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Geometric Photoisomer Investigation of Bilirubin (Z,Z)-IXα

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GEOMETRIC PHOTOISOMER INVESTIGATION OF BILIRUBIN (Z,Z)-IXα

by

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ABSTRACT

GEOMETRIC PHOTOISOMER INVESTIGATION
OF BILIRUBIN (Z,Z)-IXα

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Numerous investigations of bilirubin have been reported in the literature. Correlations among these investigations can not be made because of multiple behavioral changes characteristic of the solubilized pigment. This research was initiated to evaluate bilirubin and its associated isomers in identical chemical environments.

Two different solvent systems were experimentally selected to study chemical behavior of bilirubin and occuring photoisomers. A ternary solution consisting of 1:3:1 chloroform:methanol:water was used exclusively in visible spectrophotometric analyses. Also, a pH 10.00 buffer was utilized. This latter system has successfully been used in spectrophotometric and voltammetric work.

Four different photochemical processes have been detected spectrophotometrically, one of which has not been previously reported. In addition, voltammetric potentials of photobilirubin have been determined and compared with bilirubin and biliverdin. This was made possible by a novel extraction procedure permitting investigation of photobilirubin by itself.

I would like to dedicate this work to my mother, father and Stacy, my wife, for all their love and support.

ACKNOWLEDGEMENTS

I would like to thank Dr. John D. Van Norman, my research director, for his enthusiasm, patience and helpful suggestions throughout the undertaking of this project. His time given to me has been invaluable. I would also like to thank Drs. Kenneth G. Brown and Myung H. Kim for their comments while sitting on my research committee.

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CHAPTER 1

INTRODUCTION

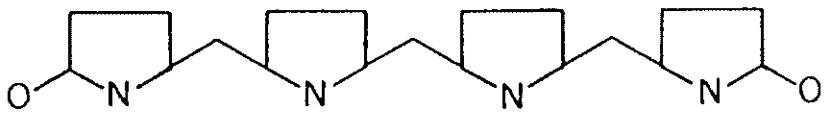
Bile Pigment Nomenclature

There are three basic criteria that must be adhered to in order for a macromolecule to be considered a bile pigment (1). First, the molecule must be a linear, open-chain tetrapyrrole. Second, an oxygen atom must be bound to each of the two terminal pyrroles. Third, bile pigments must conform to a specific skeletal structure (Figure 1A).

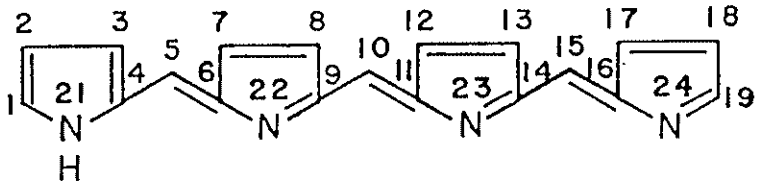
The nomenclature currently used in identifying bile pigments is based on the 21H-biline system (1). According to this system, a numbering scheme is used to identify different molecular positions (Figure 1B). Locating substituent positions on bilirubin is crucial to understanding its complex behavior.

Carbon atoms at positions C-5, C-10 and C-15 are called meso carbons, which are not to be confused with meso compounds or structures. Meso carbons C-5, C-10 and C-15 are also called a, b and c respectively. Meso compounds are molecules containing a chiral center, or a carbon atom with four different attached groups. Meso compounds are superimposable on their mirror images. In contrast, meso carbons refer to carbon atoms that link adjacent pyrroles through methyne or vinyl chains (=CH-). Bile pigments with meso linkages at carbons C-5 and C-15 are called 5,15-bilidienes (a,c-bilidienes) or rubins. An

Figure 1. Basic Structural Considerations of Bile Pigments. From top to bottom: (1A) Basic Skeletal Structure, (1B) 21-H Biline Numbering System and (1C) Lactam versus Lactim Tautomers.



IA



IB

LACTAM LACTIM

IC

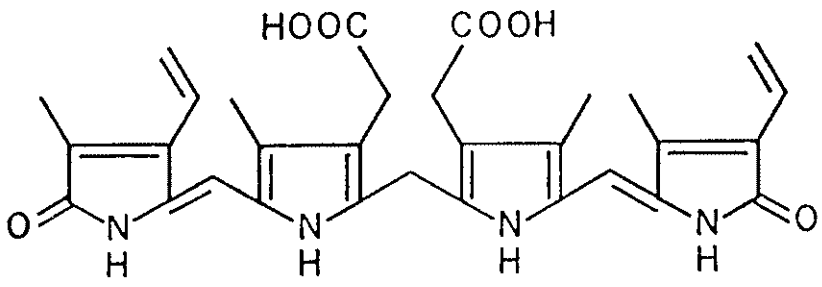
example of a rubin is bilirubin. Similarly, if a methyne linkage occurs at each of the three meso positions, this is referred to as a 5,10,15-bilitriene (a,b,c-bilitriene) or verdin of which biliverdin is an example.

Tautomerism, or proton migration, exists on oxygen atoms bound to carbons C-1 and C-19 of bile pigments. Tautomeric possibilities include both the lactam and lactim types (Figure 1C). The present consensus, supported by infrared studies, is that both bilirubin and biliverdin exist in the more stable bislactam form (1,2). This holds true regardless of whether the molecule is in solution or in the solid state.

Several options exist as to the type and arrangement of substituents that bind to carbons C-2, C-3, C-7, C-8, C-12, C-13, C-17 and C-18. The regions occupied by these carbons are collectively known as β positions. Usually β positions are bound to methyl, vinyl and propionic acid side chains. If, however, the β positions are bound to eight different substituents, a multitude of isomers which vary in moiety sequence is possible. Bilirubin has four methyl, two vinyl and two propionic acid moieties bound to β carbons (Figure 2A). Taking only this into consideration, a total of 52 structural isomers is possible for bilirubin (1).

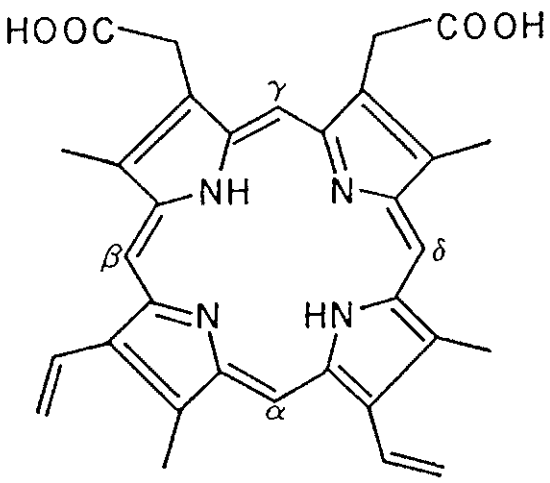
A Roman numeral system ranging from I-XV is used to designate the porphyrin isomer with the same sequence of β substituents from which a particular bile pigment was synthesized. This system is refered to as the Fischer system. Accordingly, bilirubin IX is the product of protoporphyrin IX degradation (Figure 2B).

There is no position 20 on linear structures of bile pigments.



BILIRUBIN (Z,Z)–IXα

2 A



PROTOPORPHYRIN IX

2 B

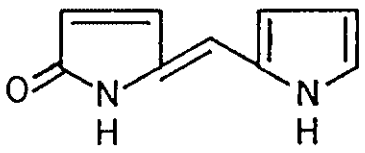
Position 20 is reserved for an imaginary carbon atom required to convert an open-chain tetrapyrrole back to its originally closed, porphyrin structure. A Greek lettering system using α, β, γ and δ indicates the point of porphyrin cleavage where carbon 20 is lost in going to a linear arrangement. Thus, bilirubin-IXα is synthesized by catabolism of protoporphyrin IX at the α meso bridge.

In explaining bile pigment nomenclature, one final area need be mentioned. Bile pigments can exist in one of several geometric forms. Meso bridges are used as reference points in determining whether a molecule has a cis or trans configuration. In making this determination, the Cahn-Ingold-Prelog sequence must be employed because bile pigments do not have simple cis-trans configurations (3).

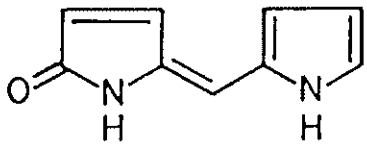
According to the Cahn-Ingold-Prelog sequence, the letters Z and E are used to differentiate between cis and trans isomeric forms (Figure 3A). If a higher priority species is on the opposite side of the meso bridge and molecule relative to another higher priority species, then a trans configuration is present and is represented by the letter E. The letter Z is used for doubly bound carbon atoms attached to higher priority species on opposite sides of the meso bridge, but on the same side of a molecule. Thus, in the Z configuration, the terminal, lactam nitrogen is cis to the endo pyrrole (Figure 3B).

Stereoisomerization

Stereochemistry is the science of molecular structure determination in three-dimentional space. Stereoisomerization is a branch of stereochemistry concerned with spatial arrangements of bonded substituents. Molecules having the same molecular formula but not necessarily the same arrangement of bonded substituents are called



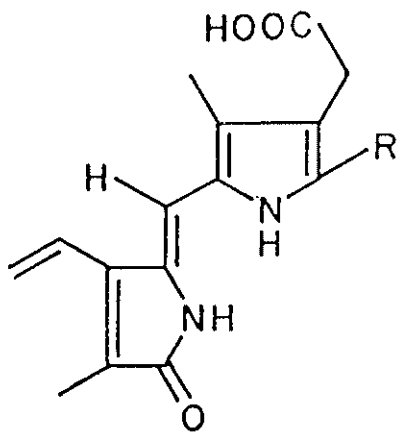
Z—CONFIGURATION



E—CONFIGURATION

LINEAR REPRESENTATIONS

3A



NON-LINEAR REPRESENTATION
OF Z—CONFIGURATION

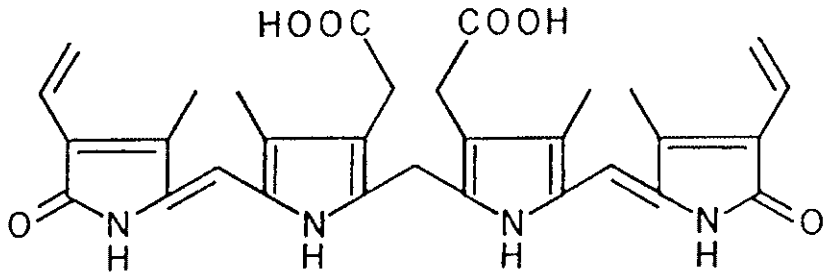
3B

isomers. Two isomers that differ from one another in size and shape are called structural isomers. If, however, two isomers differ only in the way their atoms are positioned in space, they are termed stereoisomers. Hence, stereoisomers are types of isomers which have different substituent orientations.

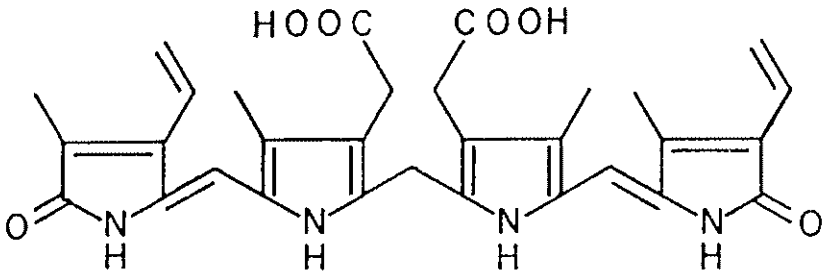
Stereoisomers can be classified as configurational or conformational. Configurational isomers are characterized by difficulty in interconverting from one form to another. Interconversion takes place by inversion or turning inside out at chiral centers, which always results in covalent bond breaking (3). Furthermore, the change between isomeric forms is slow due to the large energy barrier that must be overcome. Conformational isomers are relatively easy to interconvert from one form to another. This is because their energy barrier is small. Interconversion of configurational isomers simply requires rotation about single bonds.

Configurational isomers can exist in pairs and each pair can be further subdivided into two categories. Enantiomers are configurational isomers that are not superimposible on their mirror image. Many forms of bilirubin can exist as an enantiomeric pair. Diastereomers are similar to enantiomers only in that each species of the pair contains exactly the same atoms. A diastereomer, however, is not a mirror-image isomer since it has different arrangements of atoms. Bilirubin IIIα, bilirubin IXα and bilirubin XIIIα are all diastereomers (Figure 4).

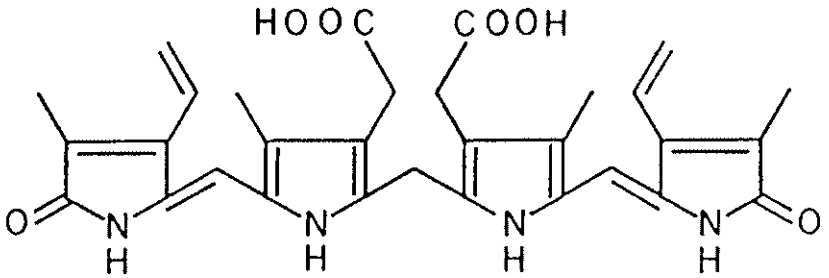
Geometric isomers are a further subdivision of diastereomers and, consequently, configurational isomers. A geometric isomer is characterized by two carbons doubly bound to each other. The



BILIRUBIN IIIα



BILIRUBIN IXα



BILIRUBIN XIIIα

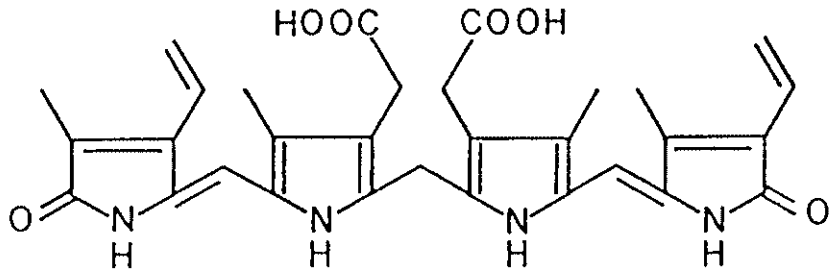
carbon-carbon double bond accounts for hindered rotation about the bond. Bilirubin (Z,Z)-IXα, bilirubin (Z,E)-IXα, bilirubin (E,Z)-IXα and bilirubin (E,E)-IXα are all geometric isomers (Figure 5).

Naturally occuring bilirubin has a Z configuration at both C-5 and C-15 meso bridges. Accordingly, the parent pigment is represented as bilirubin (Z,Z)-IXα. However, for simplicity, all references to bilirubin (Z,Z)-IXα will be referred to as bilirubin.

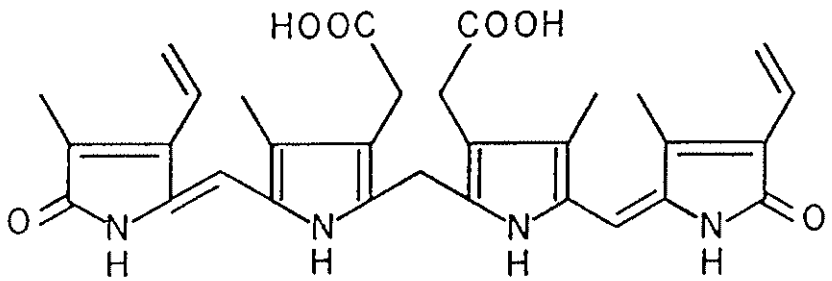
In general, configurational isomers interconvert from one form to another through the expense of covalent bond breaking. Unlike enantiomers and diastereomers that interconvert by inversion, geometric isomers rotate about carbon-carbon double bonds. Thus, a π bond is broken in the process and a new bond will have to immediately form in order to regain molecular integrity.

When bilirubin is interconverted to an E-configuration, individual isomeric forms can be resolved, indicating a relatively slow event. This is configurational isomerization by definition. However, a claim has been made that Z--->E interconversion takes place by inversion instead of rotation about carbon-carbon double bonds, as would be the case for typical cis--->trans processes (4). Thus, even though bilirubin and photobilirubin are routinely identified as geometric isomers, explainations of their interconversion are not characteristic of truely geometric processes. If bilirubin does unfold to E-configurational products, the event is actually more typical of diastereomers. That being the case, it is more appropriate to use general termonology of configurational isomerization when describing Z--->E interconversions.

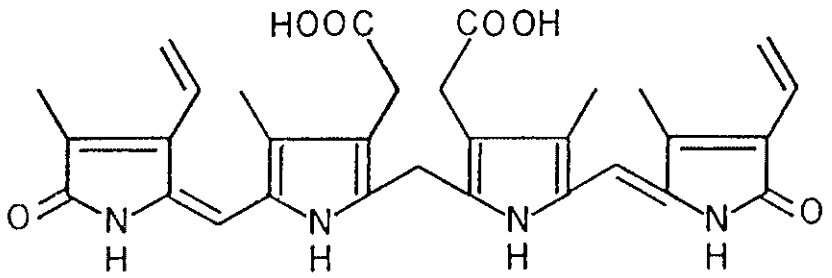
Bilirubin Chemistry



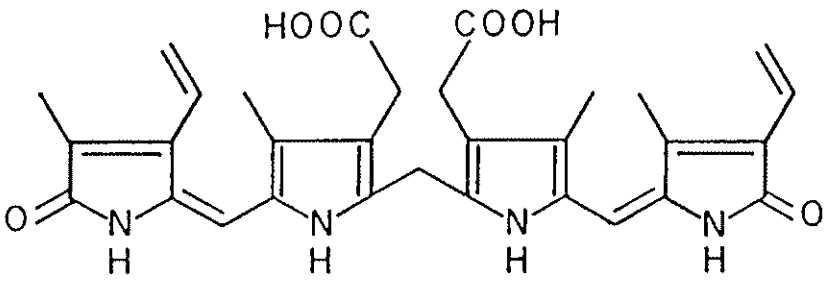
BILIRUBIN (Z,Z)–IXα



BILIRUBIN (Z,E)–IXα



BILIRUBIN (E,Z)–IXα



BILIRUBIN (E,E)–IXα

Physical constants and crystallographic data for bilirubin are listed in Table 1. Biliverdin data is given for comparison. Some differences are present in the table which are most likely due to different analytical techniques. Although the table is by no means complete, it makes a good starting point in explaining bilirubin chemistry.

Bilirubin is an unsymmetrically substituted, linear tetrapyrrole that contains three different functional moietys. First, bilirubin has four amino nitrogens, one for each of the four pyrrole rings. Second, there are two terminal oxygens on each molecule conforming to lactam tautomerism. Third, there are two carboxylic acid groups bound to β positions of the endo or internal pyrrole rings. This gives bilirubin its dipropionic acid character. Together, these moieties contribute to bilirubin's complex solubility and reactive nature.

The reactivity and solubility of bilirubin can be partially explained by the pigment's ability to internally hydrogen bond (Figure 6). Within each molecule, there is a maximum of six intramolecular hydrogen bonds possible (8,9,10). Four hydrogen bonds are relatively stronger than the other two, though (1).

All intramolecular hydrogen bonds involve the carboxyl groups (9). The pyrrolic hydrogens bond to carboxyl oxygens and the carboxylic acid hydrogens bond to lactam oxygens. This extensive network of hydrogen bonding occurs when bilirubin is in the solid state. Intramolecular hydrogen bonding also occurs when bilirubin is dissolved in nonpolar solvents even though the pigment ruptures all six hydrogen bonds by rapidly alternating between one enantiomeric form and a corresponding mirror image (11).

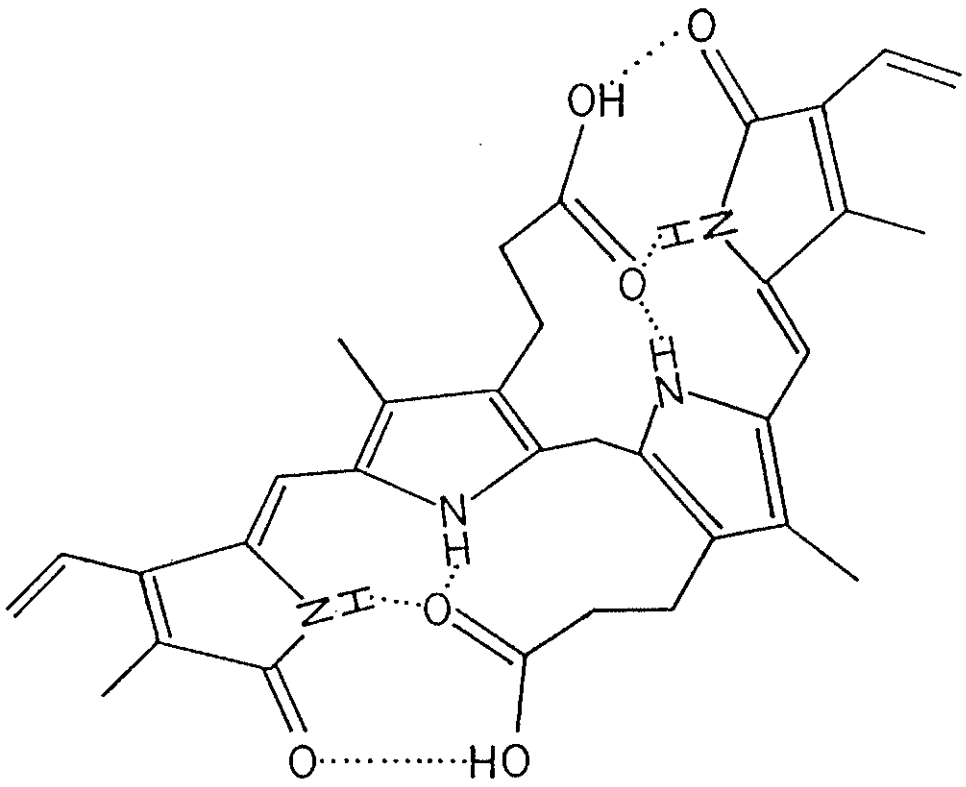
Table 1. Physical Constants of Bilirubin and Biliverdin. Note the following abbreviations: d (decompose), AL (methanol), AT (acetone), BZ (benzene), CF (chloroform), CS (carbon disulfide), DF (N,N-dimethylformamide), ET (ethyl ether), AC (acid, unspecificed), BS (base, unspecified), WR (water), I (insoluble), SS (slightly soluble), S (soluble) and RS (readily soluble).

BILIRUBIN: 2,17-DIETHENYL-1,10,19,22,23,24-HEXAHYDRO-3,7,13,18-TETRAMETHYL-1,19-DIOXO-21H-BILINE-8,12-DIPROPANOIC ACID; 5,15-BILADIENE; a,c-BILADIENE

FORMULA	MW	m.p.,°C	b.p., °C	λ max	ε,mM	SOLUBILITY AL	AT	BZ	CF	CS	DF	ET	AC	BS	WR	REF
$C_{33}H_{36}N_4O_6$	584.68	- - -	- - -	437,pH8	46.8	-	-	s	s	s	-	ss	-	-	i	5
	584.65	d	- - -	453,CF	60.7	ss	-	s	s	s	-	ss	s	s	i	6
	584.7	d>234	- - -	453-5,CF	62.6	i	ss	ss	s	-	ss	i	ss	RS	i	1
				448,DF	58.7											
	- - -	192	- - -	- - -	- - -	ss	-	s	s	-	-	ss	s	s	i	7

BILIVERDIN: 2,17-DIETHENYL-1,19,22,24-TETRAHYDRO-3,7,13,18-TETRAMETHYL-1,19-DIOXO-21H-BILINE-8,12-DIPROPANOIC ACID; 5,10,15-BILATRIENE; a,b,c-BILATRIENE

$C_{33}H_{34}N_4O_6$	582.66	>300	- - -	378,AL	40.7	ss	-	s	ss	ss	-	ss	-	s	i	5
				640-50	12.9											
	582.63	d>300	- - -	- - -		s	-	s	s	s	-	s	-	s	-	6
	582.7	- - -	- - -	376,AL	14.4	s	ss	i	ss	-	s	i	s	s	i	1

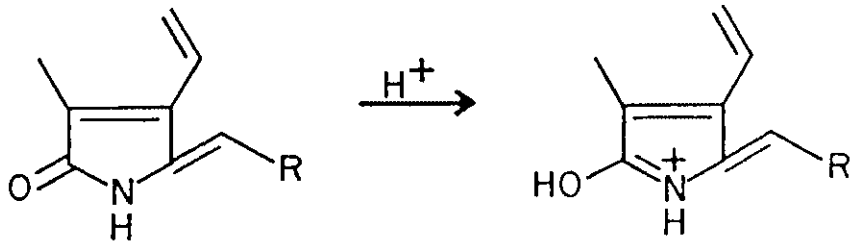

O
OH
N
O
HN
N
H
N
O
O
O
HO

When bilirubin protons are tied up through hydrogen bonding, they are not able to interact with water. Intramolecular hydrogen bonding forces bilirubin to conform to a tightly fitted structure whereby hydrophilic or water-liking moietys are not solvated by polar molecules. Even though bilirubin is amphoteric, the propionic acid side chains are unable to ionize in aqueous solution (1). Because of their weak acidity, the two carboxyl groups do not aid in water solubility.

Although bilirubin contains several polar moities, the pigment behaves more as a nonpolar species. This nonpolar behavior accounts for bilirubin's hydrophobicity or limited aqueous solubility and also its anomalously large ground state stability (4).

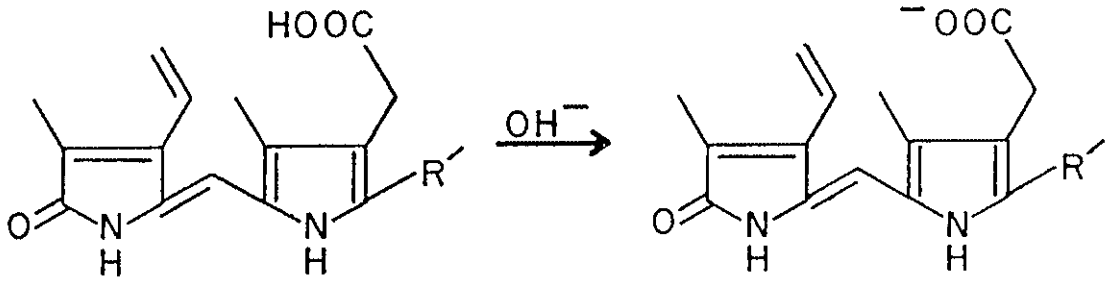
Bilirubin dissolved in concentrated mineral acids can undergo several reactions. First, ionization through proton addition to the parent molecule can take place. Protonation has been postulated (1) to occur at lactam oxygens (Figure 7A). In contrast, hydrogen ions attack biliverdin at unprotonated pyrrolic nitrogens. Second, bilirubin can be chemically oxidized in acid. If the acid is a strong oxidant, bilirubin can be oxidized to biliverdin. Furthermore, autooxidation to colorless products may occur. Both oxidative processes require oxygen. Finally, concentrated acids can catalyze the formation of symmetrically substituted bilirubin IIIα and bilirubin XIIIα from bilirubin IXα, even in the dark. This is a reversible process that does not require the presence of oxygen (8).

Bilirubin can be made to dissolve in water by increasing pH. In moderately alkaline water, bilirubin exists primarily as a dimeric or two part dianion (Figure 7B). Bilirubin dissolved in basic, aqueous



BILIRUBIN IN ACIDIC ENVIRONMENT

7A



BILIRUBIN IN BASIC ENVIRONMENT

7B

WHERE $R=C_{24}H_{27}N_3O_5$ AND $R'=C_{16}H_{17}N_2O_3$

solution is very unstable. Biliverdin, however, is remarkably more stable (1).

Bilirubin undergoes both autooxidation and rearrangement in water with added base. Autooxidation to unidentified, water soluble products is known to occur and is most rapid for dilute solutions with high pH (12). Even though oxygen is required, trace amounts are sufficient to autooxidize bilirubin. In addition, autooxidation may be catalyzed by trace metals, but it is inhibited by covalently bonded albumin. The products from autooxidation do not absorb light in the visible region, which may be due to excessive tetrapyrrole degradation (12).

Bilirubin can also reversibly isomerize in water with added base (1,12). In doing so, bilirubin looses a proton through a free radical process. This is followed by rearrangement. Isomerization of bilirubin in basic, aqueous solutions is slower than acid isomerization and requires initiators such as molecular oxygen or light. Oxygen, however, is a better initiator than light because ionization is not inhibited in aerobic solutions prepared in darkness. The products from bilirubin isomerization in basic, aqueous solutions are bilirubin IIIα and bilirubin XIIIα.

Isomerization of bilirubin in basic, aqueous solution does not proceed if bilirubin is bound to albumin. Isomerization is also concentration dependent and does not appear to occur in dilute solutions (12). Finally, isomerization is solvent dependent and occurs slowly in strongly basic, aqueous solutions, but not at all in chloroform.

Bilirubin solubility and stability are increased when the pigment is dissolved in organic solvents. Addition of acid to relatively

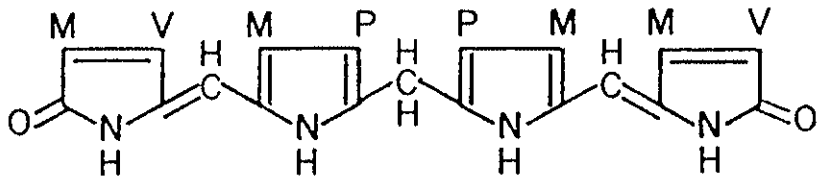
polar, organic solutions, though, does not aid in bilirubin dissolution. Instead, acid addition results in successive formation of three colored products: biliverdin, purpurin and choletelin. The colored product formation is an oxidation process known as the Gmelin Series (Figure 8). If, on the other hand, base is added to polar, organic solvents, bilirubin solubility is increased in a similar manner to increasing pH of aqueous solutions.

Besides chemical and photochemical oxidations, bilirubin can be electrochemically oxidized to biliverdin. This is an irreversible process that requires two electrons for complete conversion (13). At extremely negative potentials, bilirubin can be electrochemically reduced. Two equivalents of hydrogen atoms are needed to reduce bilirubin to unknown products (14).

Photobilirubin Chemistry

Bilirubin has already been described as undergoing multiple types of photochemical reactions. These reactions, however, have been confined mostly to aqueous solutions. In acidic, aqueous solutions, bilirubin is autooxidized to colorless products in the presence of light. In basic, aqueous solutions, bilirubin can undergo two major types of photochemical reactions. In water of high pH and, at least, with trace oxygen concentration, dilute bilirubin solutions are again autooxidized to colorless products. In concentrated bilirubin solutions of low pH and high oxygen concentration, isomerization or rearrangement to bilirubin IIIα and bilirubin XIIIα occurs.

In anaerobic, aqueous and organic solutions, absorption of blue, visible light by bilirubin results in unfolding of the parent structure (Figure 9). Recently, photoinduced Z---->E configurational

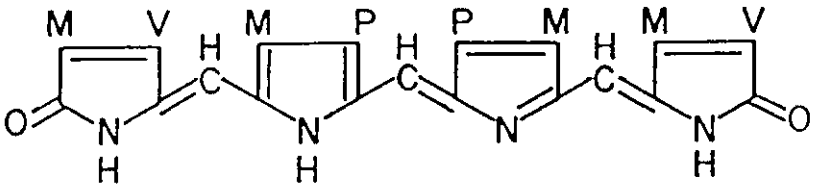


BILIRUBIN
YELLOW

↓

GREEN INTERMEDIATE MIXTURE

↓

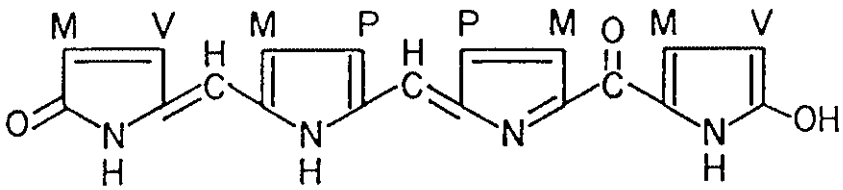


BILIVERDIN
BLUE

↓

VIOLET INTERMEDIATE MIXTURE

↓

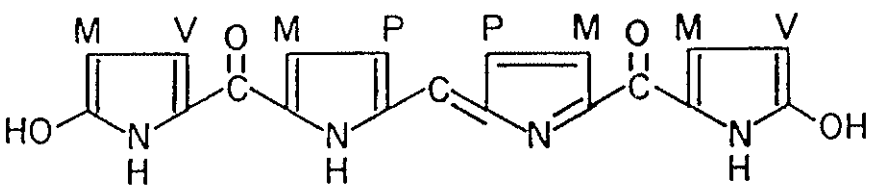


PURPURIN
PURPLE

↓

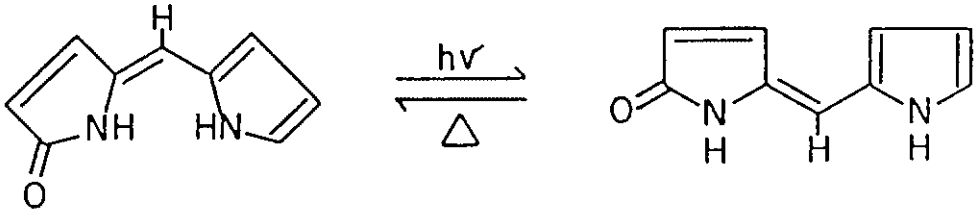
ORANGE INTERMEDIATE MIXTURE

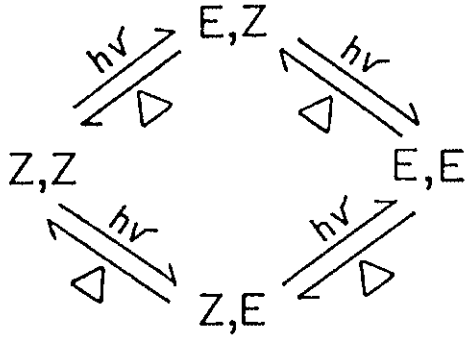
↓



CHOLETELIN
YELLOW

WHERE M=CH_3, V=$CH{=}CH_2$ AND P=CH_2CH_2COOH


H
NH
HN
O
hv
O
N
H
H
N
H


E,Z
hv
hv
Z,Z
E,E
hv
hv
Z,E

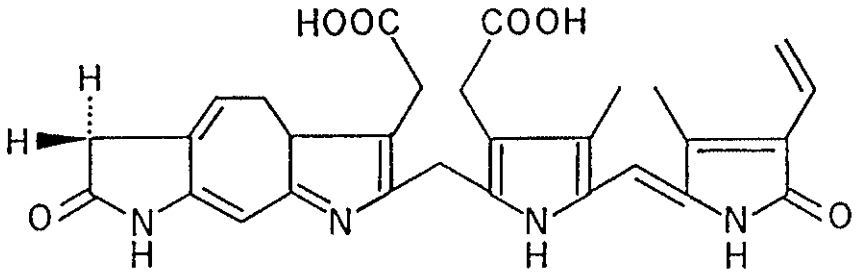
isomerization using 514.5 nm green light was effective in treatment of jaundiced newborns (15). Photoirradiation induces an equilibrium mixture of bilirubin and a group of geometric isomers known collectively as photobilirubin. At the photostationary state, [Z-Z]>>[E-Z]=[Z-E]>[E-E] (4). A recent estimate of relative concentrations reported an equilibrium mixture of approximately 80% [5]Z/15Z, 14% [5]Z/15E, 6% [5]E/15Z and 1% [5]E/15E (11).

Information about photoirradiation of bilirubin is limited. What is known is that the reaction occurs because of bilirubin's ability to undergo reversible configurational isomerization. The reaction is extremely rapid in organic solutions, occuring faster than other competing photochemical reactions such as aerobic photooxidation of bilirubin, photoaddition and photodisproportionation (4). Oxygen is not believed to be required for photolysis to occur (16). Finally, albumin does not slow photoisomerization (11). Instead, albumin stabilizes the thermal reverse reaction back to bilirubin.

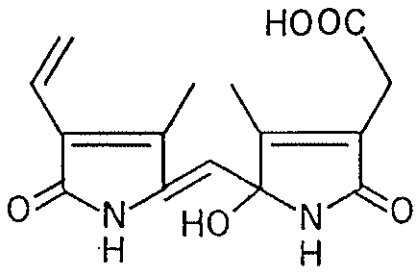
Overirradiation can lead to the formation of secondary products (Figure 10). If prolonged exposure of bilirubin occurs, structural isomers known as lumirubins will begin to irreversibly form (11). Lumirubins result from ring closure between the endo vinyl substituent at C-3 and the pyrrolic carbon at C-7. The C-15 meso bridge is unaffected and can still participate in Z--->E configurational isomerization. Further irradiation will again initiate the photooxidation process, presumably if trace oxygen is present (1).

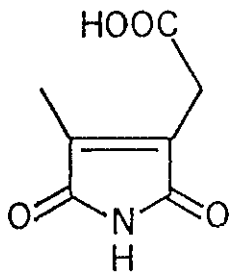
Under controlled conditions, photobilirubin can be synthesized with few isomeric impurities. The photobilirubin produced exhibits greater water solubility than bilirubin because complete intramolecular

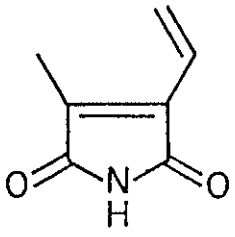
Figure 10. Photoproducts from Overirradiation. Included is Lumirubin and possible colorless products.



LUMIRUBIN







hydrogen bonding is sterically not possible. The E,E isomer has the fewest internal hydrogen bonds and, hence, is the most polar. The E isomers are thermally, photochemically and catalytically unstable products (9,16). Photobilirubin can revert back to bilirubin by addition of acid, application of heat or simply standing in dark at room temperature (11).

Statement of the Problem

The chemistry of bilirubin and photobilirubin is a complicated subject which has generated a considerable amount of confusion. The confusion first began by attempting to explain the formation and elimination of endogenous bilirubin (12). Another area of confusion stemmed from attempts to predict the structure of bilirubin (17,18). The absence of a universally accepted nomenclature system further hindered structural predictions (1). Finally, research on the behavioral characteristics of bilirubin photoisomers is in the beginning stages and presently not completely understood (11,19).

Perhaps the most perplexing aspect of bilirubin and photobilirubin chemistry has been in the reporting of results. In many cases, experimental data was compared to earlier findings which used either different or unspecified techniques (19,20). This research, however, has observed that subtle differences in technique have a significant effect on the data obtained. Consequently, perpetuated data discrepancies have been rather common and correlations between researchers have not always been clear (19,21).

The goal of this research was to clarify some confusion attributed to the complex nature of bilirubin and photobilirubin chemistry. Variations in experimental technique have been extensively

investigated. From these investigations, well defined methodologies have been established. Standardized methodologies, then, have been applied to new findings concerning the chemistry of bilirubin and photobilirubin.

Comparisons between bilirubin, photobilirubin and biliverdin have been made in identical chemical environments. Both a ternary solution composed of 1:3:1 chloroform:methanol:water and a modified pH 10.00 buffer have been used in spectrophotometrically examining the pigments. The modified pH 10.00 buffer was solely used in voltammetric analyses because of its well defined behavior. Lastly, photobilirubin was extracted into an aqueous system so that in could be evaluated without interferences from bilirubin.

CHAPTER 2

BACKGROUND

In vivo Bilirubin Production

Bilirubin is a bile pigment that is synthesized in the human body mainly through enzymatic catabolism of the porphyrin system. Approximately 20% of bilirubin production is believed to come from different sources (20). The primary synthesis of bilirubin begins when red blood cells rupture, spilling their contents into circulating plasma. This occurs mainly in the reticuloendothelial system or, more specifically, by the phagocytic cells (a tissue cell that digests other cells) of the liver, spleen and bone marrow.

Hemoglobin and other hemoproteins are two major components of red blood cells that are released from dying erythrocytes. Without cellular membrane protection, hemoglobin becomes very susceptible to physiological degradation. Consequently, circulating hemoglobin is split into two fragments, heme and globin.

Heme, an unstable iron product, is further catabolized and then oxidized by microsomal heme oxygenase into three new products. Biliverdin, a green bile pigment, is the major product. Iron, another product, bonds to a protein and the resulting complex is called ferritin. This complex will be used in future hemoglobin synthesis. Lastly, carbon monoxide is produced and this production is the main

endogenous source of the eventually respired gas.

Globin, the other product of hemolysis, is also hydrolyzed but to amino acids. Since globin synthesis and metabolism are not usually considerations of bile pigment formation, they will not be discussed further.

The biliverdin is enzymatically reduced by cytoplasmic reductase. The product from biliverdin reduction is an orange pigment called bilirubin. The overall process of hemoglobin degradation results in the daily synthesis of 300-500 mg of free bilirubin (1,22).

After bilirubin formation, an equilibrium is established throughout the pigment's circulating life between the free or naturally occurring species and a complexed form. In the complexed form, bilirubin firmly but reversibly bonds to albumin, a protein abundantly found in plasma. The covalent bond formed occurs between the propionic hydroxyl groups of bilirubin and the primary amino moities of albumin (2,20).

Due to greater water solubility, the albumin-bilirubin complex is readily transported in plasma throughout the circulatory system. Eventually bilirubin reaches the parenchymal or tissue cells of the liver and other organs. However, while in this complex form, bilirubin is unable to cross their lipophilic membranes. Instead, the complex must first dissociate, freeing the parent bilirubin molecule from the albumin protein. In the free form, lipid soluble bilirubin can diffuse across liver cell membranes.

In the cellular microsomal enzyme system of the liver, bilirubin is conjugated mainly by glucuronic acid. To a lesser extent, other acids and carbohydrates or sugar moieties conjugate bilirubin (1,20).

The primary conjugation process results in the attachment of glucuronic acid to one or both of the propionic acid carboxyl groups through ester bridges. This results in the formation of two new, weak organic acids, bilirubin mono- and diglucuronide (23). Both acids are more polar and, therefore, more water soluble than bilirubin at physiological pH. Bilirubin diglucuronide is the most polar.

After conjugation, bilirubin and other products are excreted from the liver through canaliculi or bile ducts to the intestinal tract. Here, conjugated bilirubin is hydrolyzed and subsequently reduced. The enzymatic reduction of bilirubin diglucuronide by intestinal flora results in the formation of fecal urobilinogen. Most fecal urobilinogen is further converted to fecal urobilin. Some urobilinogen, however, is reabsorbed back into the bloodstream through the intestinal walls. In this case, urinary urobilin is produced and eventually excreted from the body.

Hyperbilirubinemia and Jaundice

If bilirubin formation and metabolism do not proceed as previously described, greater than normal concentrations of bilirubin may begin to accumulate in blood. This condition is termed hyperbilirubinemia. Jaundice occurs when significant amounts of bilirubin deposit in the skin. Jaundiced individuals are often characterized by their skin turning a yellowish tint, which is due to the physical color of the solvated pigment.

Hyperbilirubinemia and jaundice are often associated with premature or low birthweight infants. Both conditions are usually the result of infant livers that are not fully developed and, hence, do not function properly. If the liver does not contain sufficient

conjugating species such as glucuronic acid, then unconjugated bilirubin will not be effectively excreted in bile. Although not as common, hyperbilirubinemia can be caused by blood incompatibilities and neonatal jaundice from excessive hemolysis followed by increased bile pigment production (12).

Regardless of how the buildup of in vivo bilirubin occurs, the physiological effects can be serious. In severe cases, unconjugated bilirubin may diffuse into the brain. Once released from albumin, bilirubin again acquires its original lipophilicity, thereby enabling it to cross the blood-brain barrier. While in the brain, bilirubin acts as a potent neurotoxin. Kernicterus and bilirubin neuropathy, both serious neurological disorders, can cause irreversible brain damage and even death.

History of Phototherapy

Prior to 1956, blood transfusions were commonly used to cure premature infants of bilirubinemia (24). Shortly after this time, two events lead to the present practice of phototherapy to rid infants of elevated concentrations of endogenous bilibubin. The first event stemmed from a registered nurse's belief that "...the combination of fresh air and warm sunshine would do [premature infants] much more good..." than accepted medical practices of that time (24). On a clear, summer morning, the nurse took a child under her care outdoors. Before long, she noticed the uncovered areas of the child's yellow skin appeared to have faded. Although the nurse mentioned this observation to the child's doctor, the observation was given little credence.

A couple of weeks after the registered nurse's initial observation, a blood sample from a severely jaundiced infant was

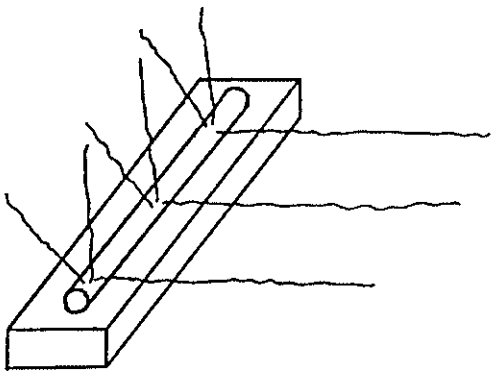
inadvertantly left on a window sill. The doctor responsible for analyzing the sample had gone to lunch. Upon the physician's return, the sample appeared to have a slight green tint instead of the characteristic yellow tint. Nonetheless, the sample was analyzed, but only after several hours of exposure from sunlight coming in the window. The analysis showed an unexpected low plasma bilirubin concentration.

Unlike the first event, a great deal of attention was given to this phenomenon. An investigation was begun and a conclusion was later drawn that exposing infants to visible light results in a lowering of in vivo bilirubin. The light source could be either natural, like the sun, or artificial, such as blue or white fluorescent lights. Irrespective of which light source was used, loss of the characteristic yellow skin was observed. Since that time, phototherapy has become an established practice of jaundice medicine routinely used in hospitals.

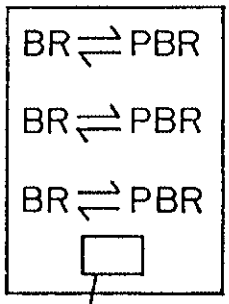
Endogenous Photobilirubin

Irradiation of unconjugated bilirubin results in rapid photoisomerization to unconjugated products known collectively as photobilirubin. Photobilirubin is an unstable configurational isomer that exists in equilibrium with bilirubin. This reversible behavior explains why unconjugated bilirubin, and not photobilirubin, is found in biliary excretions after exposure to visible light (Figure 11).

The phototheraputic process does not involve breaking and reforming of covalent bonds since unconjugated bilirubin is both the initial reactant and the final product. Instead, certain bonds appear to undergo a stereospecific inversion. This inversion or unfolding process results in the spacial repositioning of bonded substituents on

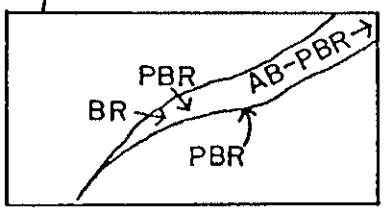


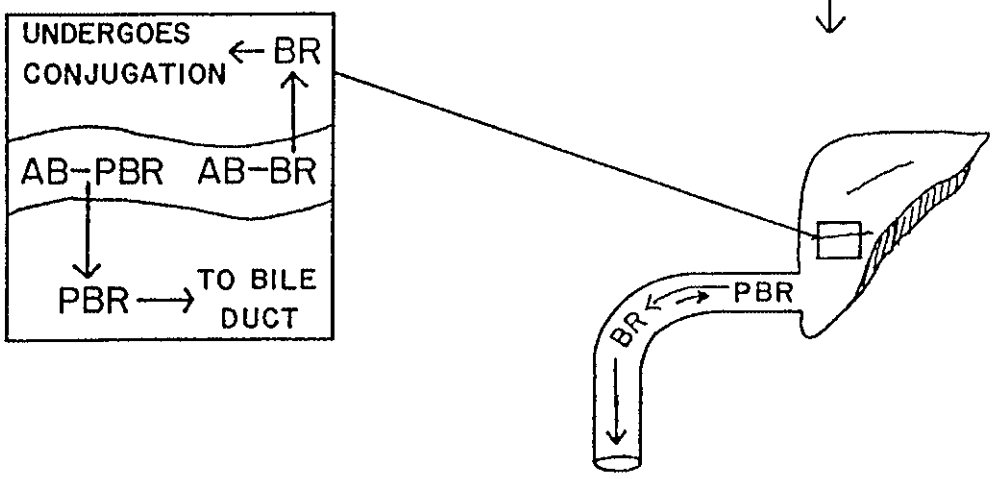
EXTERNAL SOURCE



HUMAN SKIN

PHOTOBILIRUBIN (PBR) PASSES THROUGH BLOOD VESSELS, FOLLOWED BY ALBUMIN—PHOTOBILIRUBIN (AB-PBR) TRANSVERSING ITS LENGTH TO THE LIVER





NO PBR OBSERVED IN BILE

the molecular substrate. As a result, intramolecular hydrogen bonding associated with unconjugated bilirubin is lost, or at least minimized. Hence, endogenous photobilirubin is relatively more hydrophilic than unconjugated bilirubin and is better able to pass through the circulatory system.

CHAPTER 3

MATERIALS AND APPARATUS

Materials

Bilirubin, biliverdin and bovine serum albumin were obtained from Sigma Chemical Company and used without further purification. Crystalline bilirubin was stored dessicated below $0^{o}C$. Biliverdin and bovine serum albumin were refrigerated at approximately $15^{o}C$.

With the exception of N,N-dimethylformamide, all acids, bases and organic reagents were used without further purification. N,N-dimethylformamide was vacuum distilled from anhydrous cupric sulfate to remove trace amines and water. Solvents used as mobile phases in HPLC analysis were filtered through five micron filters.

Water used in aqueous work was initially obtained from a permanently mounted spigot connected directly to a deionizer. No evaluation was made of its purity. HPLC grade water was used in subsequent aqueous work.

Buffers were purchased from Fisher Scientific and are traceable to NBS. All other reagents used in this research were of analytical grade or better.

Nitrogen and oxygen were usually obtained directly from storage cylinders. Nitrogen used in voltammetric analyses was of either an unspecified purity or 99.999% UHP purity. Oxygen was always of unknown

purity. In addition, nitrogen was used in voltammetric analysis after bubbling through an oxygen scrubbing system. The scrubbing system was an acidic solution of vanadium (II) chloride which reportedly is capable of removing trace oxygen from bottled nitrogen (25).

Apparatus

Two balances were used to measure weights of solid materials with a precision of 0.1 mg. Both balances, models AC100 and H72, were manufactured by Mettler and were periodically calibrated with Ohaus NBS class P weights prior to using. All weighings were made by difference.

Both a Rotap distillation apparatus and an ordinary still were used to purify N,N-dimethylformamide from anhydrous cupric sulfate. The Rotap distillation apparatus did have advantages of rapid distillation and ease of operation. However, extreme care had to be exercised in preventing dissolution of rubber seals.

Measurements of pH were made of aqueous solutions by an Altex pHI 43 and an Orion 221 pH meter. In each case, glass combination electrodes were used. Calibrations were made prior to use by bracketing the desired pH with standard buffer solutions.

Deoxygenation of glassware and solvents was accomplished by purging nitrogen through a Tygon tubing and borosilicate glass pipet system. The same pipet system was used in preparation of oxygenated solutions.

An Adams Analytical Centrifuge was used during sample preparation in separating suspended solute from supersaturated and undissolved bilirubin solutions.

Photoirradiation was executed with a Westinghouse Special Blue lamp, F20T12/BB, and simple light fixture. A Rayonet Reactor was also

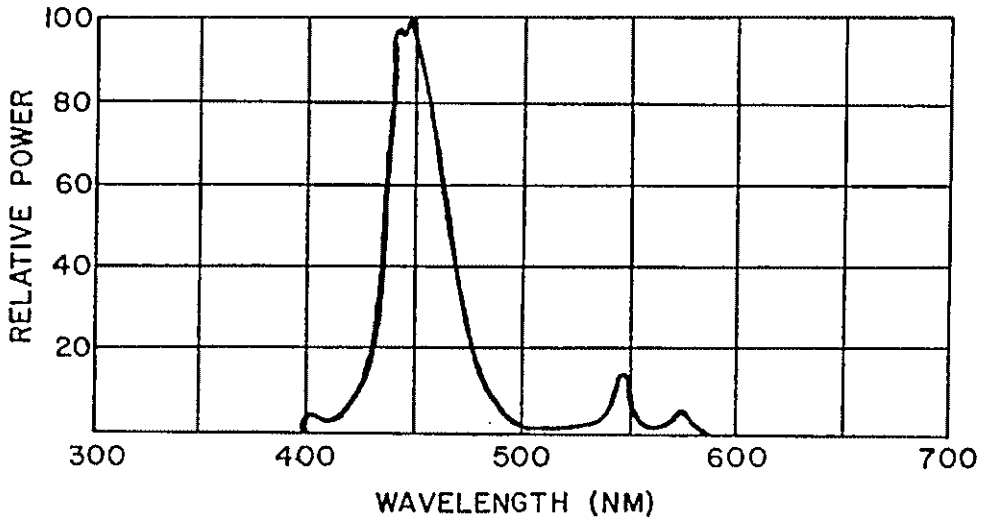
used but did not work as well. The Special Blue lamp has a narrow emission band between 400-500 nm and a nominal power rating of 20 watts (Figure 12). It was designed specifically for phototherapeutic work.

Ultraviolet and visible analyses were performed on a Varian Cary 219 Spectrophotometer. Data acquisition was performed using an interfaced Apple II Plus computer. In addition, hardware and software used were purchased from Varian Associates. Hard copies were obtained from an Epson model FX-80II printer.

Anodic and cathodic voltammetric measurements were made on an EG&G/Princeton Applied Research model 264 Polarographic Analyzer/Stripping Voltammeter. A model RE0074 X-Y Recorder was used to make hard copies of data.

A modified model 303A Static Mercury Dropping Electrode system was also used. A glassy carbon working electrode was substituted for the Hanging Mercury Dropping Electrode (HMDE) in making anodic measurements. The HMDE was used for cathodic analyses. The glass sleeve of the Ag/AgCl reference electrode was removed for analyses in N,N-dimethylformamide containing sodium perchlorate as the supporting electrolyte. However, problems with potential measurements were common and therefore, no data has been reported using that reference electrode system. The original, platinum wire of the counter electrode was not altered.

Two other reference electrodes were evaluated, but their use was discontinued. Initially, a Ag/AgCl electrode was used but the porous Vycor frit separating the electrode compartment from the bulk solution constantly cracked. After contacting the manufacture, the cracking was determined to be from changes in osomotic pressure brought about by


100
80
60
40
20
RELATIVE POWER
300
400
500
600
700
WAVELENGTH (NM)

differences in ion concentration on either side of the frit.

A non-aqueous $Ag/AgNO_3$ reference electrode was tried with a polyethylene frit instead of the glass frit. The polyethylene frit proved to be extremely unreliable, though, because electrolytic filling solution continually drained into the sample cell. Furthermore, the frit was observed to undergo a brown color change when immersed in bilirubin solutions. This same discoloration was also noticed on the Ag/AgCl reference electrode frit after closer inspection.

Direct current voltammetric measurements were made on an EG&G/Princeton Applied Research model 174A Polarographic Analyzer. Platinum disk and platinum foil were used as working and counter electrodes, respectively. Reversing these electrodes resulted in less defined peaks. A Ag/AgCl electrode with a ceramic plug was used as reference. Voltammograms were obtained on a Hewlett-Packard model 7040A X-Y Recorder.

Reverse phase liquid chromatography was performed on a Waters Associates, Inc. HPLC system complete with a model M-45 Solvent Delivery System, model U6K Universal Liquid Chromatograph Injector and model 440 Absorbance Detector. Both 254 nm and 436 nm filters were used for absorption detection. Two types of columns were used, including three different reverse phase C-18 columns and an Ultrasphere-Cyano column. A Tracor Westronics model FD10A two pen recorder was used for data acquisition.

CHAPTER 4

PRELIMINARY STUDIES

Solvent Selection

Initial investigations of this research began by preparing standard solutions of bilirubin in the "universal organic solvent", N,N-dimethylformamide (DMF). DMF is a moderately polar liquid which is miscible with water. DMF is aprotic, yet has appreciable basicity. Because of its polarity and basic properties, DMF readily dissolves nonpolar bilirubin. DMF also had the added advantage of having a low evaporation rate. However, DMF was not the sole solvent used in this research and its use was eventually discontinued for several reasons.

N,N-dimethylformamide is photochemically unstable and decomposes to amines and water. Consequently, the solvent had to be vacuum distilled from anhydrous cupric sulfate to remove photodecomposition products. Freshly distilled DMF was then protected from autodegradation by being stored in amber glass bottles, blanketed with nitrogen and refrigerated.

N,N-dimethylformamide commonly dissolved rubber seals and gummed latex tubing of apparatus used in this research. Its solvent characteristics are so powerful that DMF solutions were not injected onto HPLC C-18 columns due to potential column packing dissolution after prolonged use. Erroneous data would be anticipated from expected

column bleed.

The last reason for discontinuing N,N-dimethylformamide use was its water miscibility. Addition of water simply diluted bilirubin solubilized in DMF. Bilirubin was not forced out of solution and an orange precipitate or surface film was not observed. More importantly, a heterogeneous mixture was not formed and, thus, photobilirubin extractions could not be performed.

Methanol is another organic solvent whose use was evaluated. Many of the problems attributed to N,N-dimethylformamide were not characteristic of methanol. Methanol did not undergo autodegradation enough to require vacuum distillation. In addition, latex and rubber dissolution were not observed and injection of methanol onto C-18 columns was not considered destructive. Even with these attributes, methanol use was brief.

Methanol is miscible in water and extractions could not be performed using this liquid as the organic phase. Furthermore, since methanol is neither basic nor nonpolar, bilirubin was not soluble to the same extent as in N,N-dimethylformamide. A miscible base, 1,1,3,3-tetramethylguanidine (TMG), was added to aid bilirubin solubility in methanol.

Although added base increased bilirubin solubility, problems were encountered, especially in HPLC analysis. First, TMG slightly absorbs 254 nm radiation and could not be used in HPLC work with ultraviolet detection. Second, TMG promoted ionization of bilirubin which made the pigment less likely to interact with nonpolar, C-18 packing material. Consequently, clean separation was not observed because of interfering peaks and photobilirubin was not separated from bilirubin due to lack

of stationary phase interaction.

Chloroform was the final organic solvent tried. This solvent does not undergo significant autooxidation if protected from light and refrigerated. According to the column manufacturer, chloroform is compatible with C-18 packing material, even though it is less polar than DMF. Furthermore, chloroform completely dissolves bilirubin, even without added base. Lastly, chloroform is only slightly miscible in water, thus, enabling photobilirubin extractions to be performed under controlled conditions.

Besides organic liquids, water was also used as a solvent. Photobilirubin water solubility was evident by its tendency to extract into aqueous solution during photoirradiation. Photobilirubin extractions were the most common use of water. Water was also used in making bilirubin solutions in the ternary solution.

In order to adaquately compare data between bilirubin and photobilirubin, both species must be analytically evaluated under identical conditions. However, with the exception of photobilirubin production in the same reaction vessel, bilirubin was usually in different solvent systems. The majority of bilirubin analyses was performed with the pigment dissolved in chloroform. Photobilirubin was extracted into water. Since analytical properties of both pigments are dependent, in part, on the solvent system used, comparisons would be meaningless if made in different solvents. If, however, the two pigments were placed in identical solvent systems, then meaningful data would be acquired.

The simplest method of obtaining bilirubin and photobilirubin in identical solvent systems would be to mix the individual solutions

together. Unfortunately, a chloroform and water binary solution is not possible because the solvents are immiscible. Nonetheless, methanol added as an intermediate solvent dissolved both liquids into a homogeneous system.

The resulting ternary solutions were identical for both bilirubin and photobilirubin. A 20 mL volume of chloroform containing dissolved bilirubin was added to 20 mL of water plus 60 mL of methanol. Data from bilirubin in this solvent system was compared to results from 20 mL of water with extracted photobilirubin added to 20 mL of chloroform and 60 mL of methanol.

Another solution was also developed for analyzing bilirubin and photobilirubin in identical chemical environments. Since bilirubin was found to be readily soluble in pH 10.00 buffer, the aqueous extract could easily be added to the buffer without concern for immiscibility. To ensure that both solutions were identical, water added to pH 10.00 buffer was saturated with chloroform, just as it would be from the extraction process. Thus, 1 mL of aqueous extract was added to 19 mL pH 10.00 buffer and 19 mL of pH 10.00 buffer containing bilirubin was added to 1 mL of chloroform saturated water.

Solute Concentrations

Concentration of bilirubin in solution was limited primarily by solubility. In visible spectrophotometric analysis, the magnitude of the pigment's molar absorptivity was also considered in determining the concentration of bilirubin to be used. The average molar absorptivity of bilirubin in chloroform, ϵ, is equal to 60,000 L/cm-mol (22). According to Beer's law, the quantity of absorbed radiation is directly proportional to sample concentration. Therefore, the molar

concentration of bilirubin was $2.5X10^{-5}M$, since an absorbance of 1.5 AU was desired.

$$A=\epsilon bc$$

$$\text{or } c=A/\epsilon b$$

$$=(1.5)/(60,000 \text{ L/cm-mol})(1 \text{ cm})$$

$$=2.5x10^{-5} \text{ mol/L}$$

Biliverdin concentration was calculated in a similar manner. The molar absorptivity of biliverdin dissolved in methanol and taken at 378 nm is given as $\log\epsilon=4.61$ (5). Again, from Beer's law, the molar concentration of biliverdin was calculated to be $3.7X10^{-5}M$.

$$c=A/\epsilon b$$

$$=(1.5)/(\text{antilog } 4.61 \text{ L/cm-mol})(1 \text{ cm})$$

$$=(1.5)/(40,738 \text{ L/cm-mol})(1 \text{ cm})$$

$$=3.7x10^{-5} \text{ mol/L}$$

One other consideration in preparing biliverdin solutions was the pigment's relative purity. The label on each bottle of biliverdin stated that the pigment was approximately 80% pure. This value was used in calculating the weight of biliverdin required to obtain a $3.7X10^{-5}M$ solution.

In making up solutions of bovine serum albumin (BSA), a 1:1 molar ratio was used regardless of pigment concentration. BSA solutions were difficult to prepare because their behavior resembles surfactants in that excessive bubbling occurred during all agitating and purging. Furthermore, a literature value for molecular weight not not found. An approximate molecular weight of 70,000 g/mol was used in concentration calculations.

Since equal molar concentrations were used, no consideration was

given to molar absorptivity of bovine serum albumin in preparing solutions. Nonetheless, the molar absorptivity of BSA was calculated to be 175 L/cm-mol from information supplied by another manufacturer: Bovine Albumin, Fraction V; A<0.1 AU, b=1 cm and c=4% ($5.7X10^{-4}M$) at 411 nm (27).

All other molar concentrations of solutes were made without absorbance measurement considerations since they are transparent in the visible region of the electromagnetic spectrum.

All hydrated solutes were used after heating to approximately $100^{o}C$ for one hour, to remove externally absorbed moisture. The solutes were then immediately dessicated and allowed to cool to room temperature. Concentration calculations were based on the molecular weight of the hydrated complex.

Oxygen Removal Systems

Nitrogen was routinely used to remove dissolved oxygen from bilirubin solutions. In aerobic solutions, bilirubin was oxidized to one of several different products. Sometimes the vernoidal green color indicating the presence of biliverdin was visually observed. More often, yellow bilirubin was converted to colorless products during photoirradiation. Both oxidations were partially attributed to the presence of oxygen.

Two different methods were used to test the hypothesis that the synthesis of photobilirubin from bilirubin was catalyzed by trace amounts of oxygen. In the first method, solvents were purged for 30 minutes, the solute was added and then spectrophotometric and chromatographic measurements were taken. After data had been recorded, another series of solutions was prepared and subsequently analyzed.

The solvent vessel was evacuated at reduced pressure until the deoxygenated liquid started to momentarily boil. The degassing helped ensure that any residual oxygen remaining in solution after the nitrogen purge would be removed during the evacuation step.

The second method used to determine if photobilirubin formation was catalyzed by trace amounts of oxygen was pursued during electrochemical experimentation. Vanadium (II) chloride was synthesized in acidic solution, and this solution was used to remove trace amounts of oxygen from nitrogen. Nitrogen purified in this manner was thought to be more effective in removing oxygen from solution since it was free of residual oxygen. Thus, the probability of total anaerobic conditions was greater.

The procedure developed for preparing vanadium (II) chloride solution is as follows. First, zinc was amalgamated by adding 150 mL 0.01M hydrochloric acid to a 250 mL beaker containing 5 g of zinc metal. A stirring bar was added into the mixture and a moderate stirring rate was established. Afterwards, approximately 5 g of liquid mercury metal was added dropwise. Zinc began amalgamating immediately and after a short period of time, the mixture started to clear. However, since liquid mercury was still observed in the bottom of the beaker, zinc was introduced into the mixture several more times. Each introduction was followed by clearing, except the last. At this point, amalgamation was considered complete. The amalgamated zinc nodules were then washed with deionized water to remove powdered zinc settled onto their surface.

The vanadium (II) chloride solution was then prepared by adding 2 g of ammonium metavanadate to 25 mL concentrated hydrochloric acid in a

500 mL beaker. A slow boil was maintained to promote dissolution. The mixture turned maroon at first followed by a green color during heating. After cooling to room temperature, the solution was diluted to 250 mL.

Both the vanadium (II) chloride solution and the amalgamated zinc were added to a 350 mL Kontes gas washing bottle. The bottle inlet was connected to a nitrogen tank with Tygon tubing and the outlet was connected in series to two smaller scrubbing bottles. The scrubbing bottle closest to the Kontes bottle contained deionized water to collect acid vapors that might come over. The last washing bottle contained the same solvent in which bilirubin was dissolved. The last bottle was used to saturate nitrogen with vapor, thereby minimizing sample concentration due to solvent evaporation.

After passing nitrogen through the scrubbing system, the green colored solution turned blue and finally violet, indicating the presence of vanadium (II). The vanadium (II) chloride solution was extremely stable and was used for several months. When expired, the solution turned brown. Vanadium (II) was regenerated by adding concentrated hydrochloric acid to the Kontes gas washing bottle until the previous violet color returned.

Sample Preparation

During storage, bilirubin, biliverdin and bovine serum albumin were refrigerated to minimize decomposition and other product formation. To prevent condensation of moisture, each bottle was desiccated and allowed to reach room temperature before opening.

With the exceptions of Gmelin series evaluation, irradiation experimentation and solubility determination, all handlings of

bilirubin and biliverdin were performed in subdued light. A red photographer's bulb was used in every case.

Except for experimentation requiring oxygen, all solvents were purged with nitrogen for at least 30 minutes prior to solute addition. Even volumetric glassware used in making standard solutions were momentarily purged and subsequently stoppered. Individual solvents of ternary solutions were deoxygenated before mixing together to avoid evaporation of the more volatile liquid. After solutions were brought to volume, a nitrogen blanket was used to prevent redissolution of oxygen.

After bilirubin and biliverdin addition to a solvent, the solution was agitated, as well as deoxygenated, by bubbling nitrogen. Exactly ten minutes was allowed for complete color development or dissolution. The resulting solution was immediately analyzed.

Bilirubin and Biliverdin Solubility

The six intramolecular hydrogen bonds of bilirubin account for the pigment's hydrophobic nature. Accordingly, the solubility of bilirubin in this form was expected to be greatest in nonpolar, organic solvents. In ionized forms brought about by proton addition or removal, intramolecular hydrogen bonds are broken. Under these conditions, bilirubin solubility was expected to significantly increase in both aqueous and polar, organic solutions. This, however, was not found to always be the case.

Solubility determinations were patterned, in part, after two different systems (5,28). A weight of 1.5+/-0.1 mg of bilirubin was added to a test tube containing 10 mL of solvent at room temperature. This corresponded to a working bilirubin concentration of

2.5×10^{-4}M. If after agitating, complete dissolution occurred within five minutes of solute addition, then bilirubin was considered to be readily soluble in that solvent.

If, after five minutes, solid bilirubin was still observed, the mixture was then heated in a 75°C waterbath for ten minutes. The mixture was then allowed to cool to room temperature. If essentially complete dissolution occurred, then bilirubin was designated as being soluble in that solvent. Significant yellow, orange or red color development, though, had to be observed.

Bilirubin was defined as being slightly soluble in solvents that dissolved very little of the solute, even after heating. This was usually characterized by either orange surface films or heavy suspensions of undissolved bilirubin. If left undisturbed, a precipitate would usually settle to the bottom of the test tube if heavy suspensions were initially observed. Color development was only a fraction of solvent color in which bilirubin was moderately or readily soluble.

Bilirubin was considered insoluble in solvents that did not have any color development during heating. The solvent remained colorless and bilirubin was usually present as a surface film.

Observed solubilities in which only some of the criteria for a given designation was observed are tabulated with broken lines between two solubility designations. A decision as to which category these solubilities fall into was undecisive and therefore, the broken line represents an intermediate value.

Bilirubin was found to exhibit a wide range of solubilities in both aqueous and organic solvents (Table 2). In most aqueous

SOLVENT	INSOLUBLE	SLIGHTLY SOLUBLE	SOLUBLE	READILY SOLUBLE
HCL			X	
HNO_3				X
H_2SO_4				X
pH 2.00	X			
pH 4.00	X			
pH 6.00	X			
pH 7.00	X			
pH 8.00		X		
pH 9.00		X	X	
pH10.00				X
CH_3OH		X	X	
$CHCL_3$				X
DMF				X
TOLUENE			X	X
HEXANE	X	X		
TERNARY SOLN		X	X	

solutions, bilirubin exhibited limited solublity. Bilirubin was insoluble in deionized water as well as various buffered and unbuffered solutions ranging in pH from 1.00-7.00.

Further increases in pH enhanced bilirubin solubility in water. At pH 8.00, bilirubin was visually observed going into solution. A moderate amount of bilirubin dissolved at pH 9.00 while at pH 10.00, bilirubin was determined to be readily soluble.

Bilirubin was also readily soluble in concentrated nitric and sulfuric acids. Dissolution of bilirubin in these acids resulted in the formation of a deep red color. Addition of bilirubin to concentrated hydrochloric acid turned the colorless liquid brown. After standing for a period of time, the vernoidal green color was observed. Bilirubin added to concentrated hydrochloric acid was only soluble.

Bilirubin was only slightly soluble in polar methanol. Bilirubin solubility in methanol was observed to increase through the addition of 1,1,3,3-tetramethylguanidine. Bilirubin was readily soluble in methanol containing $1X10^{-3}M$ base.

Chloroform was also added to methanol in sufficient amounts to increase bilirubin solubility. Although changes in bilirubin dissolution were observed with increased chloroform concentration, it was not until approximately 75% chloroform in methanol that bilirubin was readily soluble.

In less polar organic solvents such as chloroform and N,N-dimethylformamide, bilirubin dissolved completely. Addition of nitric and sulfuric acids to DMF did not increase bilirubin solubility since the pigment is readily soluble in this solvent. Instead, added

acid rapidly oxidized bilirubin to biliverdin. Continual acid addition resulted in further oxidation of biliverdin to purpurin and finally to choletelin.

In determining when bilirubin was completely oxidized to biliverdin, standard biliverdin solutions were made for visual comparison in both N,N-dimethylformamide and the ternary solvent system. Standard solutions were also made in chloroform, yet added mineral acids were immiscible and Gmelin series oxidation was not observed. A visual comparison could not be made for complete conversion of biliverdin to purpurin since purpurin was not commercially available. Finally, oxidation to choletelin was considered complete when addition of excess acid did not result in further color changes.

Finally, bilirubin was not readily soluble in toluene or xylene. Bilirubin was intermediate between insoluble and slightly soluble in nonpolar hexane. This lack of solubility in nonpolar solvents was expected because of polar moities such as lone electron pairs on bilirubin oxygens that are not tied up through hydrogen bonding.

Biliverdin solubility was determined by the same method as bilirubin solubility. A weight of 2.1+/-0.1 mg of biliverdin was added to 10 mL of solvent, again giving a molar concentration of $2.5X10^{-4}M$. The same designations were used to describe relative biliverdin solubilities in various solvents. Complete color development had either a green or blue-green appearance. Table 3 lists the relative solubilities of biliverdin.

Biliverdin solubilities were similar to bilirubin, but not identical. Biliverdin was insoluble in most aqueous solutions.

SOLVENT	INSOLUBLE	SLIGHTLY SOLUBLE	SOLUBLE	READILY SOLUBLE
HCL			X	
HNO_3				X
H_2SO_4				X
pH 2.00	X			
pH 4.00	X			
pH 6.00	X			
pH 7.00			X	
pH 8.00				X
CH_3OH				X
$CHCL_3$		X		
DMF				X
TOLUENE	X			
HEXANE	X			
TERNARY SOLN				X

Biliverdin began going into aqueous solution before bilirubin, though. At pH 7.00, bilirubin was soluble in water and by pH 8.00, the pigment was readily soluble.

Biliverdin had identical solubilities in acid as bilirubin but somewhat different solubilities in organic solution. Biliverdin was only slightly soluble in chloroform, yet readily soluble in methanol and N,N-dimethylformamide. Furthermore, biliverdin was insoluble in hexane and toluene.

Photobilirubin Synthesis

In synthesizing photobilirubin in the same reaction vessel as bilirubin, two basic requirements were found necessary for successful isomerization. First, bilirubin must be sufficiently soluble for effective photoirradiation to occur. Mixtures of undissolved bilirubin and solvent are simply suspensions of solid bilirubin. Solid bilirubin is relatively stable and does not undergo isomerization as quickly as solubilized bilirubin. Second, the irradiation source must emit energy capable of bilirubin absorption. Both sunlight and a Westinghouse Special Blue bulb were used for bilirubin photoirradiation. A Rayonet reactor was also used but it appeared to promote photooxidation over photoisomerization.

Photobilirubin was synthesized in several different solvents. Chloroform, pH 10.00 buffer and the ternary solution were all used in photobilirubin production. Prior to irradiation, standard bilirubin solutions were transferred to 1 cm quartz cuvettes in which one side was normal to incident radiation. The cuvette was positioned 3 cm from the light source. The solutions were usually purged with nitrogen to maintain anaerobic conditions and also to provide convection. After

light exposure, the irradiated solution was immediately analyzed.

Photobilirubin synthesis was successfully monitored by two different analytical techniques. Absorbance difference (AD) spectrophotometry was the primary instrumental technique used to detect photobilirubin production. The difference peak obtained was not the true wavelength of maximum absorption Instead, the difference peak only approximated the type of radiation absorbed by photobilirubin relative to bilirubin under identical conditions.

Liquid chromatography was also used to monitor photobilirubin synthesis. Since chromatographic measurements were made with a fixed 436 nm absorbance detector, HPLC gave no indication of photobilirubin's wavelength maximum either. Consequently, liquid chromatographic monitoring of photobilirubin production was limited, especially after changing to newer columns.

Photobilirubin Extraction

Photobilirubin analysis in the presence of bilirubin was difficult due to large concentrations of the parent pigment. Consequently, a method was eventually developed whereby photobilirubin was extracted into an immiscible aqueous phase from bilirubin dissolved in an organic phase. The organic phase used was solely chloroform.

A weight of 14.7+/-0.1 mg of bilirubin was added to 50 mL of anaerobic chloroform in a 500 mL separatory funnel. Then, 50 mL of deoxygenated water was added. Approximately 35 cc of nitrogen per minute was purged through the mixture to maintain anaerobic conditions and mix the two phases. The Special Blue bulb irradiated the mixture.

At the end of photolysis, the orange chloroform layer was discarded, leaving a yellow aqueous layer. The remaining aqueous layer

had to be swirled several times to dislodge residual chloroform adhering to the glass. This residual chloroform settled to the bottom of the separatory funnel and was drained.

The 30 minute exposure was optimized by taking into consideration several phenomena. Short irradiation times did not result in significant yellow coloration of the aqueous layer. Longer exposures were observed to develop a slight verniodal green tint. Excessive exposure and convection by nitrogen purging turned the aqueous layer cloudy.

Acid tests were performed on aqueous photobilirubin solutions to determine if the yellow product could be repeatedly oxidized in a manner similar to the Gmelin series. First, 10 mL of extract were drained into test tubes. Hydrochloric and nitric acids were added to the extract. Regardless of volume, addition of hydrochloric acid simply diluted the yellow color. Nitric acid turned the yellow color a greenish tint. The green color was later determined to be the result of nitric acid addition to chloroform saturated water.

Oxygen stability of extracted photobilirubin was investigated next. Aqueous photobilirubin purged with nitrogen was stable for days. If, however, oxygen was bubbled through the solution, the yellow color began to fade. The bleaching process was minimized in darkness but, nonetheless, did continue. Accelerated bleaching occured when oxygen bubbling was coupled with photoirradiation.

Photobilirubin reversibility was the last evaluation made of the aqueous extract. If the extract was placed in a test tube, stoppered and left in darkness for several days, the original yellow solution began to lighten. An orange solid would precipitate in the bottom of

the test tube. As a result, photobilirubin produced from the extraction was considered to reversibly undergo configurational isomerization back to bilirubin, the starting material.

CHAPTER 5

EXPERIMENTAL AND RESULTS

Visible Spectrophotometry

Visible spectrophotometry is an analytical technique used to qualitatively and quantitatively determine the molecular constituents of a sample. Qualitative analysis is based on interactions between a molecule and incident radiation. The interactions depend on electronic structures of molecules whereby valance electrons selectively absorb narrow bands of radiation. The bands or wavelengths of radiation are inversely related to the energy required for absorption through Planck's equation:

$$E=hv$$

$$E=hc/\lambda$$

where E=photon energy, h=Planck's constant, v=frequency, c=velocity of light and λ=wavelength. The resulting absorption pattern is characteristic of chemical bonds and substituent groups, known collectively as chromophores. Unabsorbed radiation of other wavelengths simply passes through the sample.

Quantitative analysis is based on Beer's law, which has already been addressed. Briefly, Beer's law states that the amount of absorbed radiation is an exponential function of sample concentration. In addition, molar absorptivity or the absorption power of a molecular

species is related to the quantity of absorbed radiation at a given concentration.

Before qualitative and quantitative analyses were performed, a spectrophotometric method was standardized to lessen variations in data. A scan rate of 1 nm/s was selected as a compromise between minimal bilirubin light exposure from the tungsten lamp and maximum data point averaging by the interfaced computer. An accelerated scan rate of 10 nm/s was not recommended by the manufacturer because of potential peak height and position distortion. Conversely, a decelerated scan rate of 0.1 nm/s, which results in exceptional data averaging, exposed bilirubin to absorbable radiation for approximately 15 minutes during the first scan alone.

Except where noted, solutions were scanned from 600-300 nm. A spectral band width of 0.5 nm was held constant throughout the scan while the gain automatically varied. Concentrations of $2.5X10^{-5}M$ were attempted in all solvents but, because of limited solubilities, were not always obtained. Solvents in which solid bilirubin remained suspended in solution after sample preparation were centrifuged for five minutes. All computer and instrumental parameters were preset prior to scanning and solutions were immediately scanned after being placed in the spectrophotometer.

With the exception of absorbance difference spectrophotometric analysis, old solutions were discarded and new solutions were prepared following each scan. The reason being, repetitive scans of bilirubin were accompanied by minute diminutions in absorbance. This was the result of pigment irradiation from the spectrophotometer source during the actual scan. However, because of a phenomenon called color

development, this was not observed in all cases. Table 4 lists typical changes in absorbance for both an aqueous and organic solvent.

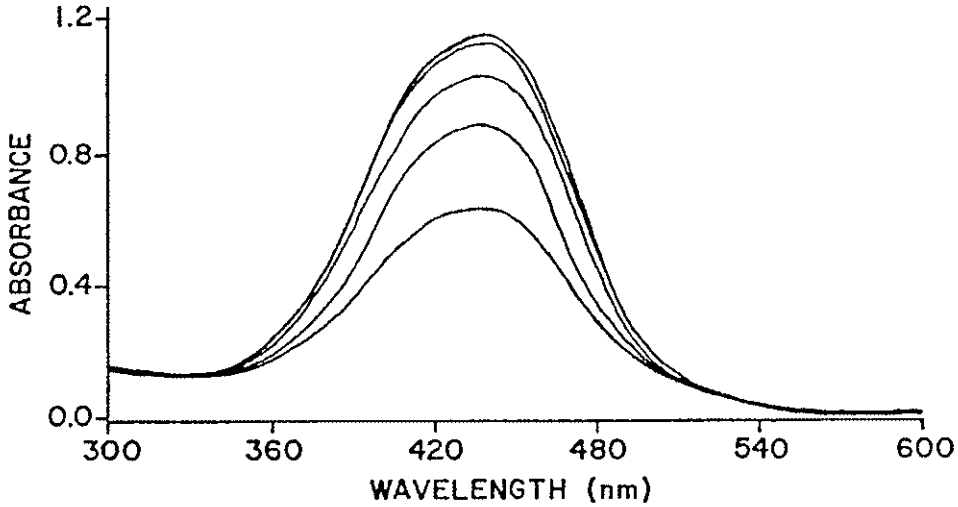
SOLVENT	SCAN 1	SCAN 2	SCAN 3	SCAN 4
DMF	1.5300	1.5269	1.5259	1.5250
pH 10.00	1.1382	1.1344	1.1338	1.1331

TABLE 4 REPETITIVE SPECTROPHOTOMETRIC SCANS OF BILIRUBIN (LISTED VALUES ARE IN ABSORBANCE UNITS)

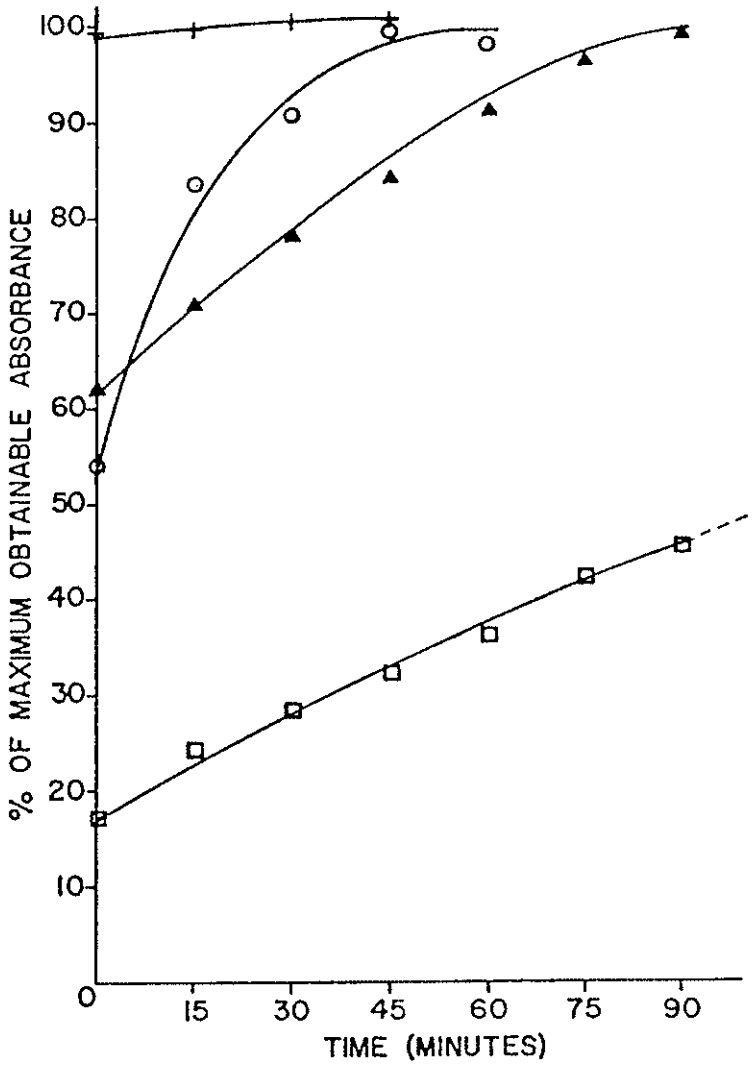
Incomplete color development is a characteristic of bilirubin that is not fully explained in the literature. Unlike most solutes, visible analysis of bilirubin could not commence immediately following dissolution of all solid material. Even though bilirubin appeared completely solubilized, absorption measurements at the pigment's wavelength maximum steadily increased (Figure 13). The rate of increase eventually stopped but a steady state equilibrium in absorbance measurements down to 0.0001 AU was never obtained. Color development was decidedly complete when a repetitive scan had a lower absorbance value than the preceding scan.

The time required to stabilize increasing absorbance measurements was found to be solvent dependent (Graph 1). In moderately polar organic solvents such as chloroform and N,N-dimethylformamide, full color development occurred rapidly, within 15 minutes of sample preparation. No change was observed in either solvent with an excess molar addition of 1,1,3,3-tetramethylguanidine. In aqueous pH 10.00 buffer, 45 minutes were required for full color development while approximately twice that was needed for the ternary solution. Finally,

Figure 13. Color Development of Bilirubin in pH 10.00 Buffer. Increasing absorbance measurements were taken at 15 minute intervals.


1.2
0.8
0.4
0.0
ABSORBANCE
300
360
420
480
540
600
WAVELENGTH (nm)

Graph 1. Color Development of Bilirubin. Note the following symbols: + (chloroform), o (pH 10.00 buffer), ▲ (ternary solution) and □ (pH 10.00 buffer containing bolvine serum albumin).


100
90
80
70
60
50
40
30
20
10
0
% OF MAXIMUM OBTAINABLE ABSORBANCE
15
30
45
60
75
90
TIME (MINUTES)

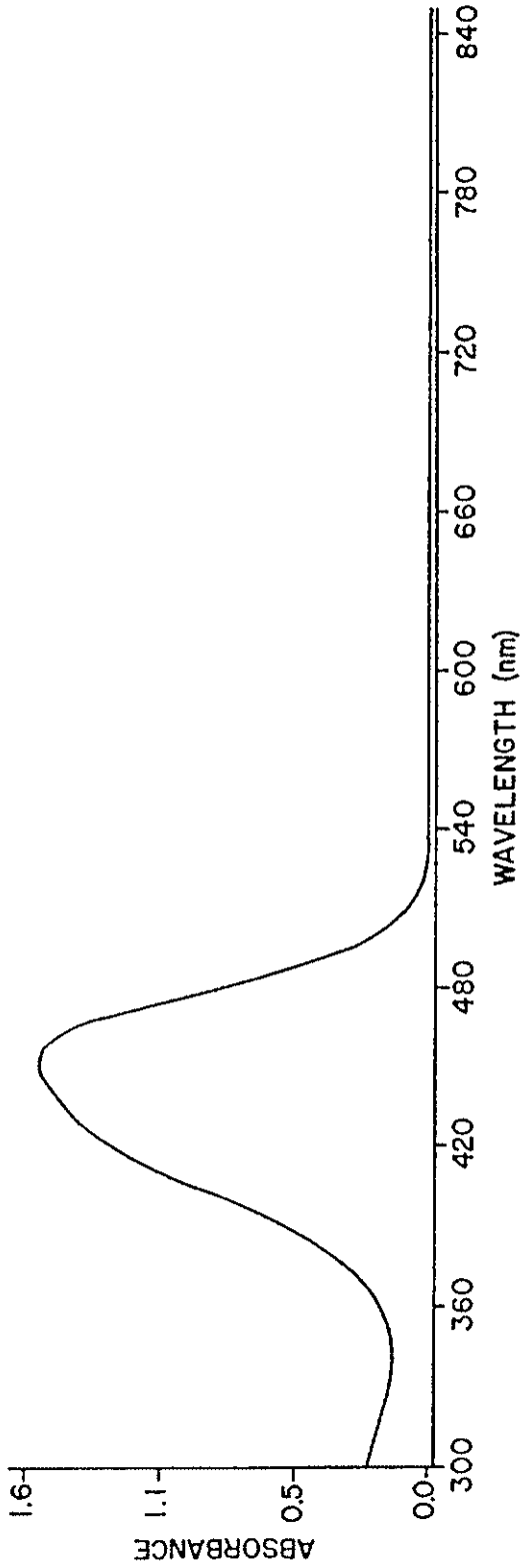
in equal molar aqueous bovine serum albumin solution, color development was not achieved until several hours after initial preparation.

Color development was hastened by heating solutions with added bilirubin in a 75°C water bath for ten minutes. Since heat could possibly accelerate both photooxidation and photoisomerization, extreme care was taken to ensure that solutions were meticulously deoxygenated and that only light from the safety lamp was present. Parafilm was tightly placed over test tubes containing bilirubin solutions to prevent concentration of pigment due to solvent evaporation.

After cooling to room temperature, bilirubin solutions were spectrophotometrically scanned. If photooxidation, photoisomerization or even thermal decomposition had occurred, alterations of bilirubin chromophores would have been observed through either color changes or shifts in wavelength maximum (1). This was not the case since heated and unheated solutions containing bilirubin appeared characteristically orange and absorbed at the same wavelength. Nonetheless, heated solutions were only used to compare data from unheated solutions.

Once understanding the effects of instrumental parameters and sample preparation, qualitative analysis was begun. Bilirubin was found to have a single, broad spectral peak in the visible region (Figure 14). The corresponding wavelength of maximum absorption was well defined for most solvents and usually varied by no more than 1 nm.

The effect of solvent on bilirubin's wavelength of maximum absorption was evaluated. Table 5 lists wavelength maxima for bilirubin in various solvents. In organic solvent systems, bilirubin absorbed a maximum amount of radiation at approximately 453 nm. Addition of TMG tended to increase wavelength maxima by 6-7 nm. In aqueous solution,


ABSORBANCE
1.6
1.1
0.5
0.0
300
360
420
480
540
600
660
720
780
840
WAVELENGTH (nm)

SOLVENT	ADDITIVE	WAVELENGTH OF MAXIMUM ABSORPTION
CH_3OH		452
$CHCL_3$		453
$CHCL_3$	TMG	459
DMF		453
DMF	TMG	460
pH 9.00		440
pH 10.00		439
pH 10.00	BSA	476
pH 10.00	$CHCL_3$ sat H_2O	439
TERNARY SOLN		454

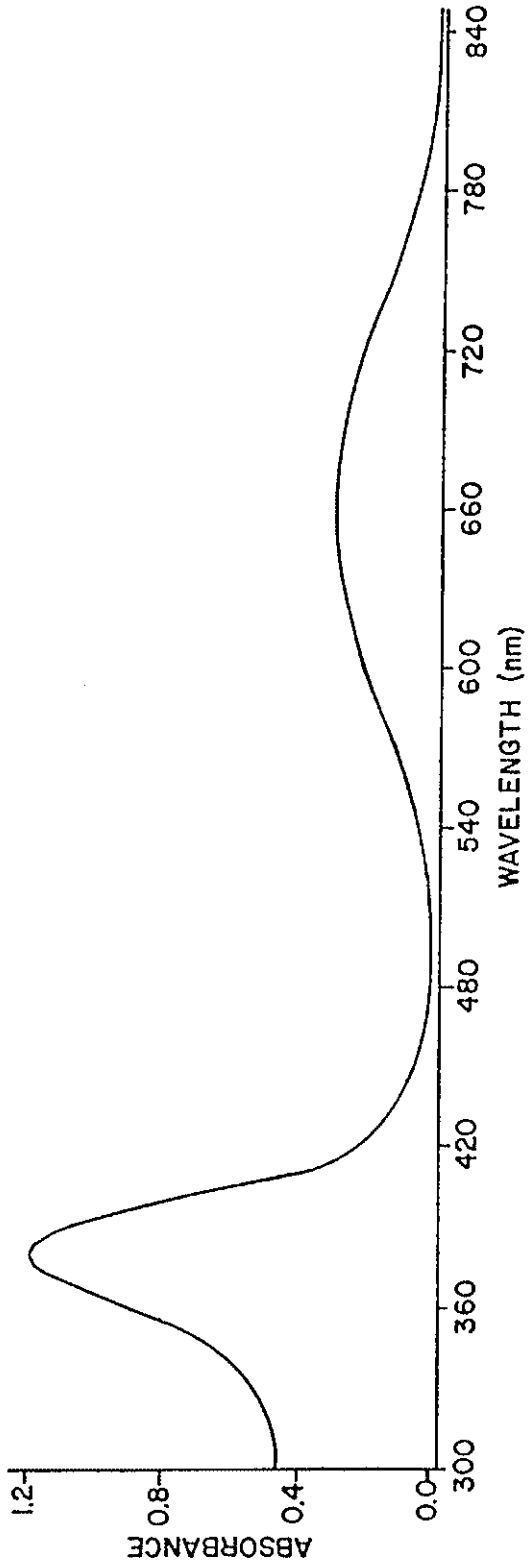
bilirubin's wavelength maxima were blue shifted relative to organic solvents by approximately 14 nm. In contrast to added base in organic solvents, the wavelength of maximum absorption in aqueous solution did not vary significantly between pH 9.00 and pH 10.00. The greatest change was in solubility.

The spectrum of bilirubin in a pH 10.00 solution containing BSA was also obtained. This resulted in a dramatic red shift of wavelength maximum relative to pH 10.00 buffer without BSA. The albumin-bilirubin complex absorbed a maximum amount of radiation at 476 nm.

Lastly, addition of chloroform saturated water to bilirubin in pH 10.00 solution had no effect of the pigment's wavelength of maximum absorption. Even though bilirubin was dissolved in a buffer and addition of water to a buffer has no effect on pH, the effect of trace amounts of chloroform on wavelength maxima was unknown. Nonetheless, the addition of chloroform saturated water resulted in only a nominal change in wavelength of maximum absorption.

The visible spectrum of biliverdin was obtained to detect any similarities between it's wavelength maximum and that of bilirubin. Similar absorption patterns between bilirubin and biliverdin were not expected because biliverdin has ten conjugated bonds compared to five for bilirubin. Therefore, biliverdin was anticipated absorbing longer wavelength radiation due to its more stable nature.

Biliverdin was observed having one major and one minor absorption peak in the visible region (Figure 15). The broad, minor peak between 600 to 700 nm is due to biliverdin conjugation and imparts the green color to biliverdin solutions. This maxima has a relatively low molar absorptivity and therefore is rarely observed during photochemical


ABSORBANCE
1.2
0.8
0.4
0.0
300
360
420
480
540
600
660
720
780
840
WAVELENGTH (nm)

experimentation. The primary absorption peak at approximately 377 nm, however, was repeatedly observed during irradiation of solubilized bilirubin. Thus, absorption of radiation in this region was of more interest. This primary maximum is the result of radiation absorption by unidentified chromophores.

Biliverdin's wavelength of maximum absorption was not found to be dependent upon the solvent to the same extent as bilirubin. Primary absorption maxima were found between 375-382 nm in all solvents, resulting in a difference of only 7 nm. This included pH 10.00 buffer containing BSA which may indicate that biliverdin does not readily bond to the protein. Table 6 lists dependencies of wavelength of maximum absorption for biliverdin in a few selected solvents.

Molar absorptivities of bilirubin and biliverdin were evaluated even though they had minimal significance relative to bilirubin and photoisomer behavior. Molar absorptivities were calculated in several different solvent systems. Table 7 lists the calculated molar absorptivies of both pigments at their wavelength of maximum absorption.

Absorbance difference (AD) spectrophotometry is a subdivision of visible spectrophotometry in which an absorption spectrum obtained during a particular spectrophotometric scan is subtracted from an earlier scan. Differences between two spectra are observed on an absorbance versus wavelength plot. The appearence of AD spectra are similar to data obtained from normal, absorption spectra, except that portions of plots may appear inverted. Furthermore, since spectral similarities cancel when taking their difference, a horizontal line corresponding to zero absorbance is commonly observed. Lastly, caution must be exercised in reporting absorption maxima obtained from AD

SOLVENT	ADDITIVE	WAVELENGTH OF MAXIMUM ABSORPTION
CH_3OH		375
CH_3OH	TMG	379
$CHCL_3$		379
DMF		381
DMF	TMG	382
pH 9.00		376
pH 10.00		376
pH 10.00	BSA	382
pH 10.00	$CHCL_3$ sat H_2O	376
TERNARY SOLN		378

Table 7. Molar Absorptivities of Bilirubin and Biliverdin (at their wavelengths of maximum absorption).

SOLUTE	SOLVENT	MOLAR ABSORPTIVITY
BILIRUBIN	$CHCL_3$	60,100
BILIRUBIN	DMF	57,400
BILIRUBIN	pH 10.00	44,500
BILIVERDIN	CH_3OH	42,200
BILIVERDIN	DMF	47,500
BILIVERDIN	pH 10.00	45,800

spectra because a positive spectral difference is not the true wavelength of maximum absorption.

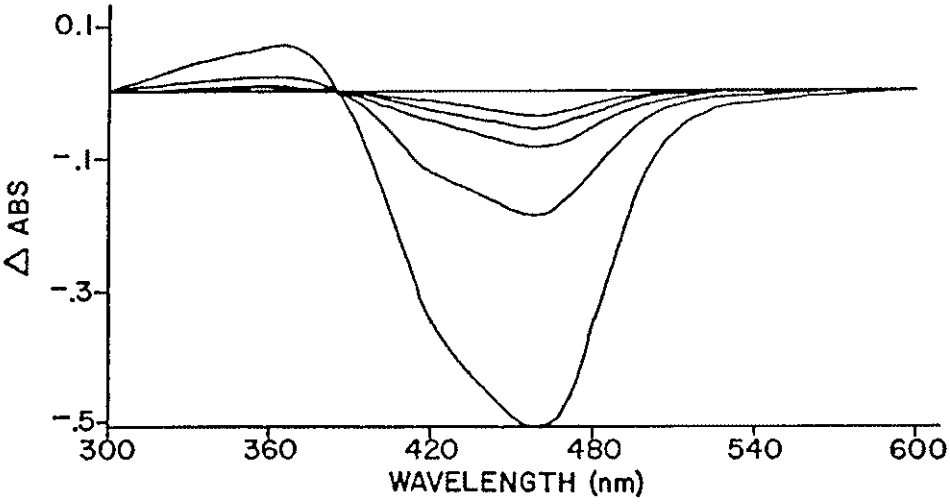
The greatest advantage in using AD spectrophotometry is its increased sensitivity over normal spectrophotometry. With the advent of computer interfacing, visible AD spectrophotometry has become an even more valuable analytical technique. Subtle differences that once went unnoticed can now be observed and recorded. Hence, photobilirubin can be detected in the presence of bilirubin with the use of computer aided AD spectrophotometry.

AD visible spectrophotometry was the first means of verifying the conversion of bilirubin into photoisomers. Initial attempts at producing photobilirubin, however, were unsuccessful. Anaerobic solutions of bilirubin in DMF were scanned and afterwards exposed to Special Blue light for five minutes. Then, irradiated solutions were rescanned under identical conditions. Instead of a positive difference peak, though, a large trough was observed in the area of bilirubin's wavelength maximum (Figure 16).

After removing the cuvette from the spectrophotometer and observing it under fluorescent room light, the originally, yellow solution appeared lighter in color. The yellow color had been bleached through photooxidation. This was found to be an irreversible process since the bleached solution never regained its original color or absorbance.

Several more attempts were made, this time varying the length of exposure. With exposure times greater than five minutes, the same phenomenon was observed: irradiation to colorless products. Continual irradiation resulted in the formation of a positive AD peak at 362 nm which was attributed to photooxidation of bilirubin to biliverdin or

Figure 16. Photooxidation of Bilirubin in DMF. Decreasing AD measurements were taken after irradiating for 15 seconds, 30 seconds, 1 minute, 5 minutes and 15 minutes, respectively. The horizontal line represents 0.0 absorbance difference.


0.1
-.1
-.3
-.5
Δ ABS
300
360
420
480
540
600
WAVELENGTH (nm)

some other vernoidal product. Even the normal spectrum displayed a secondary peak at approximately 390 nm after 30 minutes of exposure.

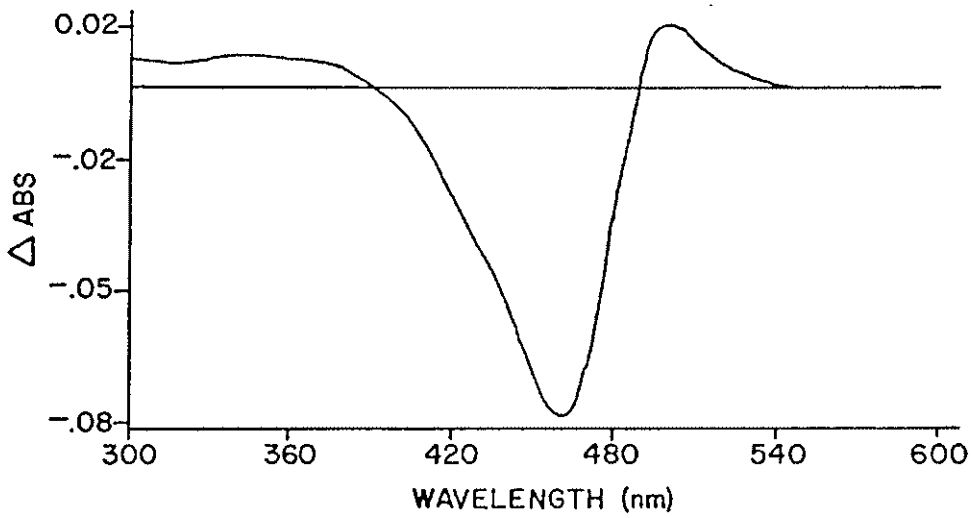
Exposure times less than five minutes were tried next. During exposures of 5-15 seconds, very small, positive AD absorbance values of less than 0.001 AU were detected at approximately 500 nm. Actual peaks, however, were not observed because of instrumental noise. A small trough at approximately 450 nm was again observed, indicating that a minute decrease in bilirubin concentration had occurred through photolysis and nominal photoisomerization. Finally, with irradiation times greater than 15 seconds, photooxidation overcame photoisomerization and positive AD values became negative.

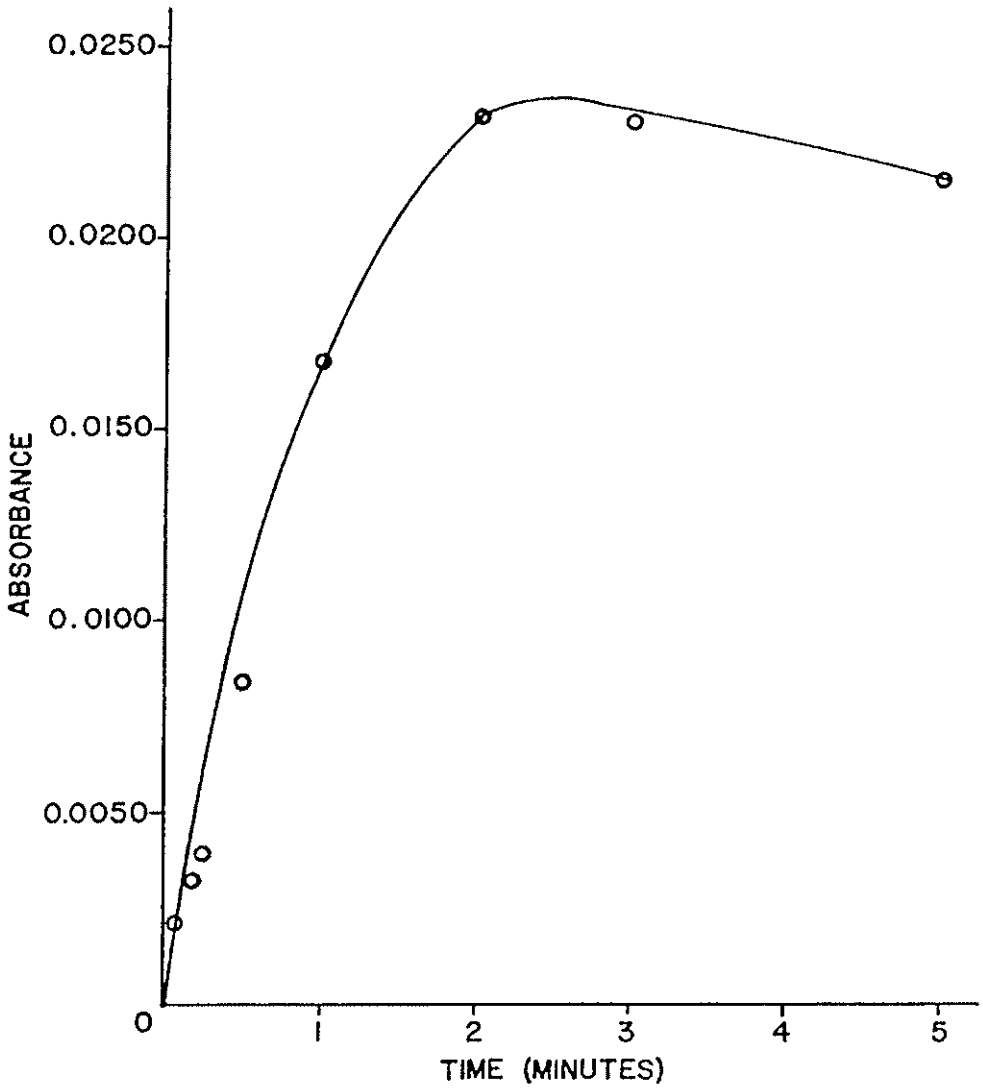
The production of photobilirubin was then attempted in chloroform, methanol and DMF with added TMG. Exposure times were begun at five seconds. In all cases, a positive peak was observed at approximately 500 nm after initial irradiation (Figure 17). The AD peak continued to grow up to a point with increased exposure to blue light. After that, absorbance peaks at 500 nm disappeared. The verdinoid peak and the photooxidation trough, though, continued to grow. Graph 2 depicts the growth and disappearance of photobilirubin in chloroform.

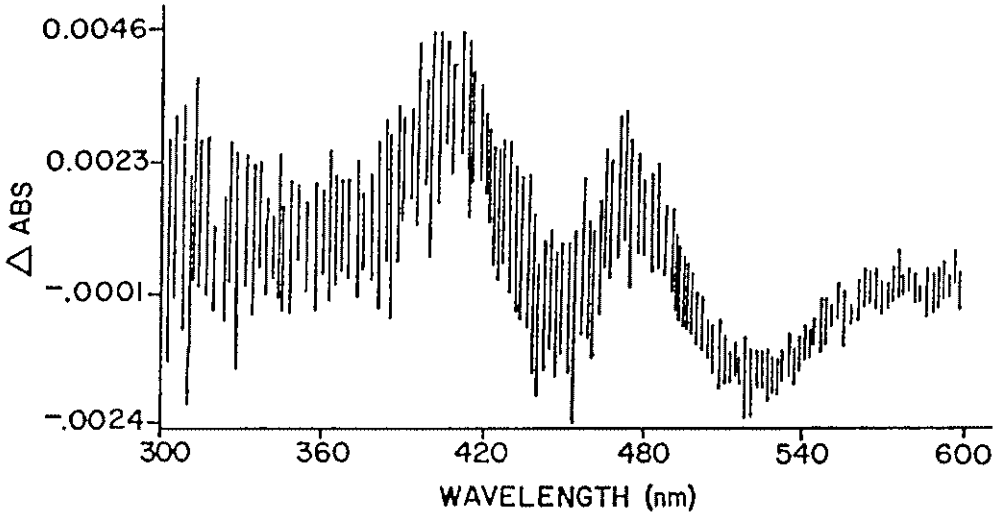
Finally, irradiation of bilirubin was performed upon pH 10.00 buffer solution and the ternary solution. In both of these solutions, an AD peak again formed, but at somewhat shorter wavelengths. In addition, a larger peak between 400-425 nm was developed (Figure 18). This new peak exhibited the same behavior as the peak at 500 nm: continual growth with light exposure followed by a decrease in height and subsequent peak loss with additional irradiation.

Biliverdin was also irradiated in a manner identical to bilirubin

Figure 17. Photoisomerization of Bilirubin in Chloroform. The horizontal line represents 0.00 absorbance difference.


0.02
-.02
-.05
-.08
Δ ABS
300
360
420
480
540
600
WAVELENGTH (nm)


0.0250
0.0200
0.0150
0.0100
0.0050
0
ABSORBANCE
1
2
3
4
5
TIME (MINUTES)

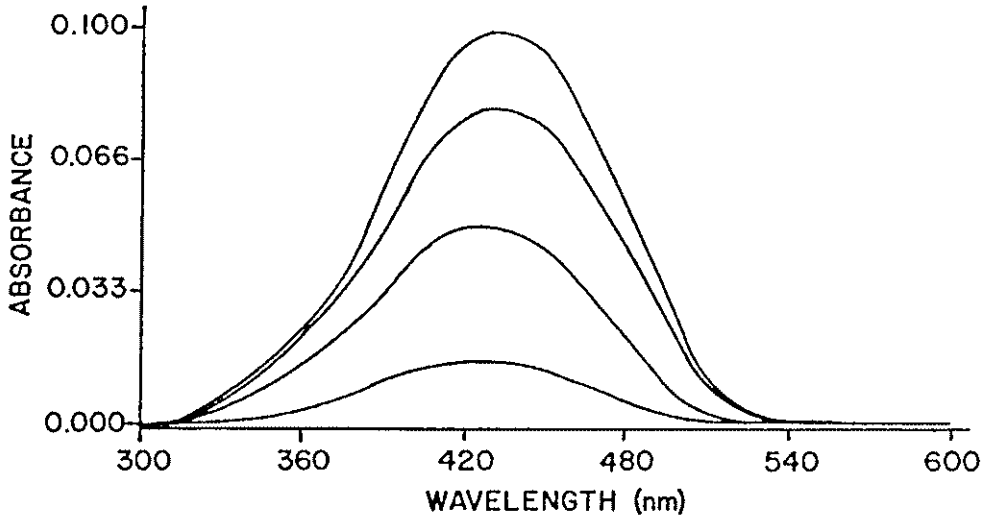

0.0046
0.0023
-.0001
-.0024
Δ ABS
300
360
420
480
540
600
WAVELENGTH (nm)

to determine if it too underwent photoisomerization. In anaerobic solutions, biliverdin did not have a positive difference peak. Biliverdin did, however, have very small, negative AD peaks at approximately 380 and 710 nm. Thus, biliverdin is believed to undergo limited photooxidation, but not photoisomerization.

The products from the chloroform/water extraction were also spectrophotometrically analyzed (Figure 19). The chloroform layer containing dissolved bilirubin was added to a methanol and water solution and the resulting solution was scanned. This spectrum was compared to a standard bilirubin spectrum in which the orange pigment was added to an identical solution of chloroform, methanol and water. No difference in the spectra were observed. Next, the aqueous layer was added to chloroform and methanol in the same proportions. This was done to ensure that behavioral correlations between bilirubin and photobilirubin would be made under identical conditions. This visible spectrum showed a broad peak between 420-435 nm, which is close to where the AD peak was observed in pH 10.00 buffer and the ternary solution.

An approximation was made of how much photobilirubin was produced and subsequently collected in the aqueous layer during the extraction process. In doing so, exactly 200 uL of the initial chloroform layer was diluted to 2 mL and spectrophotometrically scanned. After measuring the absorbance, the solution was returned to the separatory funnel. The chloroform layer, after irradiation, was drained from the separatory funnel into a 50 mL volumetric flask. The separatory funnel was swirled with clean chloroform to remove any residual bilirubin adsorbed to the glass walls. Both chloroform solutions were combined and diluted to volume. Then, 200 uL of the resulting solution was further diluted to 2

Figure 19. Visible Spectra of Extraction Products. Absorbance of aqueous extract was measured after cummulative 15 minute irradiation.


0.100
0.066
0.033
0.000
ABSORBANCE
300
360
420
480
540
600
WAVELENGTH (nm)

mL. The spectrum was obtained in an identical manner as the initial sample. As a result, approximately 24% of bilirubin was converted to photoproducts by taking the ratio of the difference in bilirubin concentration to nonirradiated bilirubin and multiplying by 100. This assumes, however that all bilirubin was converted to photobilirubin and not biliverdin or other photoproducts.

Differential Pulse Voltammetry

Voltammetry is an analytical technique that is often referred to as the "electrochemical equivalent of spectroscopy." In utilizing voltammetric analyses, the current or electron flow through an electrochemical cell is measured as a function of an electrode's applied potential. The applied potential is the electron pressure required to force an electroactive species to gain or lose electrons. At positive potentials, electrons from an analyte are lost to the anode. Thus, the analyte is oxidized in the process. Analytes are reduced at negative potentials where electrons are gained from a cathode. Both the oxidation and reduction of electroactive species occur at precise potentials in well defined systems and form the basis of qualitative analysis.

In quantitative voltammetric analyses, current is measured. The measured current is actually the sum of two different currents. The current recorded at the beginning of a voltammogram, usually to establish a baseline, is called the capacitive or residual current. The capacitive current is a measure of electrode double layer charging. A Faradaic current, on the other hand, is a measure of electron transfer between solute species in the immediate vicinity of the electrode and the electrode's surface. Thus, only analytes immediately adjacent to

the electrode's surface are electrolyzed. The solute concentration in bulk solution remains essentially constant.

During localized electrolysis, electroactive species are transported to an electrode's surface by natural diffusion. This is a consequence of the concentration gradient established between depleted solute concentration at the electrode surface and higher solute concentration in the bulk solution. Furthermore, this assumes that transport by convection is eliminated in unstirred systems and electrical migration is minimized in the presence of supporting electrolyte. The Faradaic current which is governed by diffusion processes is thus called the diffusion current.

The diffusion current can have the appearance of either a peak or a wave and it is directly proportional to analyte concentration through the Ilkovic equation:

$$i=nFAD\left(\frac{dc}{dx}\right)_{x=0}$$

where, i=diffusion current, n=number of electrons transfered, F=Faraday's constant, A=electrode area, D=diffusion coefficient and dc/dx=diffusion rate. The diffusion rate is actually a concentration gradient differential which encompasses the concentration term.

A more practical expression showing that the diffusion current is directly proportional to solute concentration is the Randles-Sevcik equation:

$$i=kn^{2/3}AD^{1/2}cV^{1/2}$$

where, k=constant, c=molar concentration and V=scan rate. The Randles-Sevcik equation also demonstrates how sensitivity in current measurements can be improved by increasing scan rate. Not shown in the

equation, though, is how increased scan rate lowers resolution. Lower resolution through increased scan rate is a consequence of capacitive current and, to a lesser extent, slow recorder response.

Differential pulse voltammetry (DPV) is one type of voltammetry that was used in this research primarily because of its increased sensitivity over classical, dc techniques. DPV differs from differential pulse polarography only in that a solid, glassy carbon electrode (GCE) or a hanging mercury dropping electrode (HMDE) was used instead of the standard, dropping mercury electrode. In DPV, fixed magnitude pulses are superimposed on a linearly increasing voltage ramp. The current difference is measured between the beginning of pulse application and near the end of pulse life.

The lowest pulse amplitude of 25 mV was selected to improve resolution between bilirubin and photobilirubin isomers. This was at the expense of forfeiting some sensitivity. A moderate scan rate of 20 mV/s was used as a compromise between resolution and sensitivity. Other instrumental parameters included a 0.2 second duration pulse and a deposition time of 000 seconds. Both the applied potential range and the current amplitude were varied and can be obtained from each individual voltammogram.

In making anodic potential measurements, a GCE was used as the indicator electrode because of its inertness relative to mercury at positive potentials. One problem associated with the GCE is insuring that the electrode responds in a reproducible manner. Removal of carbonyl formation and adsorption products on the GCE surface was attempted by holding the electrode at a conditioning potential of -0.5 V for 5 minutes prior to scanning. This, unfortunately, did not prove

satisfactory. Polishing the carbon surface with an alumina slurry on a felt pad was tried next. Although this procedure may have been tedious, it was effective.

The standard Ag/AgCl electrode was used as reference in all voltammetric analyses reported here. However, in research involving anodic measurements with DMF and sodium perchlorate, the Vycor frit of the Ag/AgCl continually cracked. DMF would then poison the AgCl electrolyte as evidenced by a visible brown discoloration. This was usually accompanied by potential shifts which resulted in undesired artifacts. New reference electrodes were evaluated for use in the model 303A.

After contacting the manufacturer, the problem was attributed to changes in either ionic strength or solvent character. As an alternative, the manufacturer recommended using a nonaqueous, $Ag/AgNO_3$ reference electrode filling solution with a polyethylene disk. Use of the $Ag/AgNO_3$ reference electrode was attempted but proved unsuccessful because of excessive, electrolytic bleeding which contaminated the test solution.

A pseudo-$Ag/AgClO_4$ reference electrode system was evaluated in which the silver wire from the original Ag/AgCl electrode was immersed in the sample cell containing sodium perchlorate. Unfortunately, problems associated with potential measurements were still present. Even though the concentration of 0.1M sodium perchlorate was greater than 400 times the pigment concentration in the cell, potential variations were still anticipated because bilirubin, biliverdin and photobilirubin would all affect the reference potential of the electrode differently. Furthermore, the magnitude of potential variations could

not be determined. Since the pseudo-Ag/$AgClO_4$ reference electrode system was unacceptable for qualitative analysis in which precise peak potentials were measured, its use was discontinued.

A counter electrode was a third electrode incorporated into the electrochemical cell. The counter electrode was constructed of a platinum wire. Its use minimized errors in potential measurements attributed to current passing though the reference electrode and also to solution resistance. The platinum counter electrode was used for both anodic and cathodic measurements.

Anodic measurements of bilirubin solutions were begun by determining oxidation potentials in DMF (Figure 20). Bilirubin was oxidized at approximately +0.42 V vs Ag/AgCl (ceramic plug instead of Vycor frit) while biliverdin, the oxidation product of bilirubin, was oxidized at approximately +0.59 V. In basic solution, brought about by addition of TMG, bilirubin was easier to oxidize as the pigment's oxidation potential was cathodically shifted 80 mV to +0.34 V.

Anodic measurements were also made of bilirubin in the ternary solution. However, in this solution, bilirubin had unstable peakforms. An approximate oxidation potential of +0.49 V was measured.

Oxidation potentials of bilirubin and biliverdin were next determined in aqueous solution. In pH 10.00 buffer, bilirubin was oxidized at +0.14 V (Figure 21). A large biliverdin peak was also observed at +0.33 V. Biliverdin was confirmed by addition of this pigment to pH 10.00 buffer and anodically scanning in an identical manner as with bilirubin (Figure 22).

Irradiation of bilirubin in DMF was performed even though photobilirubin was not detected through AD spectrophotometry.

Figure 20. Anodic Voltammogram of Bilirubin in DMF. Note the following oxidative scans: A (solvent), B (bilirubin) and C (bilirubin dianion).

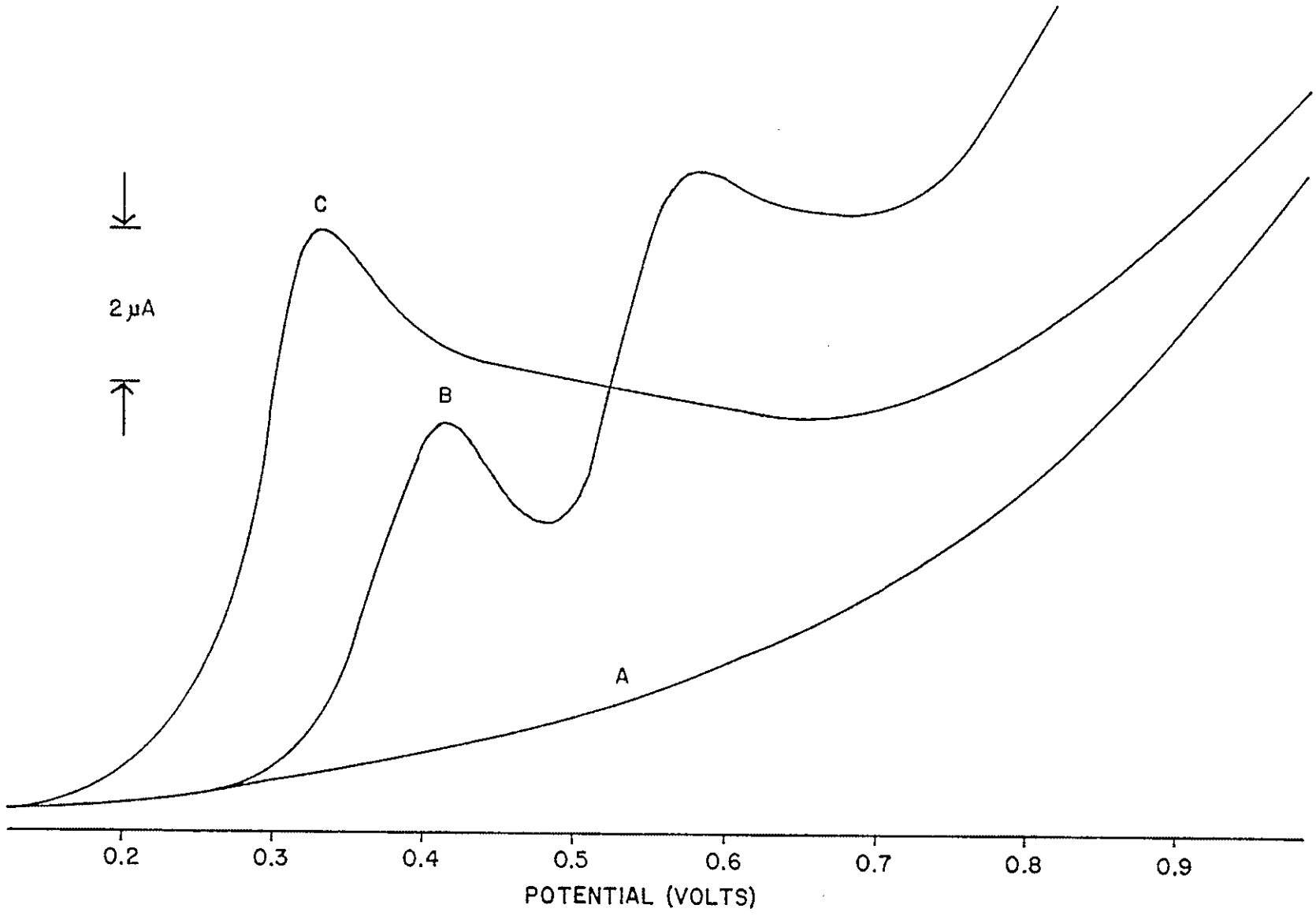

C
2 μA
B
A
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
POTENTIAL (VOLTS)

Figure 21. Anodic Voltammogram of Bilirubin in pH 10.00 Buffer. Note the following oxidative scans: A (solvent) and B (bilirubin).

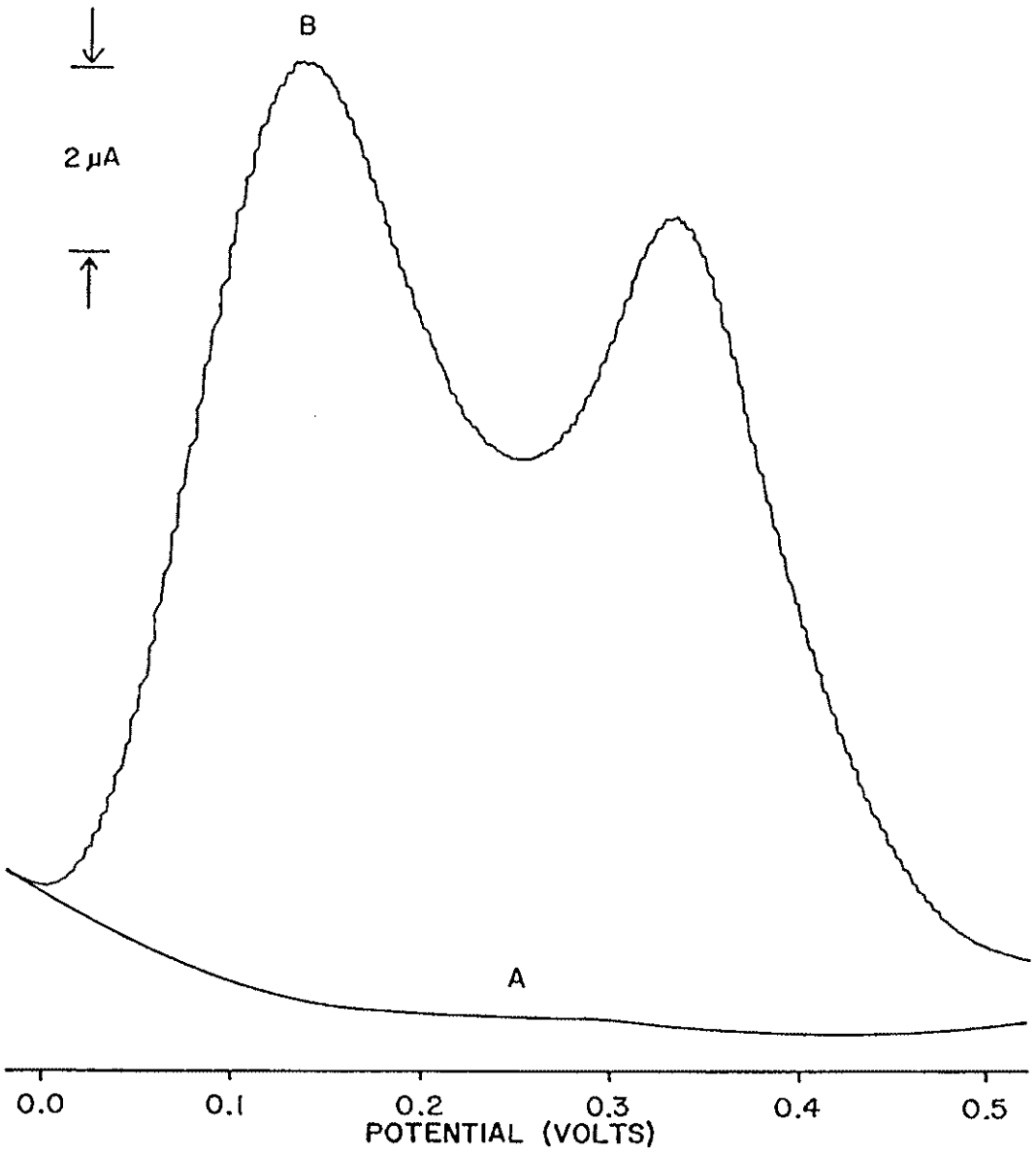
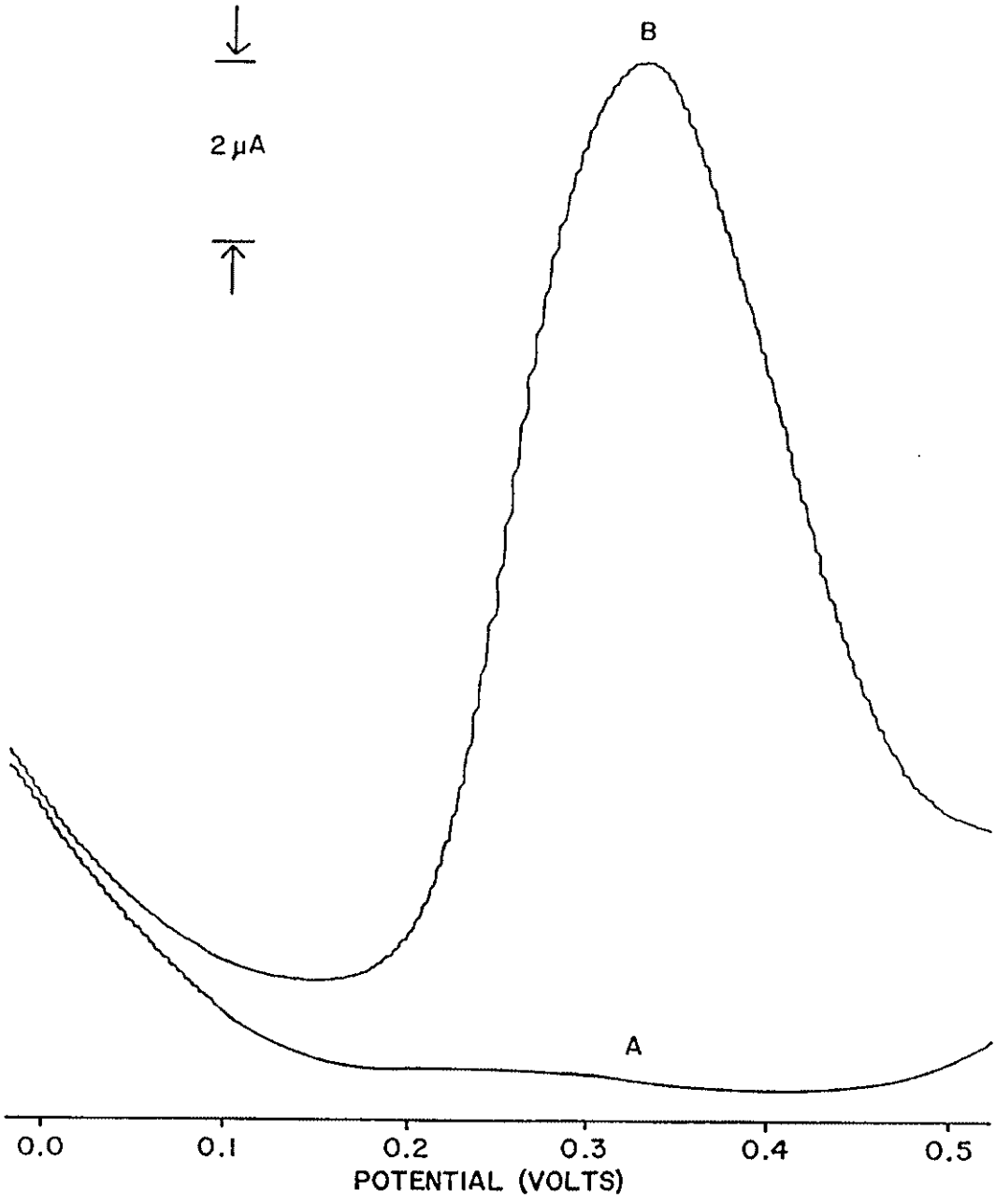

B
2 μA
A
0.0
0.1
0.2
0.3
0.4
0.5
POTENTIAL (VOLTS)

Figure 22. Anodic Voltammogram of Biliverdin in pH 10.00 Buffer. Note the following oxidative scans: A (solvent) and B (biliverdin).


2 μA
B
A
0.0
0.1
0.2
0.3
0.4
0.5
POTENTIAL (VOLTS)

Similarily, photobilirubin production was not detected here either. Irradiation was also attempted in the ternary solution despite the presence of irregular shaped peaks. Again, this did not result in any additional peaks being detected. Finally, irradiation of bilirubin was performed in pH 10.00 buffer, but this did not give rise to photobilirubin detection either.

Anodic voltammetry of the aqueous extracted photoproducts was then pursued (Figure 23). Exactly 1 mL of the extract was added to 19 mL of pH 10.00 buffer. A control using the same solvent system with added bilirubin was also analyzed, but it did not result in any potential shift relative to using pH 10.00 buffer alone. The resulting voltammogram showed a peak at +0.33 V, exactly where biliverdin is oxidized. This explains why an additional peak was not initially observed during irradiation of the sample solution. Both photobilirubin and biliverdin peaks interfer with each other. Furthermore, this implies that photobilirubin is harder to oxidize than bilirubin.

Prior to making cathodic potential scans, the GCE was replaced with the standard HMDE. Even though the GCE could have been used at negative potentials, the HMDE was selected because of the ease of operation associated with forming a new electrode surface after each scan. The reference and counter electrodes were left unchanged. Cathodic measurements were made exclusively in aqueous pH 10.00 solution. The ternary solution could not be used due to drastic shortening of the cathodic range by chloroform.

In pH 10.00 buffered solution, bilirubin reduction was found to occur at -1.43 V (Figure 24). Conversely, biliverdin has two reduction peaks. The primary reduction peak of biliverdin occured at -1.47 V,

Figure 23. Anodic Voltammogram of Extraction Products in pH 10.00 Buffer. Increasing diffusion currents of aqueous extract were recorded after cummulative irradiations of 15 minutes. Note the following oxidative scans: A (solvent) and B (aqueous extract).

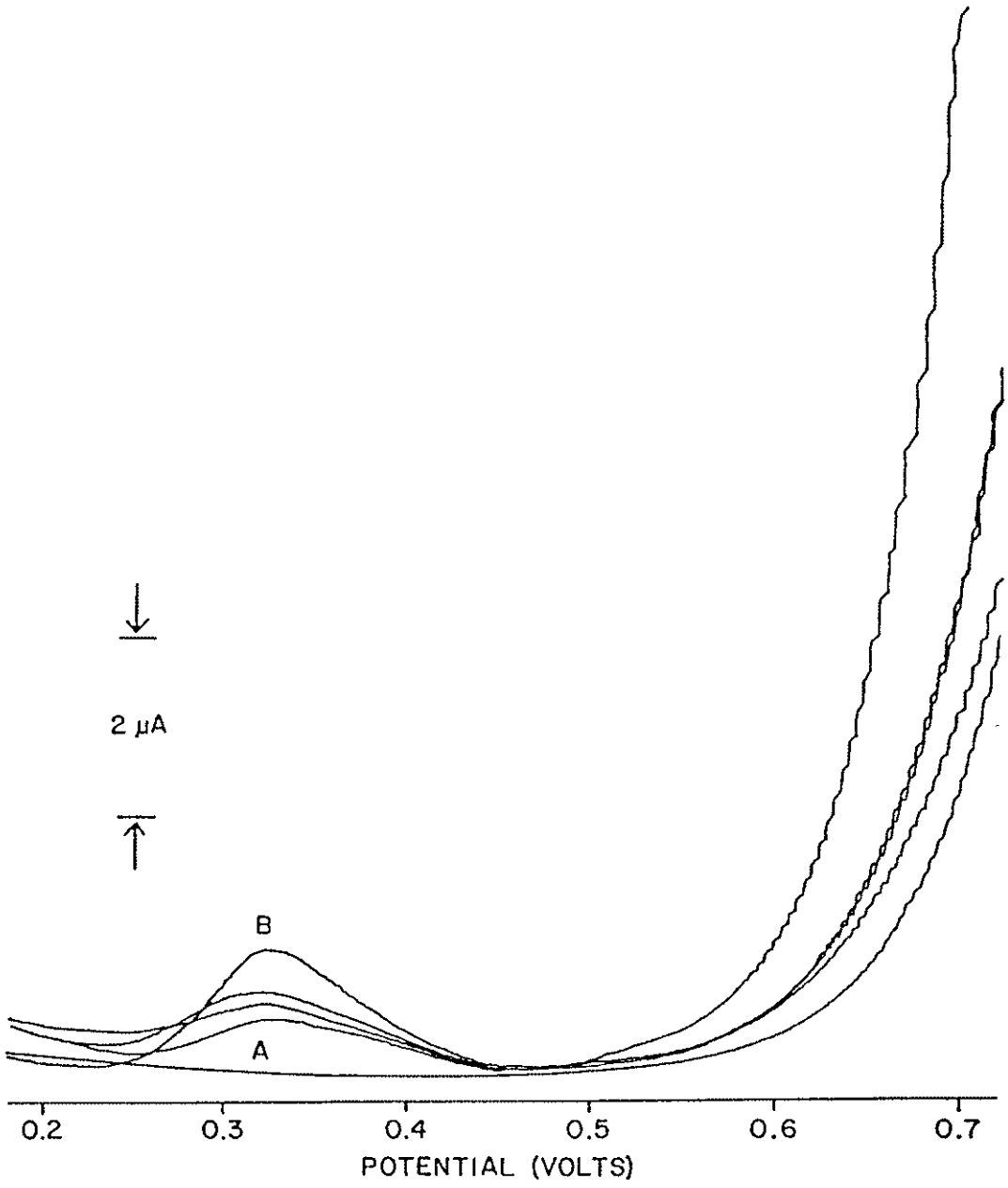
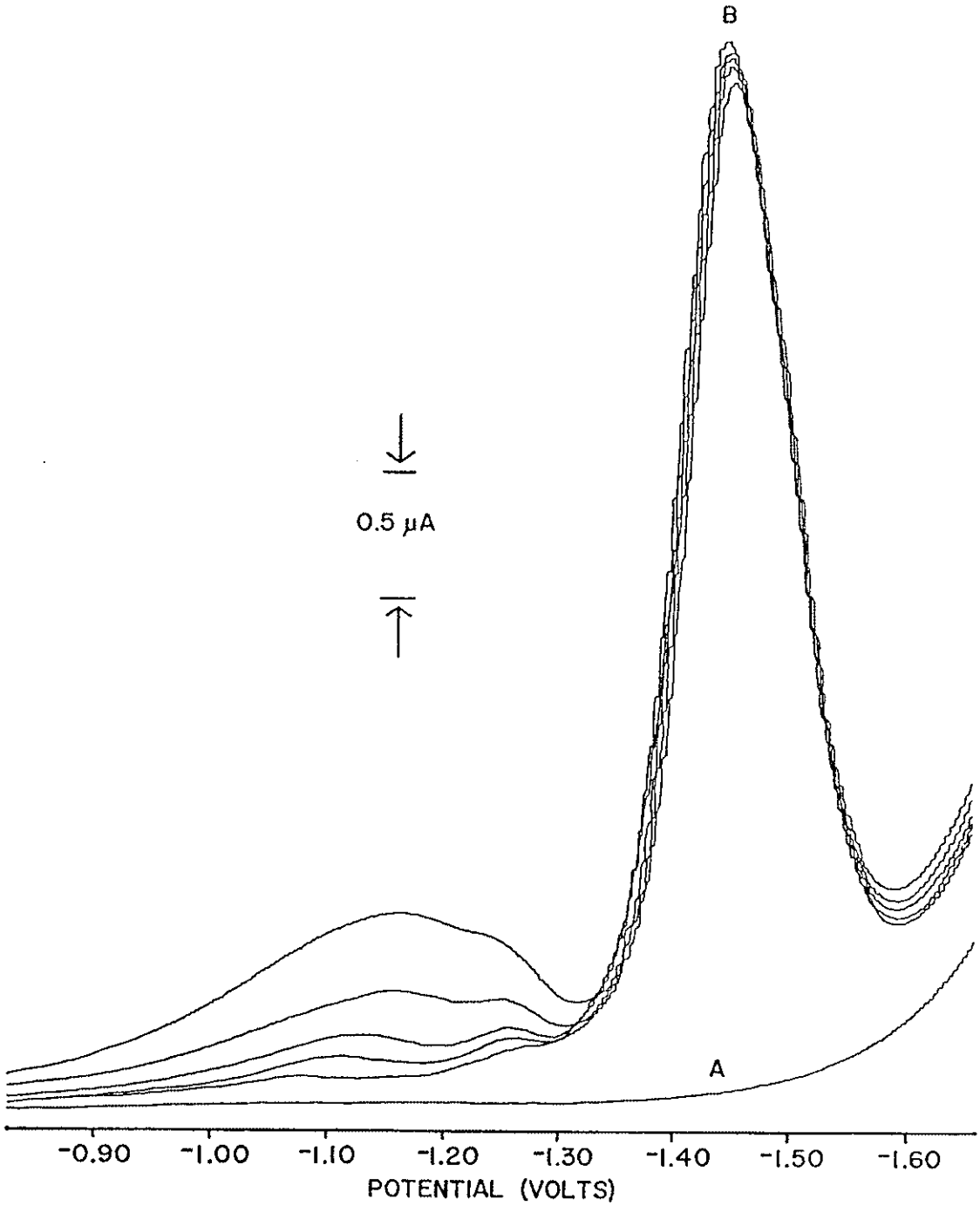

2 μA
B
A
0.2
0.3
0.4
0.5
0.6
0.7
POTENTIAL (VOLTS)

Figure 24. Cathodic Voltammogram of Bilirubin in pH 10.00 Buffer. Decreasing bilirubin diffusion current was followed by increasing diffusion current between -1.00 V and -1.30 V for irradiations of 15 seconds, 1 minute, 5 minutes and 15 minutes, respectively. Note the following reductive scans: A (solvent) and B (bilirubin).


B
0.5 μA
A
-0.90
-1.00
-1.10
-1.20
-1.30
-1.40
-1.50
-1.60
POTENTIAL (VOLTS)

almost identical to that of bilirubin (Figure 25). Not shown in Figure 25 is a secondary reduction peak at -0.78 V.

Irradiation of bilirubin in pH 10.00 buffer resulted in what appeared to be two very broad peaks. The first reduction peak was at approximately -1.10 V while the smaller, second peak was at -1.25 V. The peaks continued to grow, even with irradiation times up to 15 minutes. Furthermore, the diffusion current of bilirubin continually decreased by a nominal amount.

Addition of the aqueous extract to pH 10.00 buffer resulted in the formation of a reduction peak occuring at -1.24 V (Figure 26). This is the same reduction potential as the smaller reduction peak formed during irradiation of bilirubin in identical solution. This peak, however, was not readily evident at first. Because of trace amounts of chloroform dissolved in the extracted water, the cathodic range was shortened to the potential where photobilirubin is reduced. This problem was easily overcome though by continually purging the test solution with nitrogen. As the chloroform is eventually evaporated from the solution, the formation of a peak becomes more evident. Eventually the photobilirubin peak is completely resolved from the leading edge of the cathodic limit.

Table 8 is a summary of anodic and cathodic potentials found in this research. Potentials for bilirubin, photobilirubin and biliverdin are all included.

High Performance Liquid Chromatography

Liquid chromatography is a separation technique based on the interaction of a solute between a mobile and stationary phase. The mobile phase in HPLC is a liquid, preferrably of the same composition as that in which the sample is dissolved. This alleviates problems such as

Figure 25. Cathodic Voltammogram of Biliverdin in pH 10.00 Buffer. Note the following reductive scans: A (solvent) and B (biliverdin).

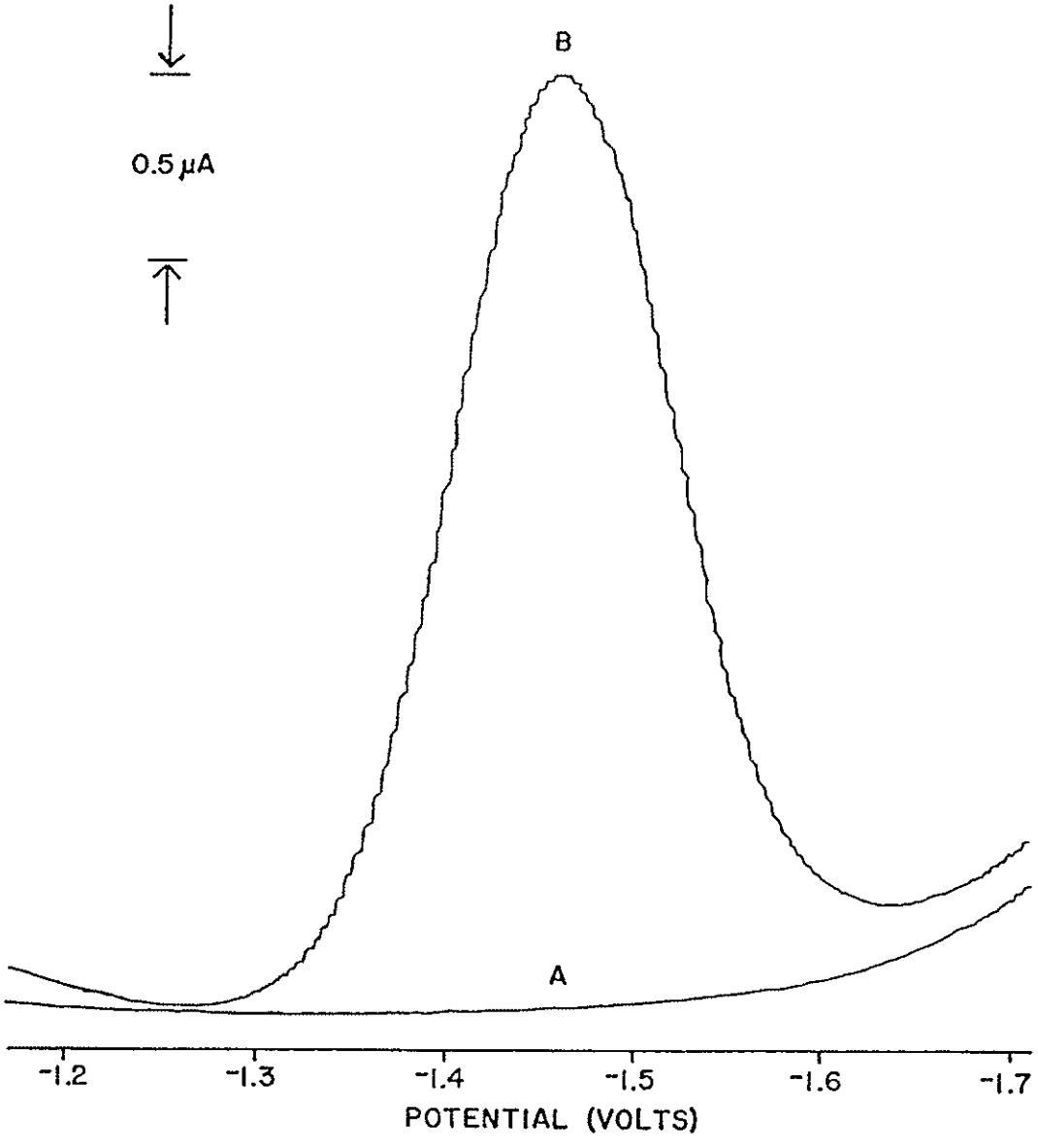

0.5 μA
B
A
-1.2
-1.3
-1.4
-1.5
-1.6
-1.7
POTENTIAL (VOLTS)

Figure 26. Cathodic Voltammogram of Extraction Products in pH 10.00 Buffer. Increasing diffusion currents of aqueous extract were recorded after cummulative irradiations of 15 minutes. Note the following reductive scans: A (solvent) and B (aqueous extract).

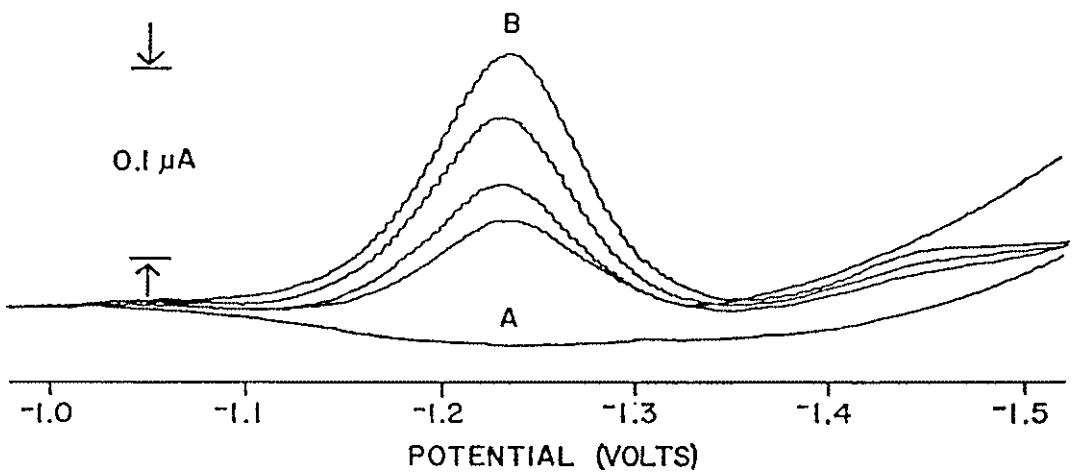

B
0.1 μA
A
-1.0
-1.1
-1.2
-1.3
-1.4
-1.5
POTENTIAL (VOLTS)

Table 8. Anodic and Cathodic Potentials of Bilirubin, Photobilirubin and Biliverdin.

PROCESS	SOLVENT	SPECIES	Ep
ANODIC	DMF	BILIRUBIN	0.42
		BILIVERDIN	0.59
	TERNARY SOLN	BILIRUBIN	0.49
	AQ pH 10.00	BILIRUBIN	0.14
		PHOTOBILIRUBIN	0.33
		BILIVERDIN	0.33
CATHODIC	AQ pH 10.00	BILIRUBIN	-1.43
		PHOTOBILIRUBIN	-1.24
		BILIVERDIN	-1.47

negative peaks which are often attributed to changes in refractive index of the mobile phase. Therefore, unless specifically mentioned to the contrary, identical mobile phase and sample solution composition were used in this research.

The stationary phase can be either a liquid or a solid. If the stationary phase is a liquid, the solute will partition between the two immiscible liquid phases. Partition or, more specifically, bonded phase chromatography was the sole type performed in this research. In the latter case, a solute will undergo selective adsorption onto a solid stationary phase based on its relative polarity.

The stationary phase chosen for bonded phase chromatography can be either more or less polar than the mobile phase. If the mobile phase is more polar than the stationary phase, then this type of chromatography is called reverse phase. Normal phase chromatography refers to a more polar stationary phase relative to the mobile phase. Both reverse and normal phase chromatography were attempted in this research.

Optimal conditions are actually a compromise between good partitioning of similar polar species and the prevention of column dissolution. The object is to choose a stationary phase of similar polarity to the solute while the mobile phase is of different polarity. This was difficult to accomplish since bilirubin is a nonpolar species and photobilirubin is very water soluble.

In selecting a chromatographic mode for separating bilirubin from photobilirubin, several guidelines were reviewed (29,30,31). Pigment solubility appeared to be the primary concern, especially since bilirubin has a molecular weight considerably less than 2000 g/mol.

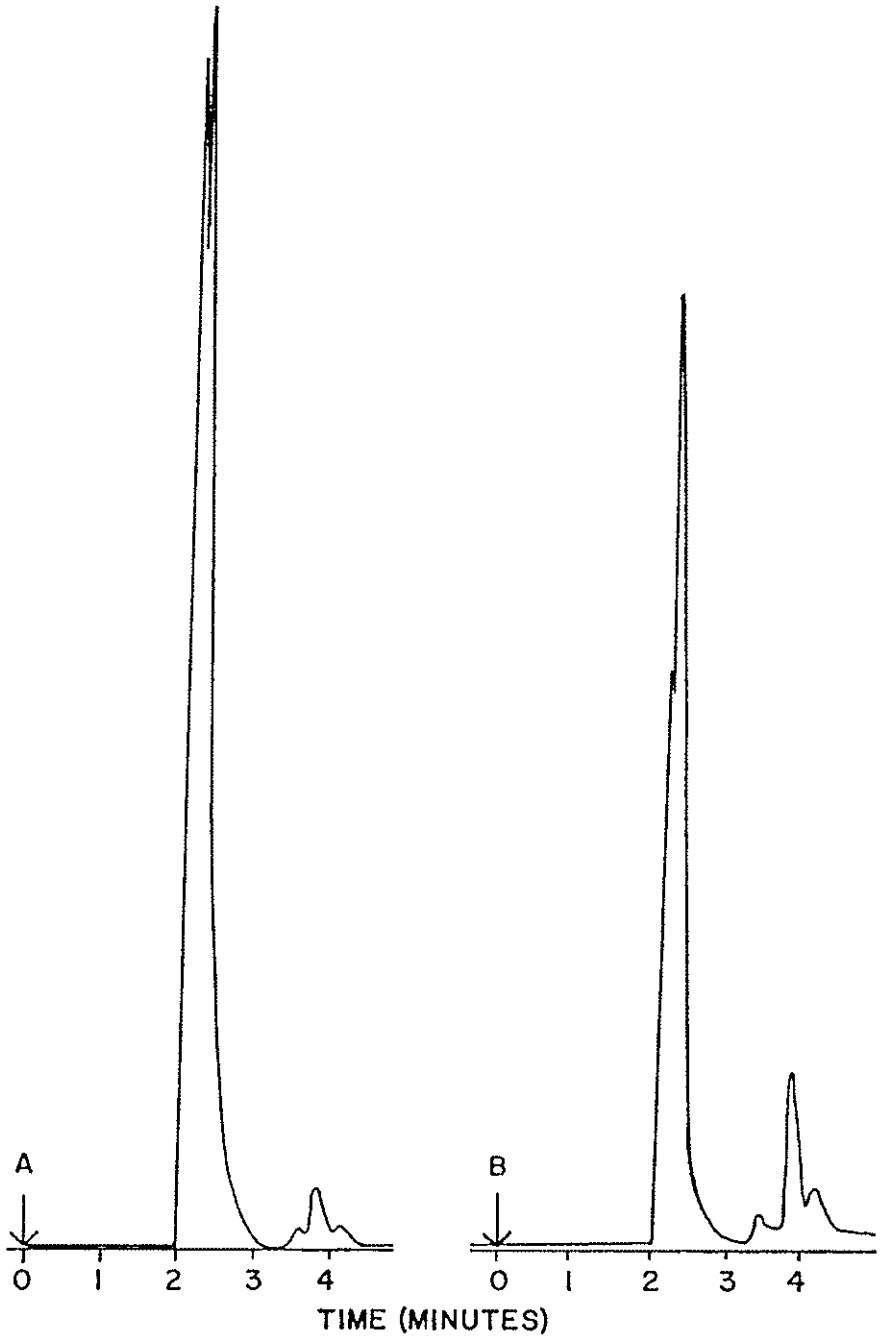
Therefore, reverse phase (RP) chromatography utilizing an aqueous or polar, organic mobile phase was selected because bilirubin solubility is greatest in moderately polar, organic solvents. Another choice, however, could have been an alkylamine (R-NH2) column which is commonly used for separating compounds displaying different degrees of hydrogen bonding.

The RP octadecylsilane (C-18) column which had been in use for several years in the instrumental lab was used first. The polar mobile phase selected was 100% methanol. A flow rate of 1 mL/min was set on the instrument and a 254 nm filter was used for absorbance detection. To prevent detection of negative peaks, bilirubin solutions were made by dissolving the pigment in methanol, even though it exhibited limited solubility. Because of this nominal solubility in methanol, detection limits of 0.05 absorbance units full scale (AUFS) and lower were used.

Bilirubin was successfully separated from photobilirubin and two of the three photoisomers were partially resolved in this system (Figure 27). Furthermore, two additional peaks were observed, most likely due to bilirubin IIIα and bilirubin XIIIα impurities. An odd observation, though, was that nonpolar bilirubin came off the nonpolar column before polar photobilirubin. Thus, the column appeared to be acting in a normal phase fashion. Nonetheless, at least partial separation was achieved and the growth of two peaks occurred with increasing irradiation.

To aid bilirubin dissolution in methanol and thus increase the concentration of photoisomers produced during irradiation, TMG was added to the solution. TMG, however, was not added to the mobile phase. In doing so, spurious results were obtained such as additional peaks coming

Figure 27. Normal Phase Chromatography of Bilirubin and Photobilirubin: A (minimal light exposure) and B (after 30 seconds of irradiation).


A
B
0
1
2
3
4
0
1
2
3
4
TIME (MINUTES)

off the column. This was later attributed to TMG as it also absorbs 254 nm radiation.

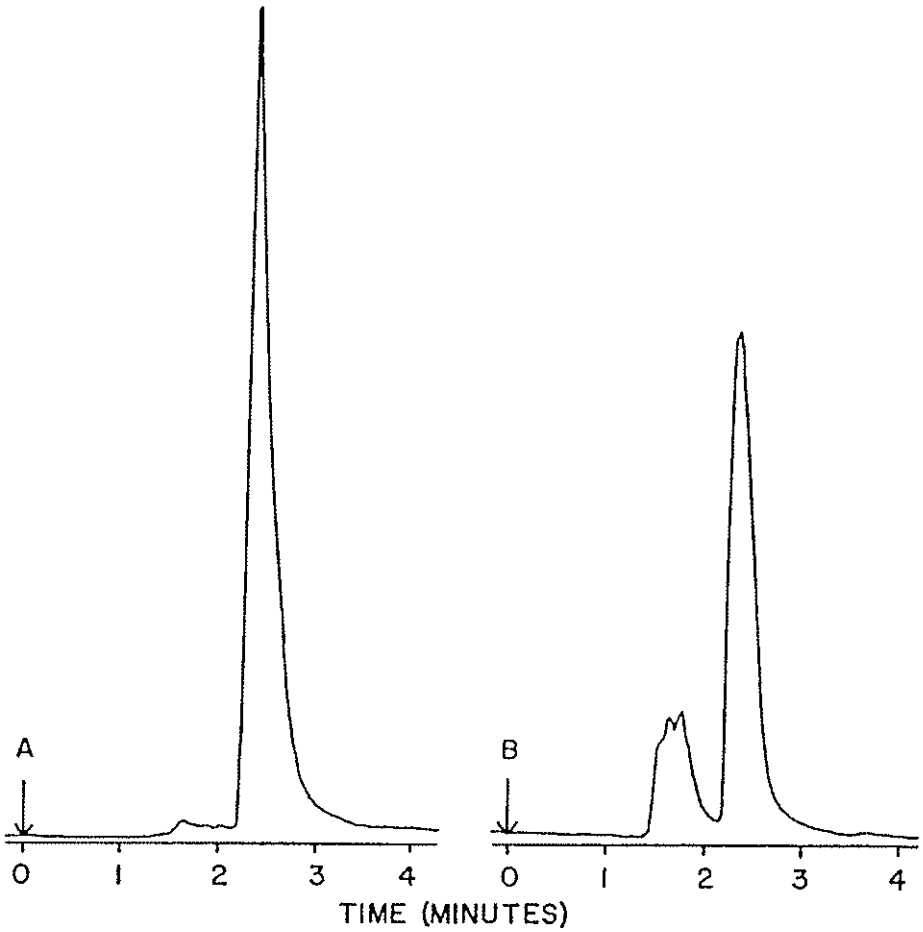
Since the presence of TMG interferred with bilirubin and photobilirubin detection using a 254 nm filter, UV detection was changed to visible detection using a 436 nm filter. Afterwards, analyses of bilirubin solutions containing methanol and TMG were resumed. As a result, loss of isomer separation was observed. This presumably occurred because the dianion forms of bilirubin and photobilirubin did not interact with the packing material and came off the column with the solvent front.

Chloroform was added to methanol next to aid in bilirubin dissolution. The concentration of chloroform in methanol was varied from 10-45%. In higher chloroform concentrations, instrumental detection was raised to 0.2 AUFS due to increased bilirubin concentration. Only two peaks were resolved. In addition, the column still acted in a normal phase mode.

In hopes of promoting increased interaction of bilirubin and possibly photobilirubin with the column packing, water was added to the mobile phase. This resulted in the column now acting in a reverse phase manner as photobilirubin eluted first (Figure 28). In 45%chloroform/ 50%methanol/5%water, photobilirubin was almost completely separated from bilirubin. The three photoisomers were resolved but eluted close to the solvent front, within 2 1/2 minutes of injection. In 13% water, the maximum concentration before the chloroform/methanol/water solution became cloudy, photobilirubin was completely separated from bilirubin but photobilirubin isomers eluted from the column unresolved.

Following these observations, a decision was made to clean the

Figure 28. Reverse Phase Chromatography of Bilirubin and Photobilirubin: A (minimal light exposure) and B (after 45 minutes of irradiation).


A
B
0
1
2
3
4
0
1
2
3
4
TIME (MINUTES)

column in hopes of removing any residues and thus promoting better interaction between bilirubin and photobilirubin with the packing material. The column was flushed with 0.015N sulfuric acid for approximately two hours. A fast and slow flush of 1.8 mL/min and 0.7 mL/min respectively were used. This was followed by similar flushing with deionized water to remove the acid and eventually the chloroform/methanol/water mobile phase to reequilibrate the column back to normal conditions.

After cleaning, the same bilirubin solutions were again injected onto the column. Although similar selectivity was observed, neither resolution nor separation were improved. In addition, more than usual peak tailing was also noticed. It appeared that the pigments were interacting with packing material more strongly now that the column had been cleaned.

A C-18 column packed by the chemistry department at Virginia Polytechnic Institute and State University was tried next to provide better separation of the pigments. Its use, however, was quickly terminated after poor chromatographic results were obtained and it was discovered that the column only had 9,000 theoretical plates, as measured by the packer.

After contacting the manufacturer of the original C-18 column and communicating to them the problem with separation, they provided a new C-18 column for temporary use. Injection of bilirubin in the same chloroform/methanol/water solution onto the new column again resulted in peak tailing, this time extremely severe. Therefore, Beckman Instruments, Inc. was contacted once more. The applications department concluded that the older C-18 column must have a residue irreversibly

adsorbed onto the packing material. This lowered the interaction between it and bilirubin while it did not affect photobilirubin because of its different polarity. Therefore, the applications department mailed a "less" reverse phase, alkylnitrile (C-CN) column.

Unfortunately, use of the alkylnitrile column did not result in any better separation or resolution. In general, retention of bilirubin was only 30 seconds quicker than retention using the C-18 column. Furthermore, since photobilirubin elutes very closely to the solvent front, hardly any change in its retention was observed. The only other difference between the two columns occurred at 97% chloroform/3%methanol where selectivities were reversed. The following is the sequential methodology used to evaluate both columns using various combinations of chloroform, methanol and water.

In 100% chloroform, both columns acted similarly in that a broad bilirubin peak was measured. Bilirubin took approximately 6-25 minutes to elute from the column. With 1% methanol added to chloroform, a broad peak was again observed. On this occasion, the peak began at four minutes and was completed at eight minutes. Finally, addition of 3-20% methanol to chloroform resulted in a relatively sharp bilirubin peak. However, with these mobile phases, bilirubin came off the column with the solvent front.

With increased concentrations of methanol, up to 100%, strong interaction again began for both columns. Within this entire range, bilirubin would begin eluting at approximately six minutes and would not completely leave the column until 5-8 minutes later. Thus, the entire range of methanol and chloroform concentrations had been explored.

Water was added to the mobile phase which had chloroform

concentrations less than 45%. This did aid in some separation of bilirubin from another unidentified peak but bilirubin was still strongly retained and took a considerably long time for complete elution.

Finally, equal molar TMG was added to bilirubin solutions and separation was attempted as before following a similar sequence. In all cases, the dianion form of bilirubin resulted in the pigment eluting with the solvent front. No separation of bilirubin and photobilirubin occurred presumably because photobilirubin was ionized also. Furthermore, negative and positive peaks were always present due to changes in the refractive index of mobile phase passing by the detector.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

Discussion

An attempt has been made to develop a system in which bilirubin and photobilirubin can be analyzed in identical chemical environments. This has been a difficult task since the chemistry of bilirubin and its associated isomers is dependent on so many variables such as light, oxygen, temperature and time. Bilirubin's chemical behavior was evaluated first using three different instrumental techniques. This was followed by analyses of biliverdin to ascertain any behavioral similarities between the two pigments, where applicable. Finally, the chemistry of photobilirubin and other photoisomers was studied in detail.

Before the commencement of instrumental analyses, bilirubin's solubility was explored in various solvents. Bilirubin has been shown to exhibit limited aqueous solubility, but this is well known. However, very little mention has been found in the literature on bilirubin's nominal solubility in nonpolar, organic solvents. Limited solubility here is most likely due to areas of the pigment's polar moities that are not involved in hydrogen bonding, such as the paired electrons of lactam and hydroxyl oxygens. Thus, bilirubin is actually a molecule of moderate polarity which is evidenced by its tendency to

dissolve in chloroform.

Bilirubin was shown to be insoluble in acidic buffered solutions down to pH 2.00. However, proton addition did occur in concentrated nitric and sulfuric acids as indicated by an immediate red color change. This was followed by the oxidative changes of the Gmelin series. The most likely area for this basic behavior is the lactam oxygens since studies indicate that the bislactam form is the predominate species (1). However, since these oxygens are tied up through hydrogen bonding, strong acid is required for protonation. Bilirubin is at best weakly basic in strong acid solution.

Bilirubin solubility increased with increasing pH. From this data, one can infer that bilirubin has acidic protons capable of dissociation. These acidic protons are from the propionic acid side chains, even though they are tied up through hydrogen bonding. The pK_1 and pK_2 for bilirubin have been estimated to be 4.3 and 5.3 respectively (1).

In sum, bilirubin is a moderately polar molecule. The degree of bilirubin's polarity is balanced between its intramolecular hydrogen bonding and the presence of electron rich moieties. Bilirubin is also amphoteric. It is, however, a stronger acid than base. All of these properties together give rise to bilirubin's complicated solubility behavior.

Oxidation of bilirubin results in the formation of biliverdin with the Z,Z configuration. However, biliverdin was not expected to have solubilities similar to bilirubin because of its greater degree of conjugation. Instead, biliverdin solubilities were shifted slightly to favor more polar solvents. Biliverdin displayed greater solubility in

pH 7.00 solution and methanol than bilirubin. In addition, biliverdin had only limited solubility in polar, organic solvents such as chloroform. Finally, biliverdin was totally insoluble in nonpolar, organic solvents whereas bilirubin was found to be nominally soluble.

Solubility of photobilirubin and other photoproducts could only be determined by indirect methods. Although to different extents, the AD peak observed at approximately 500 nm was formed in most solutions after irradiation of solvated bilirubin. Since this peak has been attributed to the presence of photobilirubin, the photoproduct must exhibit an even wider range of solubility than either bilirubin or biliverdin. What must be kept in mind, though, is that photobilirubin is a collection of three isomers, all with different polarities.

The AD photopeak occuring at 425 nm was only formed when water was present. This suggests that its formation only occurs in very polar solvent systems. The 425 nm AD photoproduct is most likely a more polar molecule relative to bilirubin.

Based on initial liquid chromatography work, photobilirubin is more polar than bilirubin. This observation was confirmed through extraction experimentation in which photobilirubin was found to prefer aqueous solution over a less polar, organic liquid.

The results of solubility experimentation lead, in part, to a method of extracting photobilirubin from bilirubin. The photoproducts produced during irradiation were successfully transferred from the organic to the aqueous phase. However, since photobilirubin had been observed through AD spectrophotometric analyses to be soluble in chloroform, not all of this photoproduct produced was expected to go into the aqueous layer. Instead, an undetermined amount probably

remained in the chloroform layer. Nonetheless, the 24% reduction in initial bilirubin concentration, coupled with the finding that no additional peaks were detected in chloroform after irradiation suggests that the majority of photoproducts were transferred to the water layer.

In extracting photobilirubin from bilirubin, several criteria must be met. First, bilirubin must be soluble in the initial solvent. Second, the leaving solvent must be immiscible with the receiving solvent or the solvent into which photobilirubin is extracted. Thus, neither N,N-dimethylformamide nor methanol could be used because both liquids are soluble in chloroform. Lastly, radiation of the proper wavelength must be used. Even though bilirubin does absorb minute amounts of near-ultraviolet radiation, the Rayonet reactor was not an effective source for irradiating bilirubin to photobilirubin. Instead, colorless product formation was the dominant reaction. The sun did a moderately good job of irradiating bilirubin, but the process took several hours. Furthermore, biliverdin formation in the organic layer always occurred, no doubt from the UV portion of sunlight. The best source of radiation was from the Special Blue lamp. This artificial light proved to be the most effective in irradiating bilirubin because its output is almost identical to the absorption pattern of the pigment.

The absence of oxygen in evaluating bilirubin and photobilirubin chemistry is very important. Oxygen was removed from solutions by purging with unspecified purity and 99.999% UHP purity nitrogen. Attempts were made to remove the last traces of oxygen by evacuating pre-nitrogen purged solutions under reduced pressure until they momentarily boiled. Further attempts were made by passing nitrogen

through an oxygen scrubbing system consisting of vanadium (II) chloride solution.

None of the methods showed any difference in the data obtained relative to using nitrogen of unspecified purity directly from the cylinder. Consequently, it appears that oxygen is not required for photoisomerization to occur. In addition, the presence of oxygen does not inhibit photoisomerization. Aerobic solutions, though, are prone to other accelerated reactions such as photooxidation.

A considerable amount of analyses was performed using visible spectrophotometry. Bilirubin was shown to have a solvent dependent wavelength maxima from 439-476 nm. The complete absorption bands of bilirubin are very broad and have an even greater range. In aqueous solutions, wavelength maxima tended to be at shorter wavelengths relative to polar, organic solvents. This trend, however, was not followed in organic liquids since bilirubin's wavelength of maximum absorption was lower in chloroform that in methanol.

Contrasting trends were also noticed for bilirubin in basic environments. Essentially no difference in wavelength maxima was observed for bilirubin in pH 9.00 and pH 10.00 buffers. This is most likely due to all solubilized species existing as the dianion. If the hydroxyl concentration was not sufficient to promote ionization, such as in the case of pH 9.00 buffer, then the remaining bilirubin did not become solubilized.

Addition of base to polar, organic liquids in which bilirubin is soluble was the best means of observing the effect of base on wavelength maxima. In this regard, added TMG to chloroform and DMF resulted in a red shift of 6-7 nm. Utilizing Planck's equation, the

dianion form of bilirubin is a lower energy molecule. Therefore, the energy required to promote an electron from a lower to a higher orbital is less for bilirubin in a basic environment.

Bilirubin's wavelength maxima was dramatically red shifted by 37 nm after complexing with bolvine serium albumin. The resulting albumin-bilirubin complex was the lowest energy form of bilirubin analyzed in this research. This may suggest that the bonds between the complexed species are relatively weak since minimal enengy is required to promote electrons to higher orbitals. If true, the low energy nature of the complex may also account for the free dissociation of in vivo bilirubin from albumin.

Compared to bilirubin, biliverdin is a lower energy molecule since its wavelength maxima due to double bond conjugation occurs at longer wavelengths. In other words, considerably less energy is required to promote an electron to a higher orbital in biliverdin than in bilirubin. Therefore, biliverdin is relatively more stable than bilirubin.

Synthesis of photobilirubin from bilirubin was initially followed by AD spectrophotometry. Although derivative spectrophotometry is more sensitive in detecting subtle changes in spectra, AD spectrophotometry proved satisfactory. Photobilirubin production was observed to be dependent on the length of exposure time. The longer an exposure time, the greater the concentration of photobilirubin produced, until an optimal amount of time was reached. Then, photobilirubin concentration began leveling off. Further irradiation caused a slight lowering of detected photobilirubin.

When the optimum exposure time was exceeded, other oxidative

reactions would occur. The predominant reaction observed was the formation of colorless products. This was evidenced by a rapid reduction in bilirubin's maximum absorbance with only a nominal increase in AD spectra. Furthermore, colorless product formation was thermally irreversible since application of heat did not regenerate the solution's original color.

Biliverdin formation, or the production of unknown verdinoid products, was the next reaction to occur from overirradiation. Uncertainity in whether or not the product formed was due to biliverdin production comes from the inability to equate maxima from AD spectra to wavelengths of maximum absorption from normal spectra.

The production of photobilirubin from bilirubin was successful in polar, organic solvents. An AD peak maxima at approximately 500 nm indicated the presence of photobilirubin. The overall stability of photobilirubin compared to bilirubin is difficult to predict because, once again, maxima from AD spectra are not true wavelengths of maximum absorption. However, in order for the true maxima of photobilirubin to be less than that of bilirubin, the AD spectra would have to be red shifted by approximately 50 nm. Assuming this was not the case, photobilirubin is a lower energy molecule than bilirubin and possibly the albumin-bilirubin complex, which is due to its lack of intramolecular hydrogen bonding. This implies that only nominal activation energy is required for photobilirubin to be converted to a lower energy state molecule such as the Z,Z-isomer or naturally occurring bilirubin.

An additional AD peak was formed in both pH 10.00 buffer and the ternary solution. In pH 10.00 buffer, this peak occurred between

410-420 nm. In the ternary solution, the AD peak was observed around 405-415 nm. Furthermore, the 500 nm peak attributed to photobilirubin was also present in both of these solutions. However, this peak was blue shifted by approximately 20-25 nm to 475-480 nm.

Comparison of the additional AD peak in pH 10.00 and the ternary solution was made with the extracted photoproduct. The extracted product was found to absorb radiation between 418-428 nm. Addition of the extracted photoproduct to pH 10.00 buffer and the ternary solution had negligible effect on the resulting wavelength maxima. Thus, the difference between maxima from AD spectra and wavelength of maximum absorption from normal spectra can again be accounted for because of differences in measuring technique.

Since the shorter wavelength peaks from AD spectra and the extraction have lower wavelength maxima than bilirubin in the same solvent system, the photoproduct must be a highly energetic species relative to the parent pigment. This statement will undoubtably meet with controversy if the new, highly energetic species were concluded to be photobilirubins. The only other plausible explanation, though, might be that overirradiation had occurred and, therefore, lumirubin was produced instead. However, since lumirubin is presumably an irreversibly formed product from bilirubin irradiation, it could not possibly be present in the aqueous extract since reversibility was observed through eventual bilirubin precipitation in the aqueous extract.

Voltammetry was the next instrumental technique used in this research. Anodic scans of bilirubin were initially made in N,N-dimethylformamide. Bilrubin was oxidized to biliverdin at +0.42 V

vs Ag/AgCl. A secondary peak was also noticed at +0.59 V and was attributed to the presence of biliverdin from the oxidation of bilirubin. Since biliverdin is a conjugated molecule and bilirubin is not, this was considered a plausible hypothesis. The alternating single and double bonds of biliverdin greatly stabilizes the molecule relative to bilirubin because of π electron delocalization. Thus, biliverdin is more stable and was expected to be oxidized after bilirubin.

The effect of added base on bilirubin's oxidation potential was also evaluated in DMF. After addition of TMG, bilirubin was easier to oxidize. Furthermore, the diffusion current associated with the dianion form of bilirubin was greater than the neutral molecule.

Anodic detection of photobilirubin was attempted by irradiating solutions of bilirubin and subsequently scanning in a positive direction. Solutions used in anodic measurements included DMF, pH10.00 buffer and the ternary solution. Photobilirubin, however, could not be resolved from bilirubin as no additional peaks were observed. Furthermore, neither potential shifts nor diffusion current diminution were noticed.

The only alternative for determining the oxidation potential of photobilirubin relative to bilirubin was to anodically scan the aqueous extract under identical conditions. In the ternary system, irregular peakforms were always observed and the validity of the results were questionable. However, in pH 10.00 buffer, well defined peaks were observed and photobilirubin's oxidation potential was determined to be +0.33 V, which was 290 mV more anodic than bilirubin and exactly the same oxidation potential as biliverdin. Consequently, biliverdin and

photobilirubin, both of which were determined to be lower energy molecules than bilirubin, are also harder to oxidize.

Cathodic voltammetry was also pursued, but only in aqueous pH 10.00 buffer. The primary reduction potentials of bilirubin and biliverdin were found to be almost identical. Biliverdin, however, did have a secondary reduction potential of -1.10 V, which occurred before bilirubin and its primary reduction peak. However, since commercially available biliverdin has approximately 20% impurities, very little consideration was given to the secondary reduction potential.

Once again, cathodic detection of photobilirubin was pursued by simply irradiating solutions of bilirubin and scanning in a negative direction. On this occasion, a very broad reduction peak was observed before either bilirubin or the primary reduction potential of biliverdin. As this peak began to grow, the diffusion current of bilirubin decreased by a nominal amount. In addition, the photopeak formed during irradiation was found to be reversible since after seven hours, its diffusion current returned back to zero. This was followed by almost complete recovery of the original bilirubin peak.

Finally, the reduction potential of the aqueous extract was also investigated, again in identical solution. In doing so, the reduction potential of bilirubin in pH 10.00 solution was found to be -1.24 V. This is approximately the same reduction potential as the smaller of the two peaks formed during irradiation of bilirubin in pH 10.00 buffer. Therefore, it appears that the smaller peak just described and the peak from the aqueous extract are due to the reduction of the same species. Furthermore, this demonstrates that photobilirubin is easier to reduce than either bilirubin of biliverdin. Relative to bilirubin,

this observation can only be due to the lack of intramolecular hydrogen bonding since both pigments are identical in all other respects.

The last area of instrumental analysis attempted in this research was liquid chromatography. HPLC was pursued for three reasons. First, separation of bilirubin from photobilirubin was desired to evaluate their relative polarities. Second, separation of the three isomeric forms of photobilirubin from one another was also desired for similar reasons. Finally, further identification of the extracted photoproducts through their relative retention times would give a better idea as to which photoproducts were actually formed.

In pursuance of the first two chromatographic objectives, photobilirubin was successfully separated from the parent pigment and partial separation was obtained among the three photoisomers. Fortunately, further separation of photobilirubin was not achieved because of the column being used at that time. This lead to the use of a new, identical column in which bilirubin was strongly retained. As a result, the original separations could no longer be reproduced. Although some researchers have used reverse phase HPLC in the separation of bilirubin from photobilirubin, other chromatographic techniques were used such as lowering packing material interaction by incorporating a surfactant in the mobile phase.

The first problem encountered in the above mentioned separations was due to the extreme difference in polarities or solubilities of bilirubin and photobilirubin. Although both pigments coexisted in polar, organic solvents, the converse was not true for aqueous systems. The second problem to overcome concerned the two solvent systems in which analyses were performed under identical conditions. In the

ternary solution, no combination of chloroform, methanol and water was found as an intermediate between strong interaction and no interaction at all. One hypothesis is that by increasing the chloroform concentration, the packing material no longer acted in the same manner as it did with lesser chloroform concentrations. In the pH 10.00 buffer system, neither column was used because pH 7.5 is the maximum recommended pH. Lastly, other isocratic solvent systems, gradient elution and even ionization through added base (which was evaluated despite the occurence of negative peaks) were undesirable because they would only add to the uncertainity of chromatography taking place.

Conclusions

Physiochemical studies of bilirubin and its associated photoisomers are extremely complex. Chemical systems of bilirubin are not stable and are susceptible to change with every passing minute. In spite of these kinetic complications, two different solvent systems, the ternary solution and the aqueous pH 10.00 buffer system, were eventually developed. These solvent systems were used in investigating the behavior of bilirubin and a few of its isomers in identical chemical environments.

The ternary solution was used in spectrophotometric and, to a lesser extent, chromatographic analyses. Voltammetric analysis using the ternary solution was pursued but found to give poor results. The aqueous pH 10.00 buffer solution, on the other hand, was used in both spectrophotometric and voltammetric analyses. However, unlike the ternary solution, the use of pH 10.00 buffer in HPLC was prohibited because of column incompatability.

In addition to confirming reported studies of the formation of

photobilirubin, a new photoproduct has been identified through AD spectrophotometry. The new product was found to have similar absorption characteristics relative to the products collected during the extraction process. Furthermore, valuable anodic and cathodic electroanalytical data were obtained from the aqueous extract. This data has not been previously reported.

Suggested future work to characterize photobilirubin more completely should include ^{1}H NMR and ^{13}C NMR. Both deuterated chloroform and D_2O solutions would be needed. These results would aid in understanding how different chemical environments effect the behavior of photobilirubin. Analyses of this kind, coupled with spectrophotometric and voltammetric work performed here, might give further insight into treating jaundiced individuals.

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