mg.%	28 mg.%
Bile after 8 days diafiltration	6 mg.%	22 mg.%

The results indicate that three chemical reactions were taking place in the ultrafiltration cell; the oxidation of free and conjugated bilirubin to free and conjugated biliverdin, and the hydrolysis of conjugated bilirubin to free bilirubin. The fact that the diafiltration was carried out at room temperature may have been responsible for these reactions.

The lyophilization process yielded 2.3 grams of material, which upon exposure to air turned green, even though it was kept in the dark or under subdued red light. A 20 mg.% solution of the lyophilization residue is ACD plasma was prepared. The Malloy Evelyn reaction showed a 0.5 mg.% direct reacting bilirubin and a 1.0 mg.% total bilirubin.

2. Amberlite XAD-2 Column Chromatography and Ultrafiltration

Bile sample 2 was human t-tube post-operative bile collected July 24,1973. It was centrifuged at 20,000 g's (11,000 rpm.) at 4° C. for fifteen minutes in a MSE-25 high speed cooled centrifuge. After centrifugation a small amount of white gray residue was discarded and the 700 ml. of bile collected were stored at -15°C. for further processing.

A 170 ml. sample of the bile was put on an 11x250 Amberlite XAD-2 column. The first 20 ml. of eluate, mostly water, was discarded. The next 150 ml. of eluate were collected. The eluate was put into the ultrafiltration cell and diafiltered with 1% $Na_2S_2O_3$ for 16 hours at 40 psi. The 600 ml. of diafiltrate collected contained no bilirubin. The pressure was raised to 65 psi. and the bile was concentrated to 75 ml. volume by forcing pure nitrogen gas into the cell. After concentration the purified bile was removed and quantitative tests for bilirubin, cholesterol and bile salts were run. The results are shown in table 13.

TABLE 13

RESULTS OF AMBERLITE CHROMATOGRAPHY WITH ULTRAFILTRATION

Assay	Cent. Bile 170 ml.	Amberlite eluate 150 ml.	Ultrafiltered bile 75 ml.
Direct bilirubin	56.1 mg.	46.5 mg.	30.4 mg.
Total bilirubin	71.4 mg.	51.8 mg.	47.2 mg.
Total/direct ratio	1.27	1.11	1.55
Cholesterol	325 mg.	315 mg.	164 mg.
Bile salts	3.1 g.	1.7 g.	1.1 g.

The Amberlite XAD-2 retained some of the bilirubin, having a greater effect on the free than the conjugated bilirubin. The 16 hours of ultrafiltration caused a greater loss of conjugated than free bilirubin, but the percentage of loss was much less than the previous run where a bile sample was subjected to eight days of diafiltration. The Amberlite resin had very little effect on the cholesterol, but it did remove considerable quantities of bile salt. This was to be expected since the manufacturer states that it will adsorb water soluble organic substances.

The loss of bile pigments during the diafiltration and ultrafiltration was probably due to a combination of hydrolysis and oxidation. The greater loss of conjugated bilirubin could be due either to hydrolysis or to the fact that it is much less stable than free bilirubin.[38]

Comparing the two runs it was concluded that concentrating the bile in the ultrafiltration cell (ie. ultrafiltration) will produce a greater reduction in bile salts and cholesterol while it will not have as great an effect on the concentration of conjugated bilirubin as long periods of diafiltration did.

3. Diafiltration of pH 7.0 Bile

A portion of bile sample 2 was adjusted to pH 7.0 with .1N HCl. Exactly 50 ml. of the pH 7.0 bile was placed in the ultrafiltration cell, and 1,550 ml. of $Na_2S_2O_3$ were washed through the cell over a 5 day period. The volume was maintained at 50 ml. and the diafiltered bile was removed from the cell. Assays for bilirubin, bile salts and cholesterol were run on a portion of the bile, while the rest was stored at -15°C. The results are shown in table 14.

TABLE 14

RESULTS OF DIAFILTRATION OF pH 7.0 BILE

Assay	Cent. bile	pH 7.0 bile	Diafiltered bile
Direct bilirubin	17.0 mg. 34 mg.%	17.0 mg. 34 mg.%	9.5 mg. 19 mg.%
Total bilirubin	21.0 mg. 42 mg.%	21.0 mg. 42 mg.%	14.0 mg. 28 mg.%
Total/direct ratio	1.23	1.23	1.47
Cholesterol	108 mg. 216 mg.%	88 mg. 176 mg.%	66 mg. 132 mg.%
Bile Salts	0.9 g. 1.8 g%	0.6 g. 1.2 g%	0.6 g. 1.2 g%

The adjustment to pH 7.0 had no effect on the bilirubin concentration, but it did cause a drop in the cholesterol and bile salt concentration. The extended period of diafiltration caused a drop in both free and conjugated bilirubin, but it had a greater effect on the conjugated. The drops in cholesterol and bile salts due to the diafiltration were not as great as when ultrafiltration was utilized.

4. Amberlite Column Chromatography

Fifty ml. of bile sample 2 were placed on a column packed with Amberlite XAD-2, and eluted with water. One hundred ml. of pigmented eluate was collected. The eluate was then assayed for bilirubin and cholesterol. The results are shown in table 15.

TABLE 15

RESULTS OF AMBERLITE COLUMN CHROMATOGRAPHY

Assay	Cent. bile 50 ml.	Amberlite eluate 100 ml.
Direct bilirubin	16.5 mg.	16 mg.
Total bilirubin	21 mg.	19 mg.
Total/direct ratio	1.27	1.18
Cholesterol	100 mg.	100 mg.

Table 15 again indicates that the Amberlite resin is not effective in the removal of cholesterol. More of the indirect (free) bilirubin was retained on the column than direct reacting. The fact that an additional 50 ml. of water were eluted through the column may have been instrumental in the greater recovery of direct reacting bilirubin.

5. Ultrafiltration of pH 6 Bile

Twenty five ml. of .1 N HCl were added to 175 ml. of bile sample 2, lowering the pH to 6.0. The acidified bile was put directly into the ultrafiltration cell and concentrated to 42 ml. total volume. This process was completed in a few hours. The concentrated bile was removed, tests were run on a small aliquot, and the remainder was stored at -15°C.

The results are shown in table 16.

TABLE 16

RESULTS OF ULTRAFILTRATION OF pH 6 BILE

Assay	Cent. bile 175 ml.	pH 6 bile 200 ml.	Ultrafiltered bile 42 ml.
Direct bilirubin	59.5 mg.	59 mg.	38.6 mg.
Total bilirubin	71.4 mg.	59 mg.	47.9 mg.
Total/direct ratio	1.20	1.00	1.24
Cholesterol	380 mg.	392 mg.	110 mg.
Bile salts	3.15 g.	2.44 g.	0.86 g.

The drop in pH resulted in the precipitation of some of the bile salts and all of the free bilirubin. This was expected since free bilirubin becomes insoluble in water at acid pH values. The pH change had no effect on the concentration of cholesterol.

Until now there was no evidence whether the drop in concentration of conjugated bilirubin in the ultrafiltration cell was due to hydrolysis or just that the conjugated form was oxidized more readily than free bilirubin. Here the sample placed in the ultrafiltration cell contained only conjugated bilirubin, and after the ultrafiltration there was a mixture of conjugated and free bilirubin. There was also a drop in the total quantity of bilirubin. This means that both processes of oxidation and hydrolysis are taking place, under nitrogen in the dark.

The loss of bilirubin in the ultrafiltration cell was offset by the fact that the resulting sample contained over 100 mg.% total bilirubin. Ultrafiltration removed large portions of both cholesterol and bile salts, but their concentrations, expressed in mg.%, had increased

due to a decrease in volume. The ultrafiltration process removed water faster than either bile salts or cholesterol.

C. Silica Gel and Silicic Acid Column Chromatography

The basic aim of the column chromatography work was not only to separate the bile pigments from the cholesterol and bile salts that are also present in bile, but to also separate bilirubin diglucuronide from bilirubin monoglucuronide and free bilirubin. The procedure used involved eluting the bile pigments with an A_{max} around 430-450 nm., evaporating to dryness, and weighing out the solid residue so that it could be dissolved in phosphate buffer and tested for purity.

The column was set up so that the eluate flowed through a Thomas Flow Cell, into a Spectronic 20 connected to a Sargent Recorder, as described in Chapter IV. The recorder monitored the relative absorbance of pigment in eluate, and if two pigments were eluted at different times, two peaks would show up on the chart.

Bilirubin assays were run on all the fractions, and bile salt and cholesterol assays were run on some of them. The solvent system used was acetone, butanol, propionic acid, water (7:4:3:1), referred to as "mixed solvent" in this paper. A solvent mixture substituting acetic acid for propionic acid was tried once, but the results were not satisfactory.

To prevent decomposition of the ultrafiltered bile to be used on the columns, the bile was divided into 5 ml. portions stored in small dark bottles and stored at -15°C. Due to repeated thawing and re-freezing, these small samples did occasionally deteriorate. It was concluded that the ultrafiltered bile should be stored as sample volumes that

would only have to be thawed three times, after which most of the bile would be used up and the remainder discarded.

The butanol and propionic acid of the "mixed solvent" esterified upon standing, so that before the column was used it was always flushed with 200 ml. of fresh solvent to wash out the butyl propionate which had formed.

If the eluate absorbance exceeded 2.0, fractions of the eluate were collected, and diluted 1:10 with "mixed solvent". The absorbances were then taken of each fraction and plotted on a graph.

1. Results with a 15x250 mm. Silica Gel Column

The first seven runs were carried out on a 15x250 mm. glass column packed with silica gel. A sample volume of 0.75 ml. was used and between 10 and 25 ml. of eluate were collected. (See tables 17,18 and 19). The flow rates ranged from 0.4 to 2.0 ml. per minute. Bilirubin assays of the eluate fraction near the region of peak absorbance, on those runs with a slower flow rate, gave equal values of direct reacting and total bilirubin, indicative that only conjugated bilirubin was present in these fractions.

Six of the elution curves showed only one peak, while one of them showed two peaks. For the run which showed two peaks the Malloy Evelyn reaction was run on eluate fractions from each peak. From the results of the bilirubin assay from each peak it was concluded that an experimental error in introducing the bile to the column was responsible for the two peaks. In all cases the pigment moved through the column as a single uniform mass, and there was no visual evidence at any time that the pigment was breaking up into two or more fractions on the column.

TABLE 17

EFFECTS OF COLUMN CHROMATOGRAPHY ON CHOLESTEROL CONTENT OF ULTRAFILTERED BILE

Column	Flow rate (ml/min.)	Sample volume(ml.)	Bile Cholesterol	Eluate Cholesterol
15x250 mm. Silica gel	0.4	0.75	1.96mg.	0.89mg.
	0.75	0.75	2.0 mg.	1.66 mg.
	0.75	0.75	2.0 mg.	2.0 mg.
25x600 mm. Silica gel	2.7	3.0	6.8 mg.	6.8 mg.
25x600 mm. Silicic acid	1.0	2.5	5.45 mg.	3.75 mg.
	3.0	3.0	7.9 mg.	8.0 mg.
	3.0	3.0	8.0 mg.	8.0 mg.
	3.0	6.0	15.2 mg.	15.0 mg.
	3.0	15.0	39 mg.	40 mg.

TABLE 18

EFFECTS OF COLUMN CHROMATOGRAPHY ON BILE SALT CONTENT OF ULTRAFILTERED BILE

Column	Flow rate (ml./min.)	Sample volume(ml.)	Bile bile salts	Eluate bile salts
25x600 mm. Silica gel	2.7	3.0	0.06 g.	0.03 g.
25x600 mm. Silicic acid	1.0	2.5	0.037 g.	0.0081 g.
	3.0	6.0	0.12 g.	0.042 g.
	3.0	15.0	0.30 g.	0.16 g.

TABLE 19

EFFECTS OF COLUMN CHROMATOGRAPHY ON BILIRUBIN CONTENT OF ULTRAFILTERED BILE

Column	Flow rate ml./min.	Bile vol. ml.	Eluate vol. ml.	Direct bilirubin of bile	Total bilirubin of bile	Direct bilirubin of eluate	Total bilirubin of eluate
15x250 mm. Silica gel	2.5	0.75	10	40.5 mg.%	63 mg.%	6.5 mg.%	6.5 mg.%
	0.4	0.75	16	60 mg.%	100 mg.%	1.8 mg.%	1.8 mg.%
	1.0	0.75	15	60 mg.%	100 mg.%	1.6 mg.%	3.0 mg.%
	1.0	0.75	25	60 mg.%	100 mg.%	1.0 mg.%	1.1 mg.%
	0.75	0.75	23	60 mg.%	100 mg.%	2.1 mg.%	2.0 mg.%
	0.75	0.75	20	60 mg.%	100 mg.%	1.0 mg.%	1.0 mg.%
	0.75	0.75	20	60 mg.%	100 mg.%	2.0 mg.%	2.6 mg.%
	0.75	0.75	20	60 mg.%	100 mg.%	1.0 mg.%	1.2 mg.%
25x600 mm. Silica gel	2.7	3.0	120	40 mg.%	60 mg.%	1.2 mg.%	1.2 mg.%
25x600 mm. Silicic acid	1.0	2.5	65	40 mg.%	60 mg.%	1.1 mg.%	1.2 mg.%
	3.0	3.0	75	60 mg.%	100 mg.%	1.6 mg.%	1.8 mg.%
	3.0	3.0	60	60 mg.%	100 mg.%	1.0 mg.%	1.2 mg.%
	3.0	6.0	60	60 mg.%	100 mg.%	1.9 mg.%	1.8 mg.%
	3.0	15.0	180	60 mg.%	100 mg.%	4.4 mg.%	4.5 mg.%

Compared to the concentration of bilirubin in the ultrafiltered bile, the concentration of bilirubin in the eluate was sharply reduced. One reason was that 0.75 ml. of bile was being collected as 20 ml. of eluate. It was noted that the elution curve dropped off sharply at the end, and then leveled off at absorbance 0.2 (the maximum absorbance of the curve being greater than 1.0). The collection of fractions was stopped at this point.

Considering that a 0.75 ml. sample containing 0.45 mg. of direct reacting and 0.75 mg. total bilirubin was being placed on the column, and close to 0.5 mg. of direct reacting bilirubin only was being collected, the indirect reacting free bilirubin was probably being eluted later. Further evidence of this was that the amount of direct reacting compared to total bilirubin dropped when some fractions were collected near the end of the elution curve.

For the first three runs the spectrophotometer was set at 450 nm. Spectral scans of these three eluates and all subsequent eluates showed that the eluted bilirubin conjugates had an A_{max} at 420 nm. in this solvent system.

The cholesterol content of the eluates was measured. A fairly large reduction in the total amount of cholesterol in the eluate when compared to the bile was only achieved in one run, where the flow rate was 0.4 ml. per minute. For this run the cholesterol content was reduced from 1.86 mg. in the ultrafiltered bile to 0.89 mg. in the total volume of eluate. Two other runs had a flow rate of 0.75 ml. per minute, and in one run there was a reduction in cholesterol content from 2.0 mg. to 1.66 mg., while in the others there was no change at all.

It was concluded that using this column it was possible to produce a mixture in which conjugated bilirubin is the only form of bilirubin present. The cholesterol level could be reduced by using a slower flow rate. The next step was to use a larger column, where larger samples of bile could be introduced, and possibly even greater purity could be attained.

2. Column Chromatography Using a 600 x 25 mm. Column

A 600 x 25 mm. glass column was packed with silica gel (.2-.5 mm.) or 100 mesh silicic acid. Because of the larger size of the column, a faster flow rate was used so that the elution times would not be prohibitively long.

The yield of bilirubin from the eluates ranged from 1.0 to 4.5 mg.% total bilirubin. Samples ranging from 0.75 ml. to 15.0 ml. were put on the column, with 30 to 180 ml. of eluate being collected. In a few cases the direct reacting bilirubin exceeded the value for the total. Ostrow and Murphy,[38] working with a purified preparation of bilirubin diglucuronide, found that the diazo reaction gave a higher extinction before the addition of methanol (direct reaction) than it did after the addition of methanol, though they did not give any explanation for this phenomenon. Again the pigment moved through the column as a single dark red brown mass. There was no evidence, either on the column or in the elution curves, that the pigment was breaking up into two bands.

After a few runs, the dark brown residue at the top of the column was noted. On the silica gel column it slowly migrated through the column after many runs, while on the finer mesh silicic acid it remained at the top of the column. After a few more days it turned a distinct green

color. The pigment may have been bilimucin, a large molecular weight bilirubin mucopolysacharride complex, reported by Gent, Haslewood and Montesdecoa.[56]

The cholesterol concentration of the eluates ranged from 4.15 mg.% to 25 mg.%. The cholesterol content of the eluates was the same as that of the bile placed on the column, with one exception. In one run in which the flow rate was 1.0 ml. per minute, compared with 2.7 to 4.5 ml. per minute for the other runs, the total content of cholesterol in the eluate was reduced when compared to the bile sample. The total amount of bile salts in the eluate was considerably less than the bile salt content of the bile, but it was most sharply reduced in the one run where the 1.0 ml. per minute flow rate was utilized. (see table 19)

The conjugated bilirubin was very stable when stored in "mixed solvent", at - 15°C. in the dark. After three weeks of storage there was no change in the direct reacting and total bilirubin values of any of the eluates. These "mixed solvent" preparations were more stable than the preparations of bilirubin diglucuronide in phosphate buffer of Ostrow and Murphy.[38] The fact that the mixed solvent was liquid at -15°C. may have been instrumental in protecting the bilirubin conjugates. It is possible that hydrogen bonding between the bilirubin conjugates and acetone produces a sterically stable form that is resistant to oxidation. Szentirmay[57] concluded from electrochemical work with bilirubin that disruption of the hydrogen bonding between the carboxylic acid protons may lead to steric changes that are conducive to oxidation.

Pure bilirubin can be stabilized by intramolecular hydrogen bonding between the carboxylic proton and the nitrogen on the pyrrole ring. This is probably not available to bilirubin diglucuronide which has to

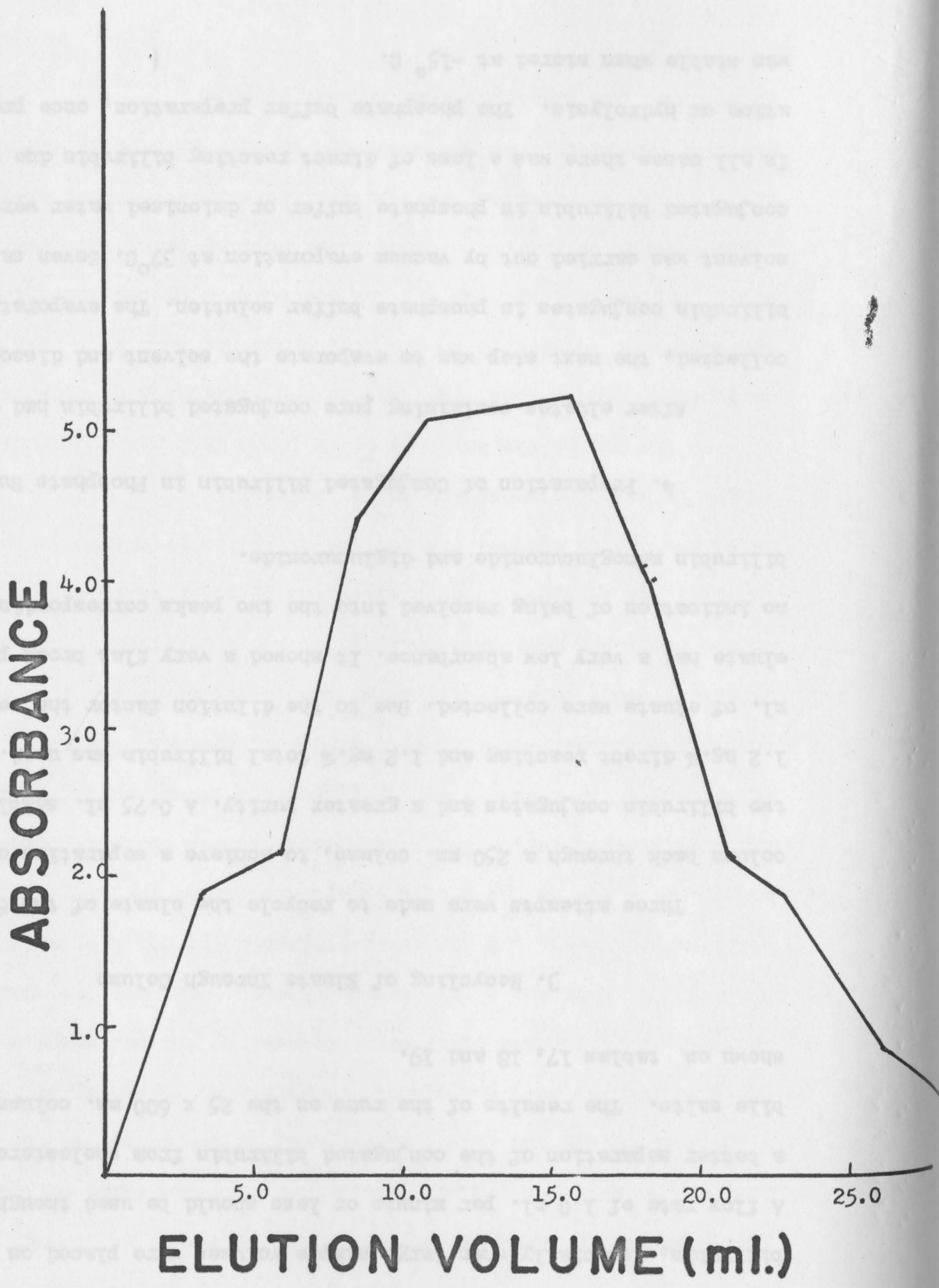


Figure 20. Elution Curve of 0.75 ml. of Ultrafiltered pH 6.0 Bile on 15 x 250 mm. Silica Gel Column

use intermolecular hydrogen bonding to solvent molecules.

The large column did provide a good total yield of conjugated bilirubin, especially when large sample volumes were placed on the column. A flow rate of 1.0 ml. per minute or less should be used though, to insure a better separation of the conjugated bilirubin from cholesterol and bile salts. The results of the runs on the 25 x 600 mm. column are shown on tables 17, 18 and 19.

3. Recycling of Eluate Through Column

Three attempts were made to recycle the eluate of the 600 mm. column back through a 250 mm. column, to achieve a separation of the two bilirubin conjugates and a greater purity. A 0.75 ml. sample with 1.2 mg.% direct reacting and 1.2 mg.% total bilirubin was used. Fifteen ml. of eluate were collected. Due to the dilution factor the recycled eluate had a very low absorbance. It showed a very flat broad peak, with no indication of being resolved into the two peaks corresponding to bilirubin monoglucuronide and diglucuronide.

4. Preparation of Conjugated Bilirubin in Phosphate Buffer

After eluates containing pure conjugated bilirubin had been collected, the next step was to evaporate the solvent and dissolve the bilirubin conjugates in phosphate buffer solution. The evaporation of solvent was carried out by vacuum evaporation at 37°C. Seven samples of conjugated bilirubin in phosphate buffer or deionized water were prepared. In all cases there was a loss of direct reacting bilirubin due to oxidation or hydrolysis. The phosphate buffer preparation, once prepared, was stable when stored at -15° C.

After all the solvent had been evaporated the residue had a dark red-brown color. After the addition of phosphate buffer the mixture immediately turned green. Oxidation by oxygen in the air was ruled out because nitrogen gas was always bled into the flask on the rotary evaporator before the flask was removed. Oxygen dissolved in the phosphate buffer was next suspected. Deoxygenated phosphate buffer and deoxygenated deionized water were prepared by the procedure outlined in Chapter IV. When the deoxygenated solvents were used the solution still turned green, but the total bilirubin did not drop significantly according to the Malloy Evelyn assay. The direct reacting bilirubin did drop though, indicating that hydrolysis was still a problem. Kuenzele, Weibel and Pelloni have recently proposed a structure for bilirubin that gains maximal stabilization from four hydrogen bonds.[58] This structure would be destabilized by the action of alkali , an ester bond on the propionic acid side chains, or the inhibition of hydrogen bond formation as a consequence of solvation by water molecules. The last mechanism may be responsible for the oxidation of bilirubin to biliverdin in water or phosphate buffer solvent.

The hydrolysis may have taken place during the evaporation. The evaporation took a long time, due to the difficulty of evaporating the last traces of butanol (b.p. 118°C.), propionic acid (b.p. 141°C.) and butyl propionate (b.p. 146°C.). If the solvent system used for chromatography could be altered so that lower boiling point solvents could be used, it may be possible to evaporate the solvent without loss of direct reacting bilirubin. As was mentioned previously though, acetic acid was substituted for propionic acid without success.

5. Yield of Solid Residue from Column Elutions

Fifteen ml. of silicic acid column eluate with 1.2 mg.% direct and 1.2 mg.% total bilirubin, were evaporated in a pre-weighed evaporation flask. It was found that minute amounts of silicic acid had been eluted with the solvent. Considering that the total amount of bilirubin in the eluate was less than 0.2 mg., any slight amount of contaminant would have had a large effect on the results. Possibly eluates containing as much as 10 mg.% bilirubin would be better for this purpose, so that the amount of bilirubin that would be present would be less subject to experimental errors.

6. Determination of Glucuronic Acid in Column Eluates

The Malloy Evelyn bilirubin assay showed that the eluates in some cases contained only conjugated bilirubin. The next step was to determine the ratio of bilirubin diglucuronide to bilirubin monoglucuronide. Samples of conjugated bilirubin dissolved in phosphate buffer or deionized water were used. The column eluates could not be used directly because of interference between the solvent system and the reagents necessary for the glucuronic acid determination.

The number of micromoles of glucuronic acid per ml. of solution was compared to the number of micromoles of direct reacting bilirubin in the original eluate before evaporation to dryness. This was done because even though there is oxidation and hydrolysis taking place during the evaporation step, these two processes will not change the amount of glucuronic acid present, while the amount of bilirubin may be reduced. The results are shown in table 20.

TABLE 20

RATIO OF GLUCURONIC ACID TO DIRECT REACTING BILIRUBIN IN ELUATES

Sample #	Direct Bilirubin	Total bilirubin	Ratio of glucuronic acid to direct reacting bilirubin
1.	1.2 mg.%	1.2 mg.%	2.0 : 1.0
2.	1.0 mg.%	1.0 mg.%	1.9 : 1.0
3.	0.4 mg.%	0.4 mg.%	1.34: 1.0
4.	1.2 mg.%	1.2 mg.%	1.77: 1.0
5.	1.2 mg.%	1.2 mg.%	1.77: 1.0
6.	1.2 mg.%	1.2 mg.%	1.77: 1.0

From the above data it can be concluded that in samples 1 and 2 almost all of the bilirubin was present in the form of the diglucuronide. Samples 4,5 and 6 contained more diglucuronide than monoglucuronide, while number 3 contained more monoglucuronide. This indicates that even though all the bilirubin conjugates came off as one peak on the elution curve, certain of the fractions may be found to contain all diglucuronide while other fractions would contain a mixture or just monoglucuronide. Complete separation of bilirubin diglucuronide from' monoglucuronide is possible by means of column chromatography using "mixed solvent".

D. Absorption Maxima of Bilirubin and Its' Conjugates

The literature gives many values for the absorption maxima of free bilirubin and its two glucuronic acid conjugates. The solvent system used is an important factor in determining the wavelength of maximum absorbance. This was demonstrated by Cole, Lathe and Billing[23], Jacobsen[39], and Ostrow and Murphy[38].

Ostrow and Murphy[38], using a solution of bilirubin diglucuronide in 50 millimolar phosphate buffer, found absorption peaks at 450 and 420 nm. The intensity of the two peaks was not changed by varying the ratio of bilirubin monoglucuronide to diglucuronide. Phosphate buffer preparations of bilirubin conjugates prepared in this laboratory consistently had an A_{max} at 410 nm. (table 21), and there was only one peak.(see fig. 22)

All except one of the silica gel and silicic acid column eluates in "mixed solvent" had absorption maxima at 420 nm. One of the eluates consistently showed an $A_{max.}$ at 440 nm. Why this one eluate whoud have an $A_{max.}$ different from all the rest was puzzling.

A deionized water preparation of bilirubin conjugates had an $A_{max.}$ at 420 nm., while a 2.5 mg.% preparation of commercial bilirubin in CPD plasma showed an $A_{max.}$ at 460 nm. (see fig. 22 and 23.)

The results of all the spectral scans are tabulated in table 21. It can be seen from table 21 that the $A_{max.}$ in the mixed solvent system is fairly constant, and it is independent of the ratio of free to conjugated bilirubin. The fact that the A_{max} is shifted to 460 nm. in CPD plasma may be due to the fact that the bilirubin is bonded to the serum proteins.

TABLE 21

ABSORPTION MAXIMA OF BILIRUBIN AND ITS CONJUGATES

Solvent system	Column used	A_{max}(nm.)	Direct reacting bilirubin	Total bilirubin
Mixed solvent	silica gel	440	1.1 mg.%	1.2 mg.%
Mixed solvent	silica gel	440	1.1 mg.%	1.2 mg.%
Mixed solvent	silica gel	440	1.1 mg.%	1.2 mg.%
Mixed solvent	silicic acid	420	1.0 mg.%	1.2 mg.%
Mixed solvent	silicic acid'	420	1.0 mg.%	2.7 mg.%
Mixed solvent	silicic acid	420	2.0 mg.%	2.6 mg.%
Mixed solvent	silica gel	420	1.8 mg.%	1.8 mg.%
Mixed solvent	silica gel	420	1.2 mg.%	1.2 mg.%
Mixed solvent	silica gel	420	1.0 mg.%	1.1 mg.%
Mixed solvent	silica gel	420	1.0 mg.%	1.1 mg.%
Mixed solvent	silicic acid	410	0.6 mg.%	3.0 mg.%
Mixed solvent	silicic acid	410	0.25 mg.%	0.6 mg.%
pH 7.4 phosphate buffer		410	0.4 mg.%	1.2 mg.%
		410	0.5 mg.%	0.8 mg.%
pH 7.4 phosphate buffer		410	1.2 mg.%	2.2 mg.%
		410	3.3 mg.%	3.9 mg.%
pH 7.4 phosphate buffer		410	0.25 mg.%	0.6 mg.%
		410	0.25 mg.%	0.6 mg.%
deionized water		420	0.5 mg.%	0.9 mg.%
2.5 mg.% bilirubin in CPD plasma		460	0.0	2.5 mg.%
2.5 mg.% bilirubin in CPD plasma		460	0.0	2.5 mg.%

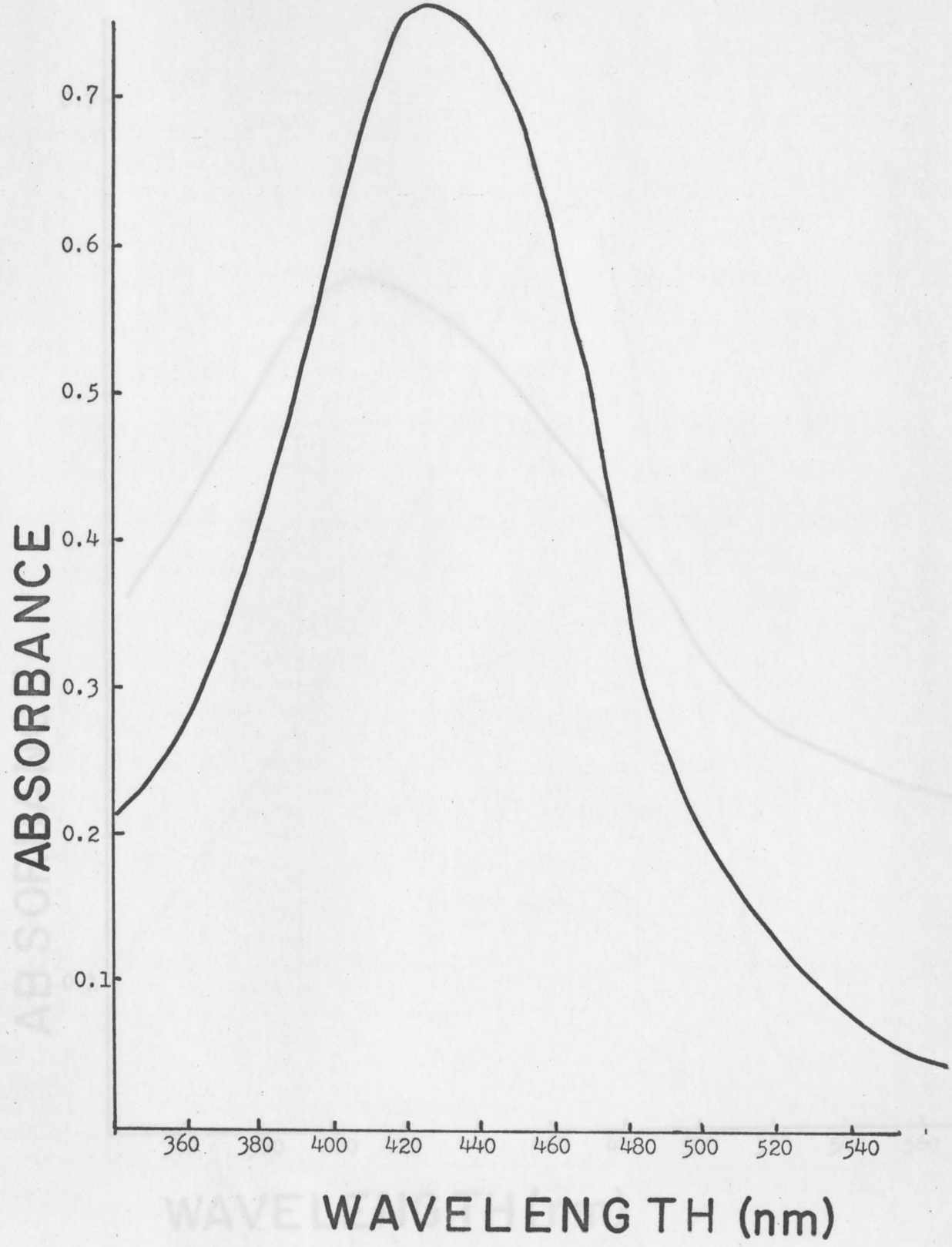


Figure 21. Absorption Spectrum of Bilirubin Conjugates in Solvent System of: Acetone, Butanol, Propionic Acid, Water (7:4:3:1)

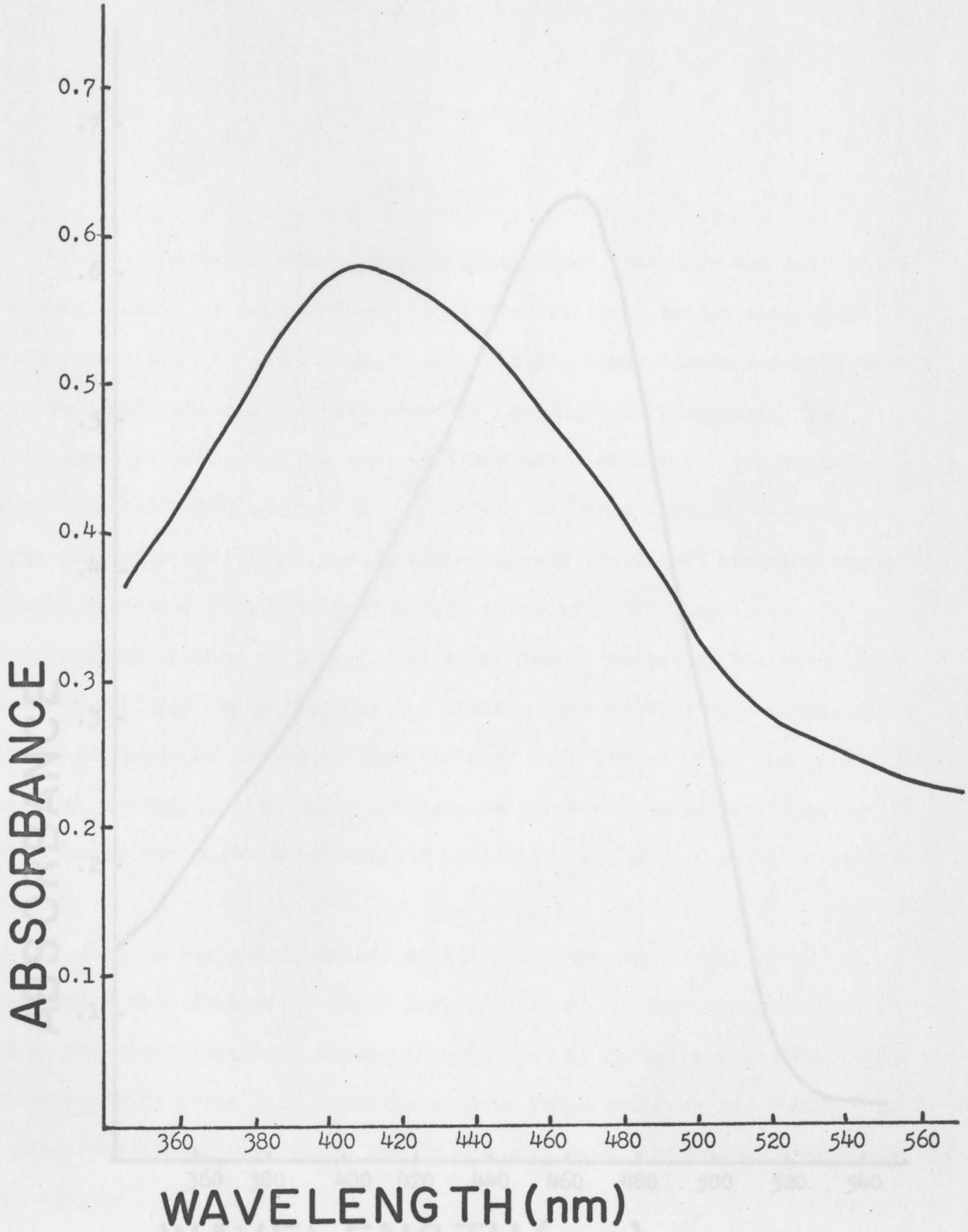


Figure 22 Absorption Spectrum of Bilirubin Conjugates in Phosphate Buffer.

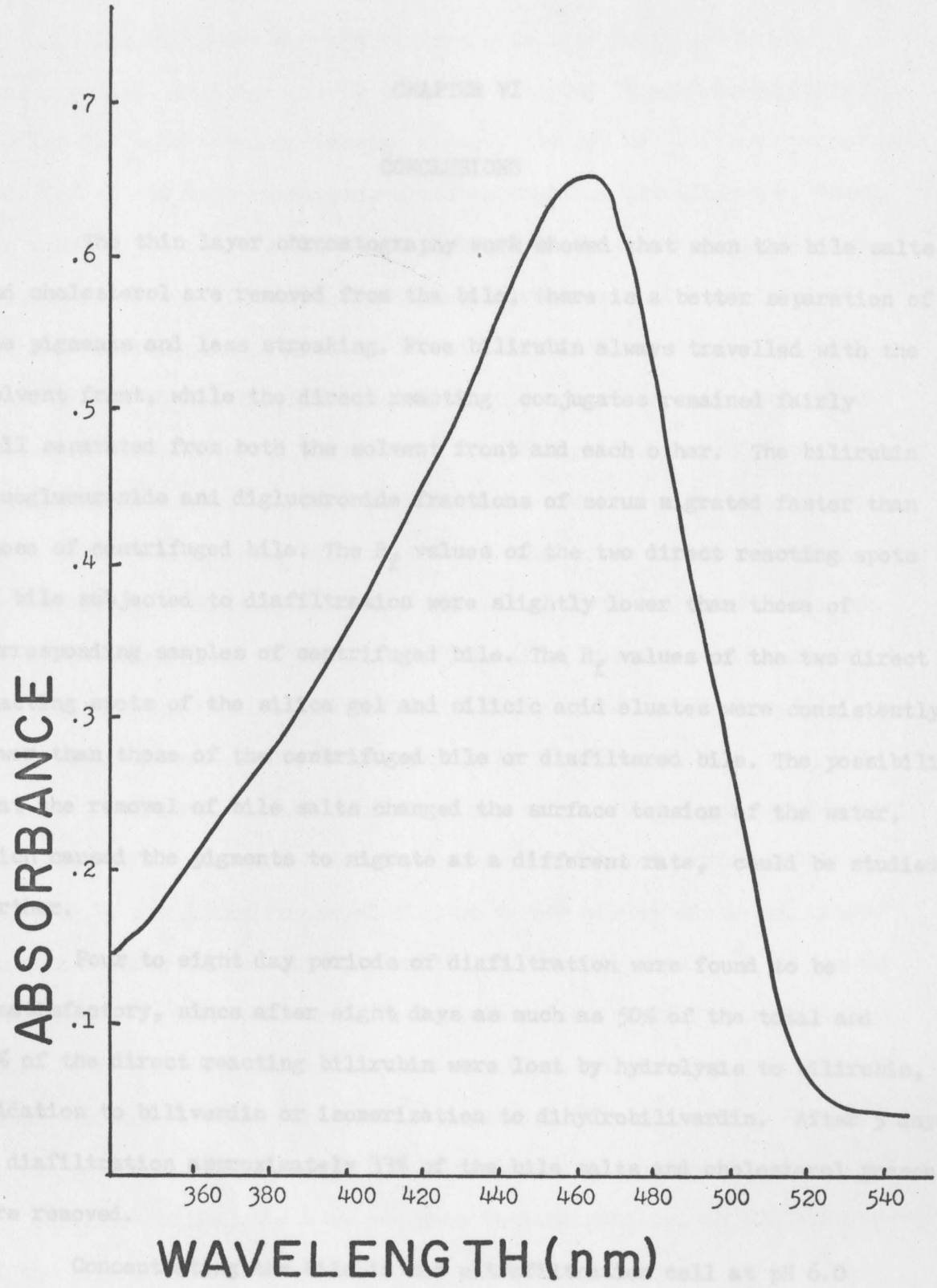


Figure 23. Absorption Spectrum of 2.5 mg.% Commercial Bilirubin in CPD Plasma-Phosphate Buffer

CHAPTER VI

CONCLUSIONS

The thin layer chromatography work showed that when the bile salts and cholesterol are removed from the bile, there is a better separation of the pigments and less streaking. Free bilirubin always travelled with the solvent front, while the direct reacting conjugates remained fairly well separated from both the solvent front and each other. The bilirubin monoglucuronide and diglucuronide fractions of serum migrated faster than those of centrifuged bile. The R_f values of the two direct reacting spots of bile subjected to diafiltration were slightly lower than those of corresponding samples of centrifuged bile. The R_f values of the two direct reacting spots of the silica gel and silicic acid eluates were consistently lower than those of the centrifuged bile or diafiltered bile. The possibility that the removal of bile salts changed the surface tension of the water, which caused the pigments to migrate at a different rate, could be studied further.

Four to eight day periods of diafiltration were found to be unsatisfactory, since after eight days as much as 50% of the total and 75% of the direct reacting bilirubin were lost by hydrolysis to bilirubin, oxidation to biliverdin or isomerization to dihydrobiliverdin. After 5 days of diafiltration approximately 33% of the bile salts and cholesterol present were removed.

Concentrating the bile in the ultrafiltration cell at pH 6.0 worked much better. Since the concentration, or ultrafiltration, could

be carried out in a few hours, only 36 % of the direct reacting and 18% of the total bilirubin were lost. Even with this loss, the resulting concentration yielded a bile solution containing 96 mg.% direct reacting and 114 mg.% total bilirubin. Seventy one per cent of the cholesterol and 73 % of the bile salts passed out through the ultrafiltrate. Since the ultrafiltration process was so sucessful, further experimentation, concentrating the bile to even smaller volumes, should be carried out.

Lowering the bile to pH 6.0 before putting it in the ultrafiltration cell caused the free bilirubin to precipitate while having no effect on the conjugated bilirubin. The pH may have changed during the ultrafiltration process, since a buffer was not used. The fact that hydrolysis of conjugated bilirubin was taking place in the ultrafiltration cell, was shown by the fact that the direct and total bilirubin values were identical before ultrafiltration, but the total exceeded the direct after ultrafiltration. The lowering of the pH caused a slight drop in bile salts and no change in cholesterol.

The removal of the bile salts was considered one of the greatest problems, since they are present in concentrations of over 1,000 to 2,000 mg.%. The Amberlite XAD-2 did not remove cholesterol, but in one case it removed 48% of the bile salts plus a small amount of conjugated bile pigment. More work could be done to determine if it would be worthwhile to recycle the eluate from the Amberlite column through a second Amberlite column. Also the use of larger Amberlite columns could be investigated. In tests with solutions of sodium cholate (a bile salt) and sodium chloride, the Rohm and Haas Company achieved an 88% retention of the bile salt on the column.

The silica gel and silicic acid columns were definitely able to achieve a complete separation of conjugated from free bilirubin when slow flow rates were used, but there was no separation of the monoglucuronide and diglucuronide conjugates as shown by TLC of the eluates. If the cholesterol and bile salt concentrations in the bile that is put on the column could be reduced to percentages that were fairly small in comparison to the bile pigments, possibly complete separation of these two lipid components from the bilirubin conjugates could be achieved.

The flow rate was a very important factor in the separation of cholesterol and bile salts on the column. With the 25 x 600 mm. column, when a flow rate of 3.0 ml. per minute was used there was no reduction in cholesterol and a 25-50 % reduction of bile salt content of the eluate as compared to the original sample of bile put on the column. When the flow rate was decreased to 1.0 ml. per minute, there was a reduction of 75% of the bile salts and a 32% reduction of cholesterol.

The migration rates of cholesterol and bile salts must be very close to those of the bile pigments under these conditions. One area of study would be to collect a group of fractions from an elution run and assay them for bilirubin, cholesterol and bile salts, to get some idea of the elution profile of each of these components. With slower flow rates a better separation of pigments from lipids would also be achieved. Fortunately the conjugated pigments are very stable in "mixed solvent", since the elution from the column may take up to a few hours.

If high pressure liquid chromatography would be used, very fine small size packings could be used, giving a larger surface area of adsorption and possibly even better separation.

Recycling the eluate back through a second silicic acid column to separate the two conjugates was unsucessful.

When the solvent was evaporated from the eluate, a red brown solid residue was left. This residue turned green and was partially oxidized to biliverdin or isomerized to dihydrobiliverdin when dissolved in phosphate buffer or deionized water. Using deoxygenated water prevented oxidation, but conjugated bilirubin was still hydrolyzed to free bilirubin.

The molar ratio of glucuronic acid to bilirubin in the eluates ranged from 1.34:1 to 2:1, which means that in one case the eluate fraction tested was pure bilirubin diglucuronide. The other eluates were probably a mixture of bilirubin monoglucuronide and diglucuronide.

Quantities of up to 15.0 ml. of bile were placed on the column, with no effect on the ability of the column to separate free from conjugated bilirubin.

The A_{max} of conjugated bilirubin or a mixture of free and conjugated bilirubin in"mixed solvent" was, with the exception of one eluate, at 420 nm. When the solvent was evaporated and the pigments were dissolved in phosphate buffer, the A_{max} consistently shifted to 410 nm. This shows that the wavelength of maximum absorbance is dependent on the solvent. Whether it is dependent on the presence of other substances in the solvent has not been determined. One of the eluates consistently showed an A_{max} at 440 nm. , and this puzzling shift may have been caused by binding to protein or some other component of the eluate.

In conclusion, these results indicate that the following steps should be followed. First the bile should be centrifuged and then passed through two large columns packed with Amberlite XAD-2. This

should remove a large protion of the bile salts present. This could be followed by a solvent extraction in chloroform to remove free bilirubin and cholesterol as Ostrow and Murphy had done.[38] This preliminary purification would probably allow a quicker passage through the membrane in the ultrafiltration process. Next the bile should be put into an ultrafiltration cell and concentrated to 1/8 or 1/10 of its original volume. This bile sample will then be quite free of lipid materials and further purification and complete isolation of the bile can be accomplished using a silicic acid or silica gel column. A very fine mesh packing, used in conjunction with a high pressure chromatography outfit, would provide for a better separation and also hold back the small amount of macromolecular and protein substances found in bile.

REFERENCES

1. M. Jirsa, "The Bile Pigments", Pigments in Pathology, ed. by M. Wollman, Academic Press, New York and London, 1969

2. T.K. With, Bile Pigments, Chemical, Biological and Clinical Aspects, Academic Press, New York, 1968

3. R. Schmid, New England Journal of Medicine, 257, 703

4. J. Routh, "Liver Function Tests", Fundamentals of Clinical Chemistry, ed. by Norbert Tietz, W.B. Saunders, Philadelphia, London and Toronto, 1970

5. A. White, P. Handler and E. Smith, Principles of Biochemistry, Mc Graw-Hill, New York, 1968

6. Reference 1, p. 172

7. Reference 2, p. 234

8. Reference 1, p. 159

9. Reference 2, p. 438

10. R. Lester and R. Troxler, Gastroenterology, 56, 1 (1959)

11. Reference 2, p. 122

12. J. Anino, Clinical Chemistry: Principles and Procedures, Little, Brown, 1964

13. C. Kuenzele, Biochem. J., 119, 395 (1970)

14. R. Broderson, H. Flogaard and J.K. Hansen, Acta Chem. Scan., 21, 2284 (1967)

15. Reference 1, p. 164

16. R. Tenhunen, Annales Medicinae Experimentalis Et Biologiae Fenniae, 43, Suppl. 6, (1965) Helsinki

17. K.J. Isselbacher and E.A. McCarthy, J. Clin. Invest., 38, 645 (1959)

18. C.H. Gregory and C.J. Watson, The Journal of Laboratory and Clinical Medicine, 60, 1 (1962)

19. J. Fevery, B. Van Damme, R. Michiels, J. De Groote and K.P.M. Heirweigh, The Journal of Clinical Investigation, 51, 2842 (1972)

20. K.P. Wong, Biochem. J., 125, 929 (1971)

21. B.H. Billing and F.H. Jansen, Gastroenterology, 61, 258 (1971)

22. C. Kuenzele, Biochem. J., 119, 387 (1970)

23. P.G. Cole, G.H. Lathe and B.H. Billing, Biochem. J., 57, 514 (1954)

24. D. Bratlid and A. Winsnes, Scand. J. Clin. and Lab. Invest., 28, 41 (1971)

25. J. Fogg and A.F. Bakken, Scand. J. Clin. and Lab. Invest., 20, 88 (1967)

26. M. Jirsa, A. Heringova and V. Jirsova, in Bilirubin Metabolism, ed. by. I.A.D. Bouchier and B.H. Billing, Blackwell, Oxford (1967)

27. J. Fogg, Proc. XIV Northern Pediat. Congr.,p.23 (1964)

28. W.V. Perelli and C.J. Watson, Clinical Chemistry, 16, 239 (1970)

29. B. Noslin, Scand. J. Clin.and Lab. Invest., 12, 49 (1960)

30. B.H. Billing, P.G. Cole and G.H. Lathe, Biochem. J., 65, 774 (1957)

31. Reference 2, p. 59

32. Reference 1, p. 161

33. P.G. Cole and G.H. Lathe, J. Clin. Path., 6, 99 (1953)

34. E. Talafant, Nature, 178, 312 (1956)

35. Reference 1, p. 160

36. D.W. Hutchison, B. Johnson and A.J. Knell, Biochem.J., 123, 483 (1971)

37. A. Cantarow and M. Trumper, Clinical Biochemistry, 3d. ed. Saunders, Philadelphia (1945)

38. D. Ostrow and N.H. Murphy, Biochem. J., 120, 311 (1970)

39. J. Jacobsen, Acta Chemica Scandanavica, 23, 3023 (1969)

40. Reference 2, p. 370

41. E. Talafant and J. Appelt, Clinical Chemistry, 14, 208 (1968)

42. R.P.H. Thompson and A.F. Hofmann, Clinica Chimica Acta, 35, 517 (1971)

43. C.F. Hayes, (Unpublished paper for senior research, Youngstown State University) 1968

44. K. Pavlicko, (Unpublished paper for senior research, Youngstown State University) 1969

45. A. Killmeyer (Unpublished paper for senior research, Youngstown State University) 1969

46. H.T. Malloy and K.A. Evelyn, J. Biol. Chem., 119, 481 (1937)

47. H.T. Malloy and K.A. Evelyn, J. Biol. Chem., 122, 597 (1938)

48. R. Kingsley, G. Getchell and R.R. Schaffert, Standard Methods for Clinical Chemistry, Vol. I, p. 11, Academic Press

49. Manual of Fluorometric Methods, G.K. Turner Associates (1970)

50. E.J. Mroszczak and S. Riegelman, Clinical Chemistry, 18, 987 (1972)

51. J. Folch, J. Lees and G.H.S. Stanley, J. Biol. Chem., 226, 497 (1957)

52. T. Bitter and H.M. Muir, Analytical Biochemistry, 4, 330 (1962)

53. B.A. Noir, E.R. Garay and M. Royer, Biochimica et Biophysica Acta, 100, 403 (1965)

54. Applications Manual; Concentrating, Desalting and Separating Solutions and Suspensions, pub. no. 427, Amicon Corp., Lexington Mass. (1972)

55. Amberlite XAD-2, Technical Bulletin-Ion Exchange Department, Rohm and Hass Co., Philadelphia, Pa.

56. W.L.G. Gent, G.A.D. Haslewood and G. Montesdecoa, Proceedings of the Biochemical Society, 122, 15 p (1971)

57. R. Szentirmay, (Unpublished Masters Thesis; Youngstown State University) (1973)

58. C. Kuenzele, M. Weibel and R. Pelloni, Biochem. J., 133, 357 (1973)