THE BINDING OF BILIRUBIN, PHOTOBILIRUBIN AND BILIRUBIN MODEL COMPOUNDS BY POLYMERIC RESINS.

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BINDING OF BILIRUBIN, PHOTOBILIRUBIN AND BILIRUBIN MODEL COMPOUNDS

Ph.D. Chemistry

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ABSTRACT

The photolysis of bilirubin IX-α in organic solvents leads to both EZ/ZE isomers and lumirubins, with the latter being formed from the former. The relative yields of the various photoproducts is markedly dependent on the solvent. Small amounts of photoproducts are also produced on irradiation of aqueous bilirubin solutions; however, the addition of human serum albumin increases the efficiency of the photoreaction, predominantly by isomerization of bound bilirubin.

The formation of photoproducts from aqueous bilirubin solutions increases the rate of adsorption onto cholestyramine but not peptide-substituted polyacrylamide resins. The photoproducts, principally lumirubins produced by irradiation of bilirubin in DMSO, are selectively adsorbed by cholestyramine.

Using a semi-quantitative model it is shown that in aqueous solution the binding of derivatives of indoles and pyrroles onto cholestyramine involves electrostatic interactions as well as hydrophobic interactions. The free energy of transfer of the small molecule from water to an organic phase permits an estimation of the role of the hydrophobic interactions.

RESUME

La photolyse de la bilirubine IX-α dans des solvants organiques produit des isomères EZ/ZE et des lumirubines, les lumirubines étant formées par ces isomères EZ/ZE. Les quantités relatives des photoproduits dépendent du solvant. Des petites quantités de photoproduits sont aussi formées par irradiation des solutions aqueuses de bilirubine. L'addition de la sérum-albumine humaine augmente l'efficacité de la photoréaction surtout par l'isomérisation de la bilirubine.

La formation des photoproduits de solutions aqueuses de bilirubine augmente la vitesse d'adsorption par la cholestyramine, mais pas celle des résines polyacrylamides substituées avec des peptides. Les photoproduits, principalement les lumirubines, produits par irradiation de la bilirubine dans le sulphoxyde diméthylique, sont adsorbées de façon sélective par la cholestyramine.

En utilisant un model semi-quantitatif, on démontre qu'en solution aqueuse la liaison des dérivés d'indoles et de pyrroles par la cholestyramine implique des interactions électrostatiques ainsi que des interactions hydrophobiques. L'énergie libre de transfert d'une petite molécule dans l'eau à une phase organique permet une estimation du rôle des interactions hydrophobiques.

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to my wife, Dvorah,

my family,

and in memory of my mother.

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LIST OF ABBREVIATIONS AND SYMBOLS

The abbreviations and symbols for the physical or chemical terms and units used in this thesis are in accordance with those adopted by IUPAC (International Union of Pure and Applied Chemistry), IUPAP (International Union of Pure and Applied Physics) and IUB (International Union of Biochemistry), published in the Handbook of Chemistry and Physics. The three-lettered abbreviations for amino acids are those recommended by the Joint Commission on Biochemical Nomenclature of IUPAC and IUB. Some of the abbreviations and symbols are listed below:

Abs	absolute
Ala	alanine
Arg	arginine
BOC	tert-butyloxycarbonyl
BR	bilirubin
BSA	bovine serum albumin
CA	cholestyramine
cm	centimeter
DCC	1.3-dicyclohexylcarbodiimide
DEA	N,N-diisopropylethylamine
DMF	dimethylformamide
deg	degree
dL	deciliter
DMG	N,N-dimethylglycine
DMSO	dimethylsulfoxide
DP	degree of polymerization

eq	equivalent
EZ/ZE	bilirubin in the EZ or ZE configuration
F	force
g	gram
h	Planck's constant (6.6262×10^{-34} Js)
HPLC	high performance liquid chromatography
HSA	human serum albumin
J	Joule
K	binding constant
kJ	kilo-Joule
LR	lumirubins
Lys	lysine
M	concentration in molarity (mole/liter)
mg	milligram
min	minutes
ml	milliliter
mol	mole
MW	molecular weight
N	concentration in normality
nm	nanometer
$^3O_2$	triplet oxygen
PL	polylysine
R	universal gas constant ($8.314\ JK^{-1}mol$)
	alkyl group
S_o	electronic ground state
S_1	first excited electronic singlet state

sec	second
T_1	first excited electronic triplet state
T_2	second excited electronic triplet state
T	temperature
T_f	heat of fusion
TFA	trifluoroacetic acid
TMG	N,N,N-trimethylglycine
UV	ultraviolet

ΔG	free energy change
ΔG_b	free energy of binding
ΔG_T	free energy of transfer
ΔH	enthalpy change
ΔS	entropy change
ε	dielectric constant
µl	microliter

CHAPTER 1

INTRODUCTION

1.1 METABOLISM OF BILIRUBIN

Bilirubin is a linear tetrapyrrole whose structure is usually represented as in Figure 1.1. However, X-ray diffraction (1) indicates that the crystal structure is, in fact, a folded structure better represented by Figure 1.2. This conformation, which also exists in some solutions (2,3), is stabilized by six hydrogen bonds between the NH/O and OH/O groups. Consequently, the hydrophilic part of the molecule, most notably the two COOH groups, are buried within the molecule, leaving only hydrophobic groups on the exterior so that the molecule exhibits hydrophobic characteristics.

Bilirubin is formed mainly from the degradation of hemoglobin released into blood plasma from decomposing erythrocytes. The hemoglobin in the red cell is protected from catabolism, but once the red cell is destroyed by phagocytosis or hemolysis the hemoglobin is rapidly converted to bilirubin and other products by the mechanism shown in Fig. 1.3 (4). This breakdown results in two toxic products, CO, which is excretable, and bilirubin, which is not excretable since it is poorly soluble in water.

The bilirubin that is formed enters the circulation where it is bound to the protein albumin, transported to the liver cells, and is taken up by a protein called ligandin (5). In the liver the bilirubin is combined enzymatically with the sugar glucuronic acid (Fig. 1.4) (6) and then excreted in the bile.

If any part of the mechanism for excretion of bilirubin is impaired an elevated concentration of unconjugated bilirubin results in the plasma, producing

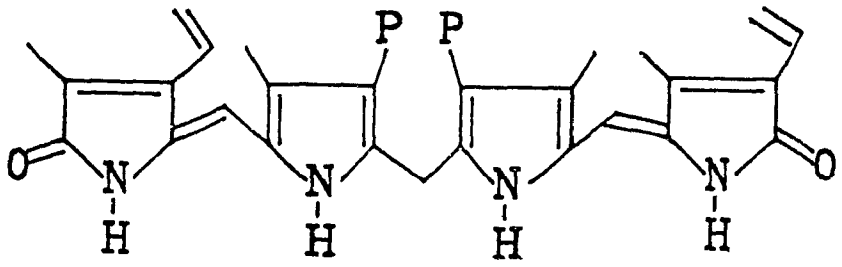


Figure 1.1 The common representation of bilirubin as a tetrapyrrole (P = $-CH_2CH_2COOH$).

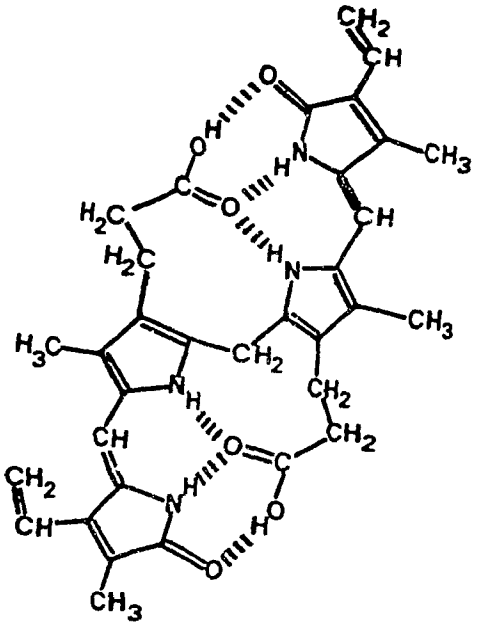


Figure 1.2 The bilirubin molecule showing the six intramolecular hydrogen bonds.

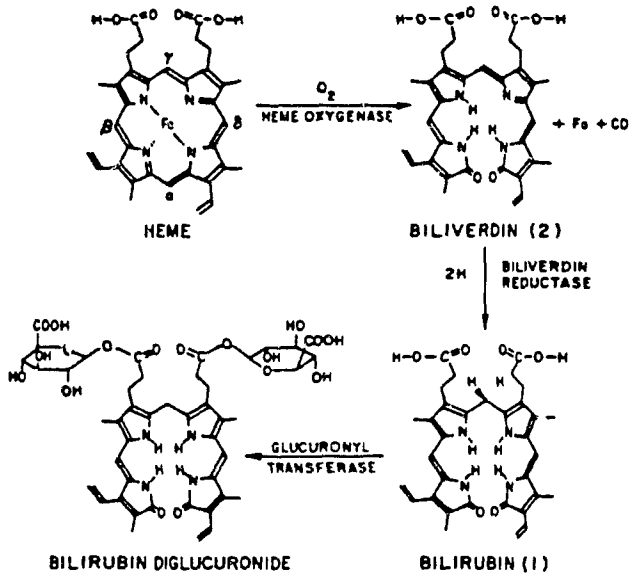


Figure 1.3 The biosynthesis of bilirubin. The heme molecule is oxidized at the α-bridge after which nonenzymatic ring opening occurs randomly at all four bridges, yielding three additional isomers. Although only one end product is shown, the glucuronyl transferase catalyzes formation of both mono- and diglucuronides of bilirubin. (4)

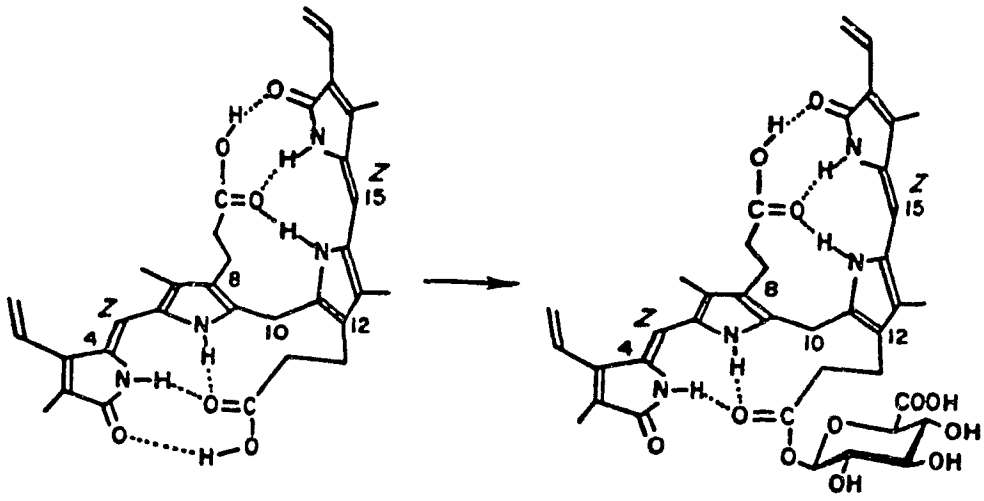


Figure 1.4 A closer look at the conversion of bilirubin to bilirubin monoglucuronide. (6)

hyperbilirubinemia. This condition can, in its extreme, be fatal since unconjugated bilirubin can cross the blood brain barrier and damage cells of the central nervous system.

The general mechanism of bilirubin excretion is accurate except in two cases: 1) the human fetus and 2) the newborn. Specifically, the bilirubin glucuronyl transferase activity in fetal and newborn liver is very low (7). Since the mother's liver is responsible for excretion of bilirubin for the fetus, this is not a problem until after birth. At this time the bilirubin begins to accumulate in the newborn due to the insufficient glucuronyl transferase and an increased rate of destruction of the red cells (8). Consequently, newborn infants often develop hyperbilirubinemia, more commonly known as neonatal jaundice. It is estimated that 50% of all infants develop at least mild jaundice during the first week of life, and that about 10% of neonates will require therapy (9). For these 10% there are basically three types of treatment: (1) exchange transfusion, (2) hemoperfusion and (3) phototherapy, the last being the most common. Before elaborating on the possible treatment of hyperbilirubinemia, it is important to understand the chemistry of bilirubin.

1.2 GENERAL PROPERTIES

1.2.1 ISOMERS

Inspection of Fig. 1.1 shows that by interchanging the substituents on the pyrrole rings a number of structural isomers of bilirubin can be formed. The naturally occurring isomer is the IX-α, so named because it is derived from the natural IX isomer of ferriprotoporphyrin by cleavage of the porphyrin ring at the α bridge position. The reaction is stereoselective because of the configuration of the enzyme and thus only the IX-α isomer is formed (10). Although the III-α and

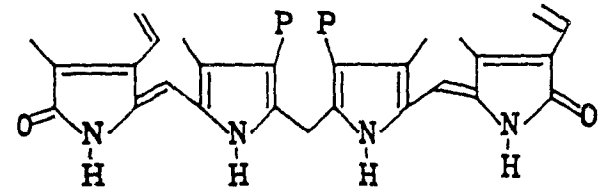
XIII-α isomers exist in commercial preparations, due to "isomeric scrambling" (Fig. 1.5) (11), in vivo the IX-α isomer predominates.

Studies by Blanckaert and co-workers (12) have shown that small amounts of the protoporphyrin IX ring are catabolized at the β-position, and smaller amounts at the gamma and delta positions. It is estimated that more than 95% of human adult bile is in the form of α isomers. The remaining non-α isomers are water soluble and may thus be considered as nontoxic (13).

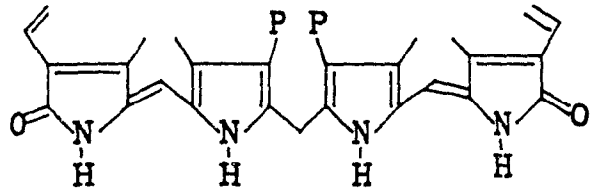
The bilirubin structure in Figure 1.1 indicates the possibility of cis-trans isomerization at the two double bonds connecting the outer rings to the methin bridges. Thus, in each of these places the molecule can assume the Z or E configuration, giving rise to four possible configurational isomers. Since the protoporphyrin IX is in the Z/Z configuration, the naturally occurring bilirubin IX-α also assumes this configuration. Illumination of the Z/Z isomer can lead to photoisomerization which gives rise to the Z/E, E/Z and E/E isomers. In addition, it has been found that these isomers can be converted to other configurational isomers known as lumirubins. These photoprocesses will be considered more fully in the discussion of the mechanism of phototherapy.

1.2.2 SOLUBILITY

The solubility of bilirubin in aqueous solution depends on the isomer that is being considered. The naturally occurring bilirubin IX-α (Z/Z) contains six hydrogen bonds at the interior of the molecule rendering it hydrophobic. At pH levels below seven it is virtually insoluble (14,15). Since the III-α and the XIII-α isomers can also exist in the Z/Z configuration, they are also insoluble. When the hydrogen bonds are broken and the molecule rotated, as is the case with the Z/E and E/Z isomers, the molecule becomes more hydrophilic and thus more soluble in water. The non-α isomers are more soluble in water for the same reason, i.e.,



(A)



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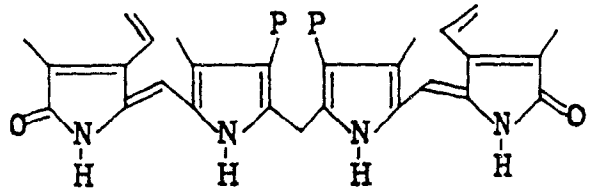

(C)

Figure 1.5 The α-isomers of bilirubin. (P = CH_2CH_2COOH) (A) IX-α; (B) III-α; (C) XIII-α.

accessibility of the carboxyl groups to the solvent.

The solubility of bilirubin in alkaline aqueous media varies as a function of pH as shown in Figure 1.6. At pH = 8.5 the solubility is 0.6 μM (13).

The solubility of bilirubin in organic media depends on the polarity and asymmetry of the solvent. As the polarity increases the solubility increases; thus n-hexane is a poor solvent (<1 μM) and dimethylsulfoxide (DMSO) is much better (10 μM) (13). Toluene and xylene, being asymmetrical, are good solvents.

It is noteworthy that the pattern of solubility is essentially different from that observed with lipophilic substances which dissolve in hexane, ether and olive oil but not in formamide and DMSO (16). Thus, the formation of intramolecular hydrogen bonds makes bilirubin hydrophobic but not lipophilic.

1.2.3 ACIDITY

The acidity of bilirubin is still under debate. A pK_a value of 7-8 has been reported by some authors (17, 18) while others have found that the pK_a to be around 5 (19,20). The main reason for the discrepancy is the shape of the pH curve. According to Brodersen (18), the shape of the curve indicates that the process is not a simple titration of the two protons but also involves an equilibrium between dissolved and undissolved bilirubin. However, many authors have shown that a pK_a of 4.4 is most probable (21).

1.2.4 SPECTROSCOPIC PROPERTIES

In organic solvents bilirubin absorbs in the visible region at ca. 460 nm, and in the UV at ca. 290 nm. In aqueous solution an absorption maximum is observed at 438-440 nm with an extinction coefficient of 4.7×10^4 l/mol-cm (22).

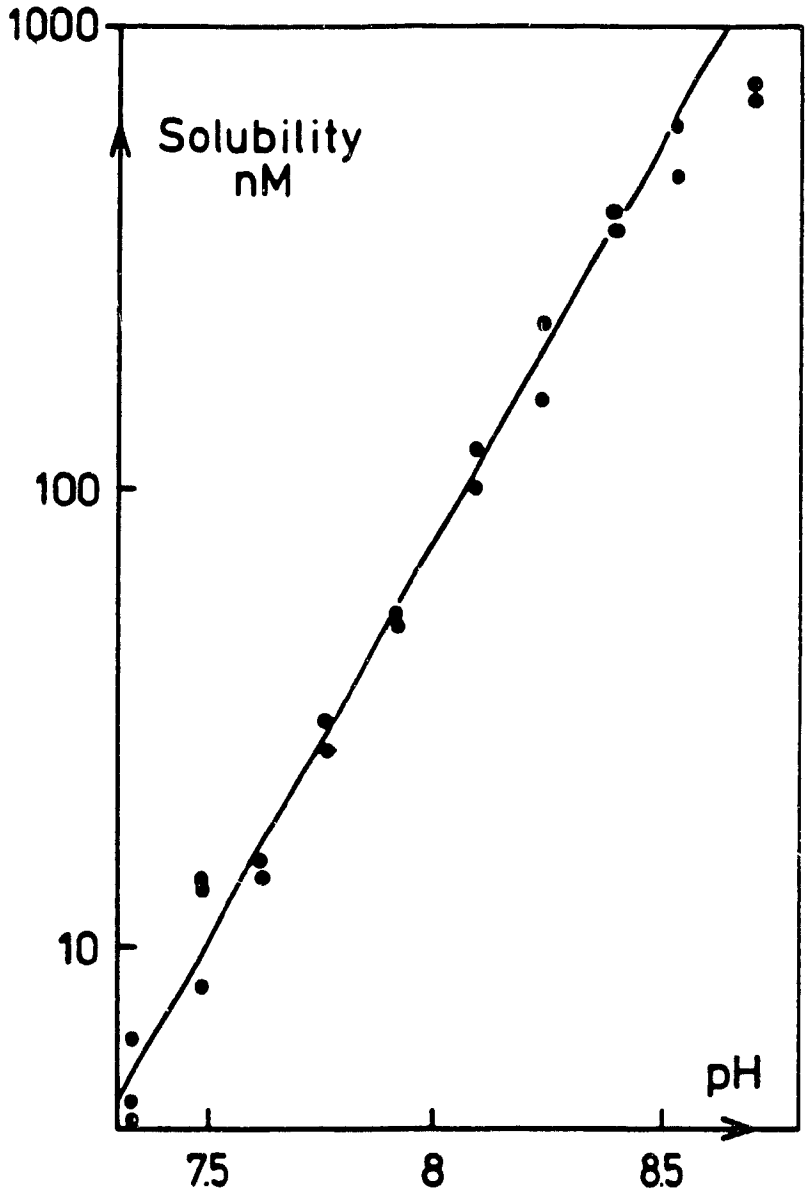

Figure 1.6 The solubility of bilirubin in aqueous buffer (37°C) as a function of pH. (13).

The absorption spectrum obeys Beer's Law at concentrations up to 10 mg/dl in aqueous solution at pH values > 7.8.

The electronic spectra of bilirubin will be considered in detail later after a discussion of the photoisomerization of bilirubin.

1.3 TREATMENT OF HYPERBILIRUBINEMIA

Regardless of what is causing the hyperbilirubinemia, once the condition is diagnosed it must be treated. In the adult it is a sign of liver failure and must be treated accordingly. In the neonate it is usually a temporary condition which must be dealt with until such time as the infant's system functions properly. This must be done as soon as the bilirubin level exceeds about 9 mg/dl in the premature infant and 15 mg/dl in the full-term infant, since these levels are potentially damaging to the central nervous system (23-28).

1.3.1 EXCHANGE TRANSFUSION

One possible form of treatment, and the first to be used on a large scale, is exchange transfusion. It has been used in newborns since the early 1950's (29), primarily for the prevention of kernicterus, although it has been shown that some infants have suffered brain damage even after the transfusion (30). The main advantage of exchange transfusion lies in the fact that it is the quickest way to reduce serum bilirubin levels. Within one hour of the start of exchange transfusion the serum bilirubin level can be lowered by 50% (31).

However, this form of therapy is not without cost and risk. Special care is needed for the infant prior to the transfusion, including regular monitoring of the infant's temperature (which may include the use of a radiant heater), heart rate and respiratory rate. In addition, the infant must be kept without food for a period of 3-4 hours prior to the transfusion, thus delaying the procedure. Once the child

has gone through the preparatory steps, the actual exchange transfusion requires a minimum of two physicians and one nurse, thus increasing the cost of the treatment.

Complications include heart failure, cardiac arrest and intestinal perforation, to name a few. The mortality rate is estimated between 1% and 4%, for term and preterm infants, respectively. Thus, although it is a very efficient method in terms of time required to reduce the serum bilirubin levels, exchange transfusion for the treatment of neonatal hyperbilirubinemia remains, for most hospitals, a last resort.

1.3.2 HEMOPERFUSION

Another possible treatment is hemoperfusion, which refers to a method of cleaning the blood by passing it through a column that binds the substance to be removed. As early as 1948 Muirhead and Reid (32) carried out resin hemoperfusion in experimental studies. The original studies had problems with ion exchange resins since these resins tended to reduce blood platelets, leukocytes and electrolyte compositions (33).

The first non-ion exchange resin to be used was Amberlite. This is a non-ionic, polymeric adsorbent made up of a styrene-acrylic ester copolymer (Amberlite XAD-7) and a styrene-divinylbenzene copolymer (Amberlite XAD-2), which had the advantage of not affecting the electrolyte compositions in the circulating blood; however, platelet depletion was still a problem (34).

Activated carbon (charcoal) has been used successfully as an adsorbent in hemoperfusion as well. It was used in patients as early as 1964 by Yatzidis (35). Charcoal has the advantage of having a large adsorption capacity but it suffers from limited selectivity. Despite this, charcoal is still used for the removal of endogenous and exogenous toxins in hemoperfusion (36).

Some of the problems associated with the use of charcoal as an adsorbent have been alleviated by placing the enzymes, ion exchange resin and activated charcoal inside artificial cells (37). In this manner the problems of blood cell depletion and particulate embolism have largely been solved. Further work in this area led to a successful clinical application using albumin collodion coated activated charcoal (ACAC) which does not effect the platelets or white blood cells (38-40).

For the removal of protein bound toxins, such as unconjugated bilirubin, many adsorbents have been investigated, but problems such as low capacities and blood incompatability still remain (41-44). A hemoperfusion column containing an anion exchange resin which is both biocompatable and efficient in removing unconjugated bilirubin has been tested in vivo by Sideman, et al. (45-47). It consisted of a macroreticular anion exchange resin including Amberlite, coated with albumin, chemically linked to the particles by the reaction with glutaraldehyde (48). Using this system approximately 70% of the original unconjugated bilirubin was removed from jaundiced dogs.

Possible resins to be used for hemoperfusion or ingestion have also been investigated by this laboratory (49, 50). Small peptides, designed to mimic the binding site for bilirubin on HSA, have been attached to water swellable polyacrylamide resins. These resins proved to be very effective for the removal of bilirubin in vitro.

Thus, hemoperfusion has been shown to be effective in removing various toxins from the body. When albumin is coated onto the resin, protein bound toxins such as bilirubin can also be removed. However, problems still exist, such as the complications in the procedure and the high cost compared to phototherapy, which make this procedure a less popular choice for the lowering of bilirubin levels in the newborn suffering from hyperbilirubinemia.

1.3.3 PHOTOTHERAPY

Each year thousands of newborn babies undergo phototherapy to reduce the concentration of bilirubin in the plasma and thus diminish the possibility of neurotoxic damage. The efficacy of using light to reduce bilirubin levels owes its beginnings to the observations of J. Ward, a nurse who supervised a premature baby unit. After taking her infants outdoors in the sunlight she noticed that the skin of a jaundiced infant had become bleached where it had been exposed to the sunlight. Further observations by the doctors showed that the bilirubin concentration also decreased (51,52).

Phototherapy is now the treatment of choice and has been for the last 25 years. Phototherapy units are banks of 8 - 10 fluorescent lamps, covered with a plexiglass sheet to absorb any UV radiation, placed about two feet above the skin surface of the infant The infant is placed in these incubator-like devices wearing only protective coverings on the eyes. The geometrical configuration of these units is not based on any experimental study, rather, it has simply been assumed that the largest number of lamps which could be placed above the incubators without interfering with the nurses would be fine. The only change in the unit over the last 25 years has been the development of special fluorescent lamps.

The light sources used originally for phototherapy had an output mainly in the blue region, as this is where bilirubin itself has a high absorbance. However, with additional knowledge came a rethinking concerning the wavelength that would lead to the desired result, i.e., the lowering of the serum bilirubin level. Between 1958 and 1978 lamps with spectral emission matching the bilirubin absorption maximum were thus used. With the discovery of photobilirubin between 1978 and 1983, the use of special blue and white lights was continued despite the fact that a different spectral distribution would have been expected. Ennever, et al. (53) showed that the optimum light for EZ/ZE

formation is in the UV which, due to safety considerations, is not clinically feasible. Thus, blue lamps have continued to be used, despite some controversy concerning the relative efficiency of blue and green light (54-60).

The optimum light for phototherapy is as yet undetermined, as is the entire irradiation procedure (continuous irradiation, intermittent irradiation, optimum light power density at the body surface, etc.).

Initially phototherapy was used to reduce the demand for exchange transfusion. There was no evidence that this treatment actually detoxified the patient, and in fact, phototherapy was used for years before the mechanism began to be understood. The possible side effects and long term effects of phototherapy, discussed later, are still under debate.

There is still no firm agreement on certain details of the mechanism of phototherapy. Until 1978 it was thought that the main process leading to the decrease in bilirubin level during phototherapy was autooxidation (61), which will be discussed in more detail in the section on electronic spectra. This mechanism was accepted until it was shown that substantial quantities of unconjugated bilirubin are found in the bile during phototherapy. Accordingly, degradation of bilirubin could not be the only mechanism which leads to the decrease in the overall serum bilirubin level (62,63). The research which followed led to the discovery of many products, commonly referred to as photobilirubin.

1.3.3.1 PHOTOBILIRUBIN

McDonagh, et al. (64) were the first to suggest that the presence of unconjugated bilirubin in the bile during phototherapy is consistent with a mechanism in which bilirubin is first isomerized and then excreted in the bile (Fig. 1.7). The isomerized bilirubin then reverts to its native form in the bile. He

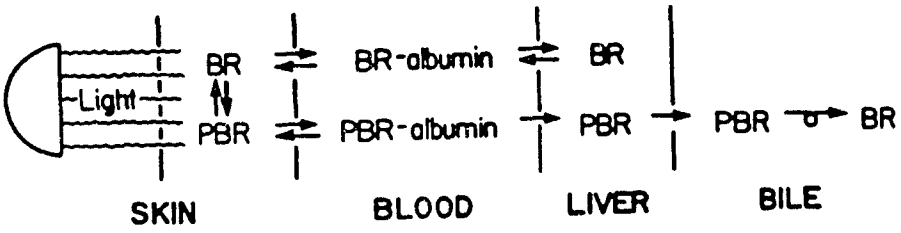

Figure 1.7 The mechanism of bilirubin excretion during phototherapy of neonatal jaundice. (PBR = photobilirubin) (66)

supplied evidence for (59) photoisomerization of bilirubin in vivo by showing that Gunn rats excreted photoproducts within minutes of the start of phototherapy. (Gunn rats, named after the geneticist Charles Gunn who discovered them in 1934, are rats which are unable to glucuronidate bilirubin and thus develop life-long jaundice - if they survive the toxic effects of bilirubin (65)). The mechanism proposed by McDonagh involves the light-induced conversion of bilirubin to unstable polar intermediates, which were first called "photobilirubin". These could be excreted by the liver and bile without conjugation Once in the bile they revert to bilirubin. Subsequently, photobilirubin was detected and identified spectroscopically in the serum of photoirradiated Gunn rats (66).

Stoll, et al. (67) verified photoproduct formation when they showed that, in the absence of oxygen, two pairs of photoisomers are produced by irradiation of bilirubin in chloroform. Finally, Lightner (68-70) showed that photooxidation is not the important step in phototherapy when he proved that photoisomerization is almost instantaneous and thus precedes photooxidation.

The two pairs of photoisomers found by Stoll were separated by chromatography and analyzed by mass spectrometry to verify their isomeric character. One pair of isomers, IA and IB, were unstable and reverted spontaneously to bilirubin. This reversion could be retarded by the addition of ethylenediaminetetraacetic acid (EDTA), albumin or nonpolar solvents. The IA isomer seemed to be the major product while IB and the other pair were only minor products However, photoisomers IIA/B were formed in abundance when bilirubin was irradiated in solvents which interfere with hydrogen bonding, as in the case of dimethylsulfoxide, DMSO. The major difference between this pair of isomers and the other pair is that the photoisomers IIA/B do not revert to bilirubin, though they do interconvert.

In 1979 Onishi, et al. (71) started studying the photoproducts produced by the irradiation of bilirubin-albumin solutions. Using HPLC they obtained three main peaks corresponding to photoproducts that are more polar than bilirubin, and labelled them photobilirubin, unknown pigment and simply peak 1. The product labelled photobilirubin reverted back to bilirubin, like Stoll's IA/B isomers, while the unknown pigment did not. Onishi acknowledged that these products could be the same as those discussed by Stoll.

In 1981 Ostrow confirmed much of Onishi's work (72). He produced and purified chromatographically a pair of unstable (I) and a pair of stable (II) photobilirubins. He also used mass spectrometry and azopigment analysis to prove that these derivatives are neither photoadducts nor the XIII or III isomers of bilirubin. Stoll and Bonnett (73) showed the same phenomena as Ostrow and Onishi but they also found another product they called photobilirubin III.

The product called "photobilirubin" by McDonagh and Onishi, and IA/B by Stoll refers to the isomers obtained from the ZZ -->EZ photoisomerization of bilirubin, shown in Figure 1.8. This isomerization leads to a mixture of 4Z/15E, 4E/15Z and 4E/15E bilirubins from the original 4Z/15Z bilirubin. This mechanism was subsequently agreed upon by others (74-77).

McDonagh then reported (78) that prolonged irradiation of bilirubin can lead not only to the configurational isomers but also to structural isomers which he called lumirubins (Figure 1.9). At about the same time, Onishi assigned an endo-vinyl cyclized product to his unknown pigment (79) and Stoll and Bonnett also postulated that their photobilirubin IIA/B were derived from the same type of cycloaddition reaction. Studies by HPLC and NMR indicated that the bilirubin molecule undergoes an intramolecular cycloaddition reaction (80-82). At present it is uncertain whether lumirubin is formed from bilirubin or from the 4E/15Z photoproduct.

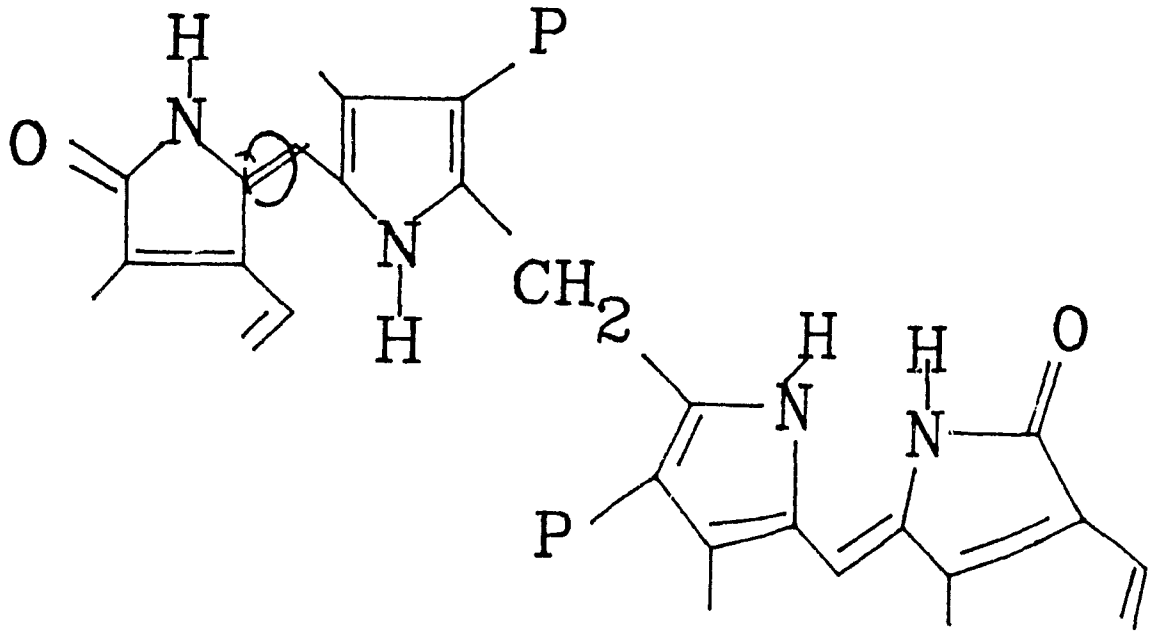

Figure 1.8 The photoisomerization of bilirubin, giving the EZ isomer.

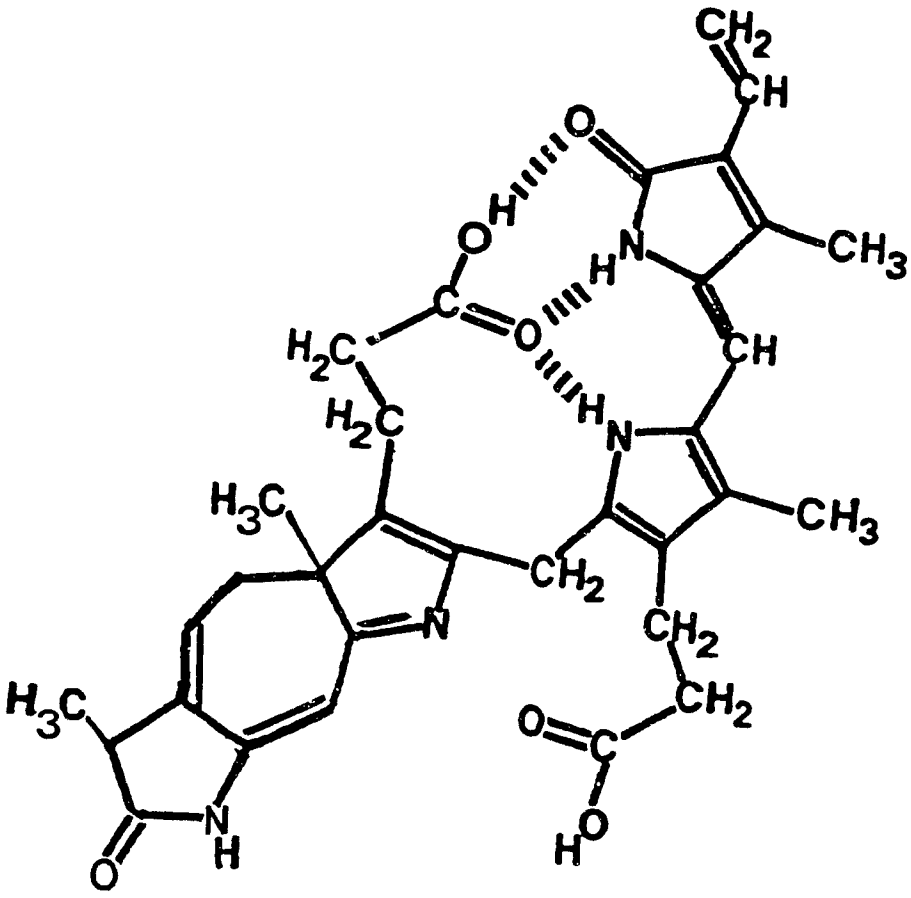

Figure 1.9 The proposed structure of lumirubin.

Additional studies showed that lumirubin can also undergo further isomerization in much the same way as bilirubin does. Light can isomerize lumirubin to more polar E isomers, reaching a ZZ<-->EZ equilibrium without detectable reversion to the parent bilirubin (83).

After the initial discovery of the lumirubins they were found to exist in much lower concentrations than the photobilirubins (0.5-2.0% of the total bilirubin compared to 18-20% (84)) in the serum of infants exposed to phototherapy. However, the clearance of lumirubins from the serum was found to be much more rapid. Thus, the efficient excretion of this photoproduct may play a quantitatively important role in the therapeutic effectiveness of phototherapy (85,86).

In summary, at the present time the mechanism of phototherapy is thought to be as follows: The unconjugated bilirubin in the skin of the neonate is both isomerized and cyclized to form at least two pairs of isomers, which in this thesis will be called the EZ/ZE isomers and the lumirubins, respectively. These products are partitioned to the plasma, taken up by the liver and excreted in the bile. In the bile the unstable EZ/ZE isomers rapidly revert to the bilirubin in its ZZ configuration. This accounts for the increment in the amount of unconjugated bilirubin found in the bile of infants undergoing phototherapy. Also, the more stable photoproducts, known as the lumirubins, account for the major polar photoproducts which are also found.

Recently, there has been much concern over the safety of phototherapy. It has been suggested that (87) potential mutagenic and/or carcinogenic long term side effects are associated with phototherapy.

1.3.3.2 ELECTRONIC EXCITED STATES

Figure 1.10 shows the electronic absorption spectrum of bilirubin in

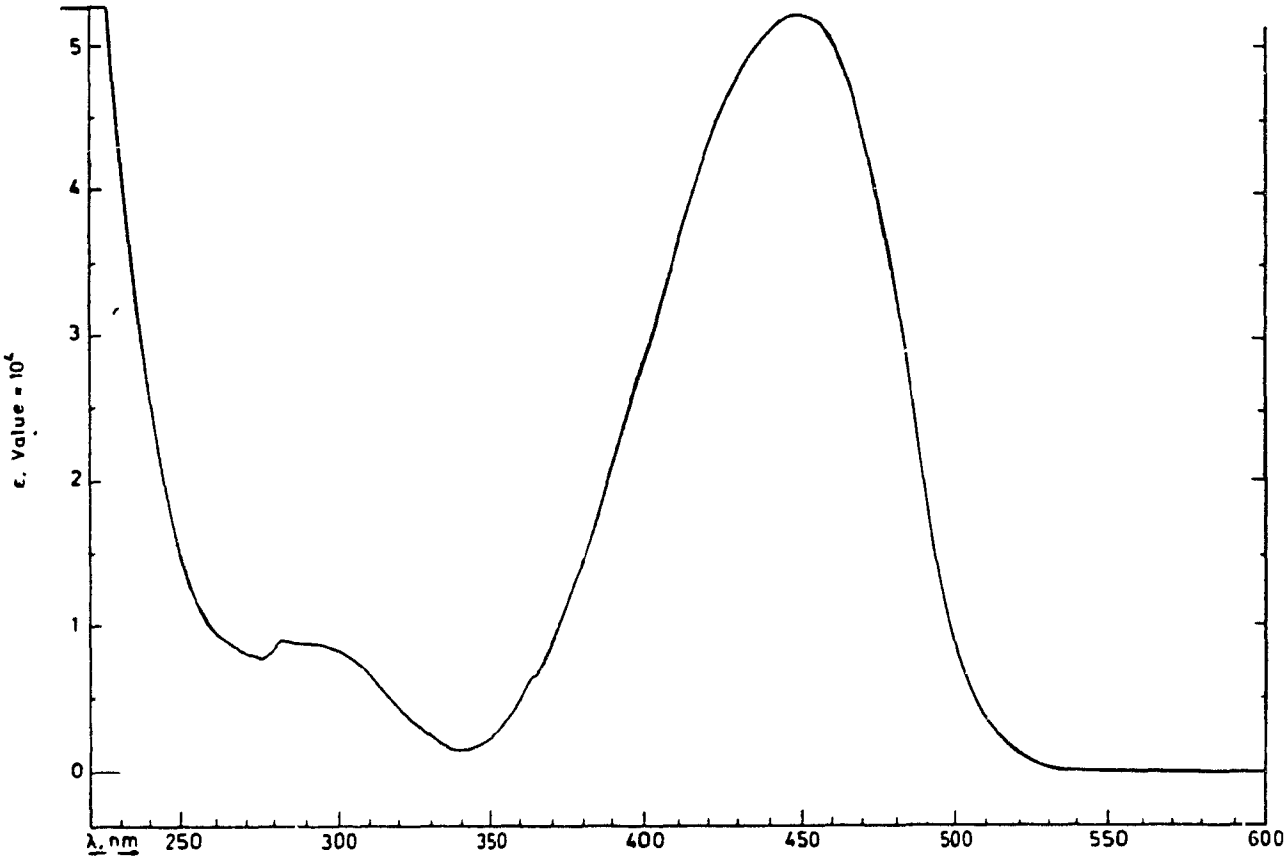

Figure 1.10 The visible-ultraviolet spectrum of bilirubin IX-α (9.0×10^{-6} M) in acetonitrile at 25°C. (88)

acetonitrile (88). There is one large peak with an extinction coefficient of ca. 60,000 near 450 nm and two shorter wavelength maxima near 300 nm and 200 nm, with an inflection point near 220 nm. Excitation with visible light containing some energy in the 450 nm region should lead to the lowest lying excited state (87). From here it can fluoresce or phosphoresce, although it is still unclear whether or not the latter process occurs in bilirubin (89-92). Bilirubin does fluoresce weakly in $CHCl_3$ (93) and other organic solvents (94). The lowest lying singlet excited state of bilirubin is 264 kJ/mole above its ground state, as seen in Fig 1.11. It is important to note that the formation of T_1, the lowest lying triplet state, is not readily accessible by direct irradiation due to a very small probability for intersystem crossing (92).

Studies using 347 nm laser flash photolysis (92) were not successful in identifying any bilirubin transients in organic media. However, in bilirubin/human serum albumin (BR/HSA) solutions a short-lived transient was found leading to the following hypothetical mechanism for isomerization:

$$BR\ (S_0) \text{----> } BR\ (S_1) \text{---> } BR^* \text{---> } BR\ (S_0)$$

where BR^* is a transient which may or may not be a triplet state.

However, Pratesi (83) and Tran and Beddard (90) showed that near room temperature electronic relaxation of bilirubin in the excited singlet state proceeds predominantly via a radiationless pathway that does not include intersystem crossing, which suggests that there is no triplet state. On the other hand, triplet state properties have been reported by Land (95), based on studies by the pulse radiolysis technique and anthracene sensitized energy transfer.

Thus, most authors agree that photoisomerization proceeds via the following mechanism: light absorption results in excitation of bilirubin from S_0 to

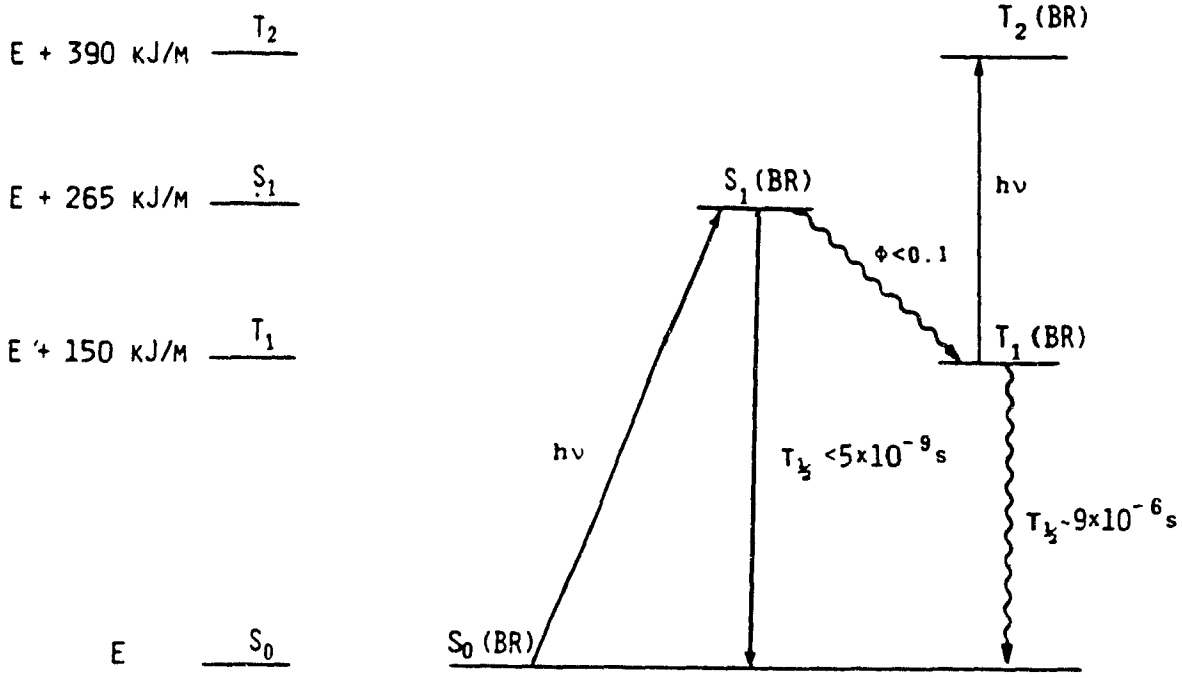

Figure 1.11 The photochemical reaction cycle for electronic excitation of ground state bilirubin IX-α (S_o) to its first excited singlet state (S_1) which can decay to (S_o) with fluorescence or undergo intersystem crossing to the lowest lying triplet excited state (T_1). (93)

S_N excited states, from which fast internal conversion processes leave the molecule in the lowest S_1 excited state. A twist about one of the C = C bonds gives rise to an excited state intermediate with twisted geometry (Fig. 1.12). A radiationless transition from this state yields bilirubin or the EZ/ZE isomers.

The mechanism of the second most important photoprocess, photooxidation, has also been explained using the electronic excited states. Absorption of visible light promotes an electron to a higher energy state (Fig. 1.13) without change of spin. This excited state now has no net unpaired spins but two singly occupied orbitals. It is possible that a spin inversion can take place leading to an excited triplet state with two unpaired spins and two singly occupied orbitals. Both the T_1 and S_1 states now contain an electron further away from the nucleus and thus less tightly bound, consequently they can be removed by oxidizing agents. In addition, the "hole" left in the excited state would be expected to bind an electron more strongly than the S_0 state. Consequently the S_1 and T_1 excited states are more easily oxidized and reduced than the ground state.

Photooxidation reactions have been proposed to proceed via the singlet or triplet state. The latter possibility includes either a Type I or a Type II mechanism (96). The S_1 and Type I mechanisms involve the reaction of excited bilirubin or bilirubin directly with $^3O_2$, usually with one electron or H atom transfer, to give BR^+ or BRH which may lead to biliverdin and other oxidation products. The Type II mechanism involves quenching of the T_1 to give singlet excited O_2 and ground state bilirubin, which, by electron transfer, can yield products. However, it is difficult to distinguish between these mechanisms.

The mechanism may be in doubt, but the products of the photooxidation are well known and vary in amount and type with solvent (93, 97) (Fig. 1.14). Some of the products include biliverdin, various propentdyopents (ca. 60% of the

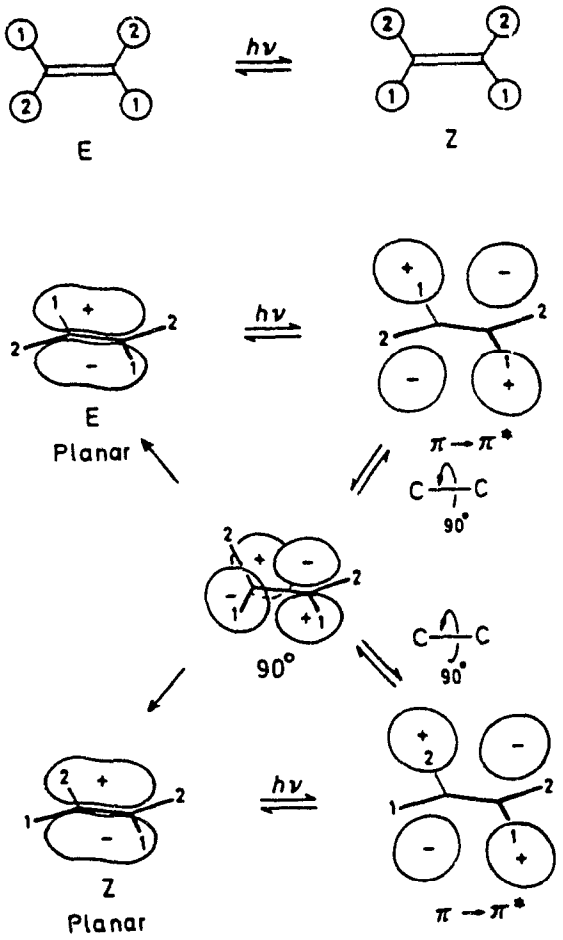

Figure 1.12 (Upper) Diagrammatic representation for E <----> Z carbon-carbon double bond isomerization. The E isomer is determined by having its two higher priority groups on opposite sides while the Z isomer is determined by having the two higher priority groups on the same side. (Lower) Diagrammatic orbital representation for photochemical breaking of the olefinic π-bond, first by excitation to a π-π* excited state (antibonding π orbital), then rotation about the carbon-carbon single bond to give a 90° twisted intermediate. The intermediate can collapse by rotation to starting planar olefin or to the planar isomer. (93)

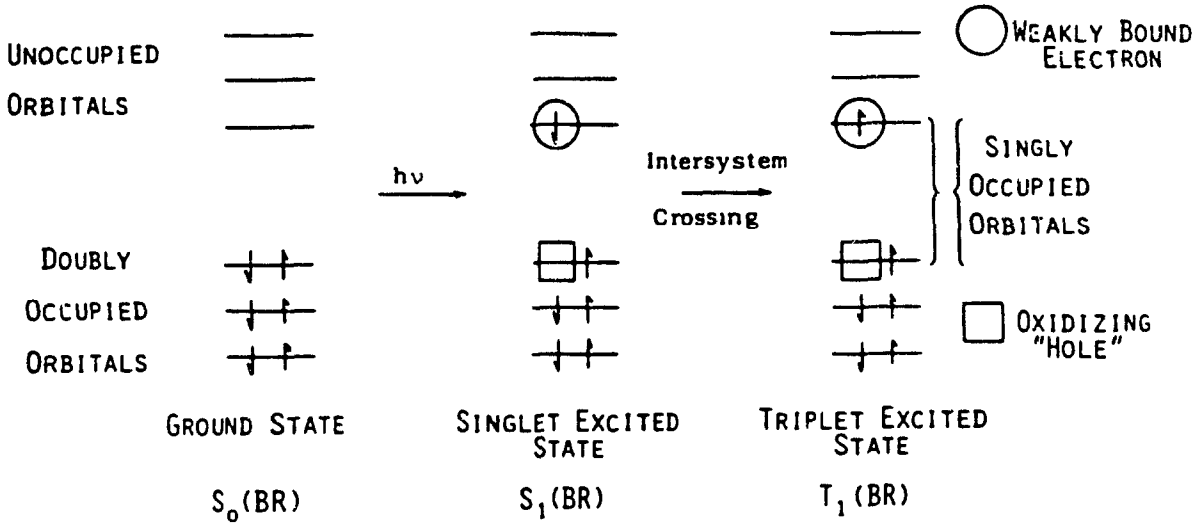

Figure 1.13 Schematic diagram illustrating electron excitation in bilirubin and oxidizing and reducing properties of the excited states (93).

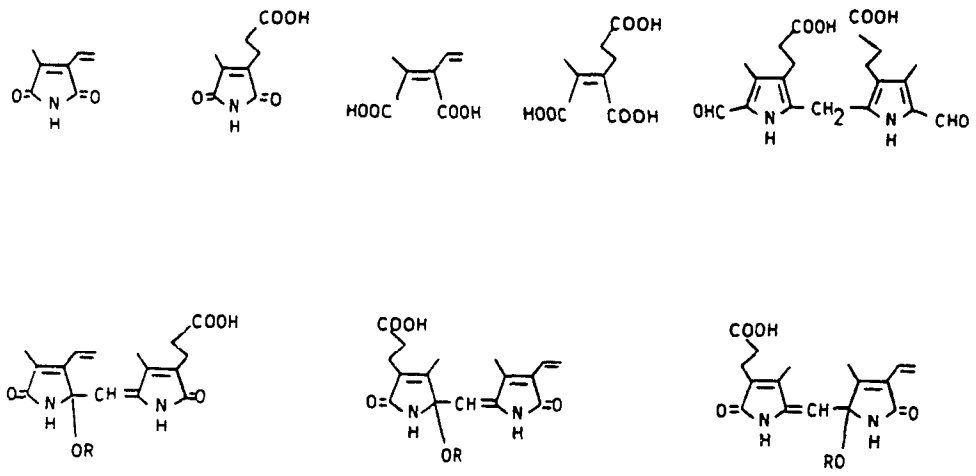

Figure 1.14 The photodegradation (via photooxidation) products of bilirubin IX-α. R = alkyl or hydrogen. (93)

products), methylvinylmaleimide as well as cyclic imides.

1.3.3.3 SIDE EFFECTS OF PHOTOTHERAPY

Phototherapy remains the treatment of choice in lowering the serum bilirubin concentration because it is simple, effective, and no permanent or serious side effects in vivo have been unequivocally identified. Nonetheless, the actual dangers of the method are still being rigorously debated. It is thought that phototherapy was accepted fairly rapidly as a treatment for hyperbilirubinemia because light could not be seen as harmful to the infant. It is very possible that since no possible side effects were expected, none were found (98). It was not until 1972 that possible hazards of phototherapy started to appear in the literature. Since then many articles arguing for and against these hazards, have appeared. For the most part the suggested dangers are related to the use of broad-band spectrum fluorescent light, and are discussed in several review papers (99).

Recently several authors have challenged the current practice of using phototherapy for the treatment of physiologic jaundice in otherwise healthy newborns (100,101). Phototherapy is currently used when the bilirubin level reaches about 15 mg/dl despite the belief by some that phototherapy may be more damaging to the infant than moderate jaundice (102,103). In Sweden, for example, normal term neonates receive phototherapy only when the bilirubin level exceeds 20 mg/dl (104). Detailed follow-up data on these infants did not identify any psychological or neurological effects (105). Concerns range from effects upon organ systems and the influence on intrinsic biologic behaviour as well as upon cellular and molecular events, to the possibility that phototherapy is physiologically stressful and potentially hazardous to the developing infant (102,103,106-109). Constant light can interrupt the natural biological rhythm and

has been shown to adversely affect the plasma somatotrophin (human growth hormone) level (110).

Although the potential toxic effects of blue light are recognized (111), it has been shown that infants can undergo treatment with phototherapy for as long as 2.5 years with no known deleterious effects (112,113). Thus, although in vitro studies have shown that wavelengths between 350 and 450 nm are carcinogenic and mutagenic (114) it seems that these studies cannot easily be extrapolated to the human neonate (115).

Finally, there still remains concern that even small amounts of retained photoderivatives may be toxic, especially to the brain (116). The neurotoxicity of the photoisomers and hydroxybilirubins produced during phototherapy has not been assessed. However, patients with Type II Crigler-Najjar syndrome chronically carry serum bilirubin concentrations that range from 6-22 mg/dl; yet they exhibit normal survival and no intellectual impairment, despite repeated exposure to sunlight and intensities of up to twenty times that of phototherapy lamps (117). Also, infants that develop cholestasis during phototherapy exhibit a gray-brown discoloration of the skin, serum and urine due to the inability to excrete photoderivatives in the bile. Such "bronze babies" show normal development and no known neurologic impairment after the syndrome subsides (118).

Despite the theoretical complications discussed, phototherapy for neonatal hyperbilirubinemia has been used extensively for many years with very few serious complications. However, long-term follow-up of treated infants is needed to study possible effects on cancer rates and sexual function, and until such time there will be some reservations about the ultimate effects and safety of phototherapy.

1.3.4 TIN-PROTOPORPHYRIN ADMINISTRATION

During the preparation of this thesis a new approach to the problem of hyperbilirubinemia was reported by A. Kappas, et al. (119). They studied the effects of administering Sn-protoporpyrin, a potent competitive inhibitor of heme oxygenase, to term infants with direct Coombs-positive ABO incompatibility. This group was chosen since these infants have a significant and well-defined risk of substantial hyperbilirubinimia. The use of this enzyme inhibitor, in theory, should moderate the degradation of red blood cells and thus decrease the formation of bilirubin. It is the first proposed treatment that involves inhibition of the formation of bilirubin rather than clearing the pigment after it is formed.

The results of the study by Kappas, et al. show that a dose of Sn-protoporphyrin (0.75 μmol/kg of body weight) injected intramuscularly two or three times at 24 hour intervals significantly decreases the plasma bilirubin concentration and thus decreases the number of infants requiring phototherapy compared to a control group. No short term side effects were noted and follow-up studies of the treated infants is now in progress.

1.4 BINDING OF BILIRUBIN TO ALBUMIN

The important step in the hemoperfusion process is the binding of bilirubin to the resin in the column. Similarly, binding of bilirubin is also the first important step in the elimination from the body - it must first bind to the protein albumin. Thus, an understanding of the properties of bilirubin binding is important.

Before a discussion of bilirubin binding is possible, some understanding of the protein albumin is essential. Albumin is the most abundant protein in human plasma and it accounts for 60% of the total protein concentration, with a concentration of 42 g/l (120). Its most important function is the binding of

hydrophobic ligands such as bilirubin, fatty acids, steroids, lysolecithin, thryoxine, drugs and dyes. Thus, any toxic effects these substances may have are nullified when bound, since it is the unbound drugs which give rise to the physiological effects.

The amino acid sequence of human serum albumin has been reported previously (121,122). It is a chain of 584 residues, cross-linked by 17 disulfide bridges into a series of nine loops, six large and three small (Fig. 1.15) (120).

The structure of bovine serum albumin, BSA, has also been elucidated (126) (Fig. 1.16). It contains 581 residues and is different from HSA in 135 places, although these differences are generally small since they are polar to polar and nonpolar to nonpolar changes. Both HSA and BSA contain more acidic than basic residues and are thus overall acidic with a net charge of -18.

Once the amino acid sequence was determined, a pattern of three repeated homologous units was noted. This led to the thought that these similar sequences could be folded similarly into distinct structural units or domains. In fact, early physical and chemical studies of the protein had already led to the concept (123-125) of domains, interconnected by peptide chains. More recently, Brown's model (126) has six subdomains, each containing 3 α-helices, X, Y, and Z. Each helix has about twenty-two amino acid residues which give about six turns of the α helix. The sequence of twenty-two amino acids is terminated by restrictions imposed by the disulfide bridges. The inner face of the trough-like structure of the subdomain contains predominantly hydrophobic residues (127). This is consistent in all six subdomains, leaving the outside proteins hydrophilic, as would be expected for a surface that is in contact with the solvent. Thus, each subdomain has a hydrophobic face, clusters of basic residues, and proline residues at the tips of the long loops. However, each subdomain is also unique in sequence, and probably exhibits a certain degree of specificity in binding.

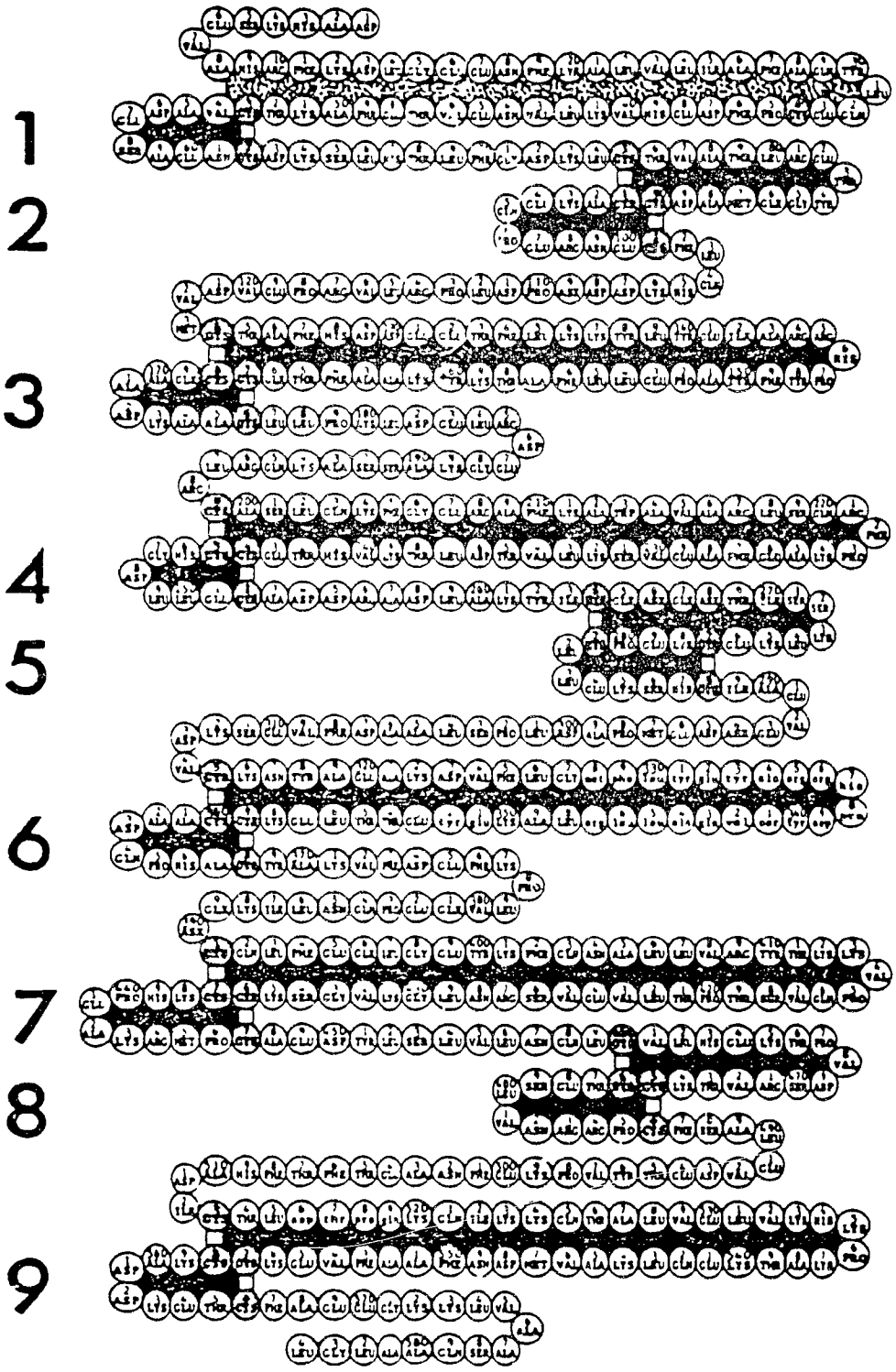

Figure 1.15 Amino acid sequence and 9-double-loop structure of human albumin. (120)

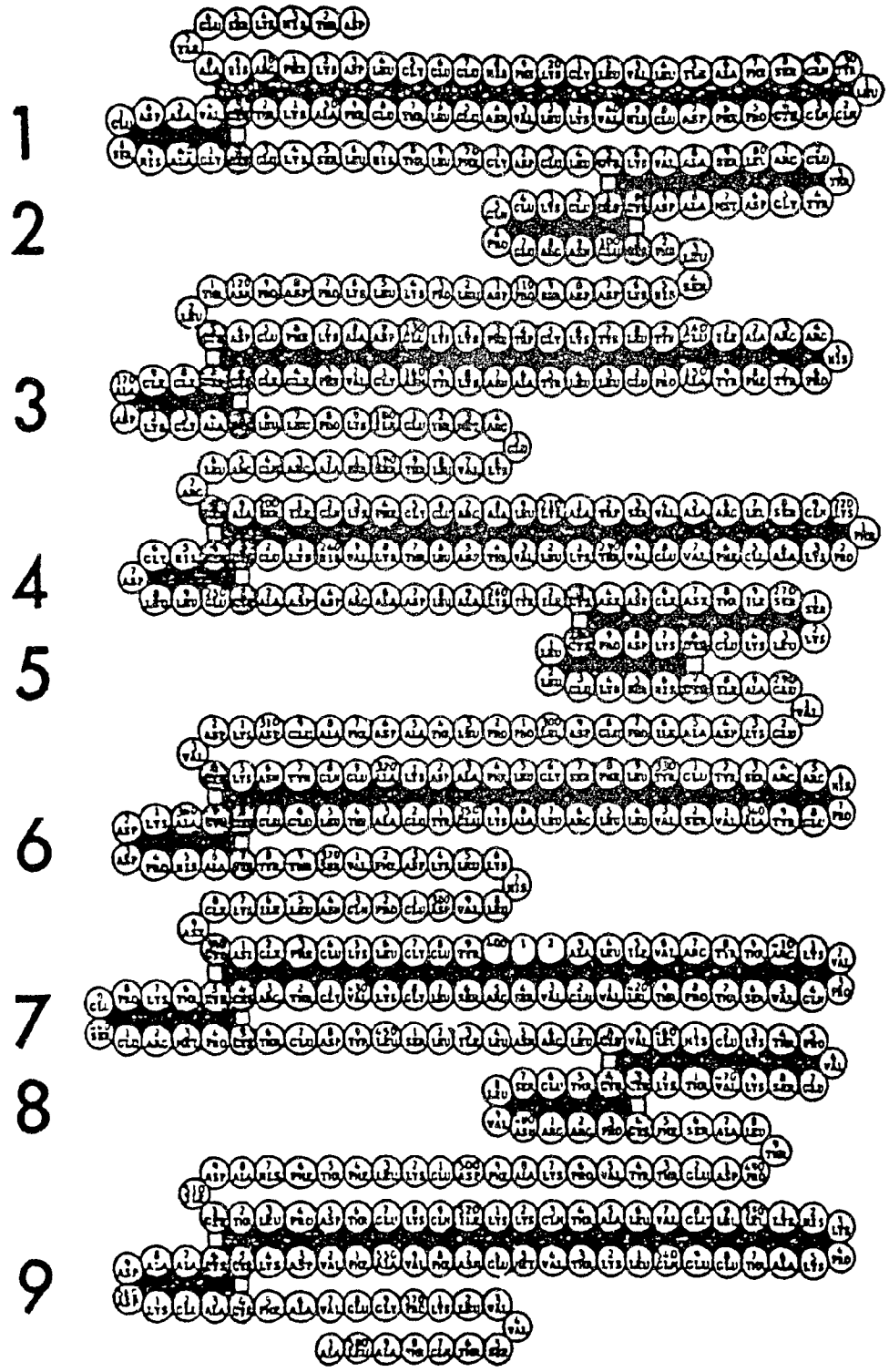

Figure 1.16 Amino acid sequence of bovine serum albumin. (127)

Many of the ligands that bind to albumin can occupy more than one binding site. There are high affinity sites and low affinity sites, each of which vary in number, depending on the ligand. Fatty acids, for example, bind to albumin at six high affinity sites and between 20 and sixty low affinity sites (127). In addition, more than one ligand can bind to a specific site, giving rise to competition between ligands.

In the blood plasma, bilirubin is present almost exclusively bound as the dianion to one site on the albumin molecule with smaller amounts located at one or two secondary sites (13). This is an equilibrium process and therefore a small concentration of free pigment exists. In situations where the bilirubin concentration is increased greatly, as in icterus or acidosis, there is a tendency to form bilirubin albumin aggregates.

Jacobsen (128) was the first to present a binding isotherm showing that one molecule of bilirubin is bound to albumin with high affinity and that two additional molecules are bound with lower affinity. However, it has been difficult to show the binding of the third molecule. It is presently held that although many molecules of bilirubin can bind to albumin with weaker and weaker affinities, the higher affinity sites include one very strong and one slightly weaker binding site. Thus, two molecules of bilirubin can be bound to one molecule of HSA. The reported numerical values of the binding constants vary greatly with method and author (13).

It is also not yet clear where the primary binding site for bilirubin is located on the albumin molecule. Many researchers have tried to isolate the specific site; yet no definitive results have been presented. The following is a brief review of some of the work done to date:

Reed, et al. (129) used cleavage of albumin, by enzymatic hydrolysis and by cyanogen bromide, to show that several fragments do and several do not bind

bilirubin. One fragment which did bind bilirubin with high affinity was found to contain the residues 186 - 306. This fragment is in the subdomain 2-AB which includes Lys-220 located at the mouth of the binding site.

Jacobsen (130) prepared an albumin derivative with covalently attached bilirubin. He argued that bilirubin is bound to Lys-240 in HSA (Lys-238 in BSA), still in subdomain 2-AB.

Kuenzle, et al. (131) used activated bilirubin enol ester to show that bilirubin is attached to two peptide fragments. These involved the residues 124 - 293 and 446 - 547.

Using photoproducts of bilirubin, Jori et al. (132) and Hutchison and Mutopo (133) found that the fragment 187 - 397 of BSA and 1 - 124, respectively, are important. Hutchison and Mutopo also found that peptides 125 - 297 bind bilirubin to a lesser degree.

C. Jacobsen (134-136) used chemical modifications of various amino acids to deduce which ones were involved in the binding process. He showed that lysine, arginine, histidine and tyrosine are involved in the high affinity sites while tryptophan and cysteine are not.

Work done in this laboratory (49) revealed that bilirubin adsorbs readily onto polyamide resins with lysine and arginine containing pendants. Comparatively, the adsorption was much less for resins containing tyrosine, histidine and alanine. A resin containing the peptide sequence Arg_5-Ala_3-Support (the trialanine acting as a spacer) competed favourably with albumin for the binding of bilirubin. It was suggested that the binding sites on albumin for bilirubin contain two accessible arginine residues, and thus the tip of loop 4 may be a possible binding site.

Further work on polypeptide-substituted crosslinked polyacrylamide resins revealed that the sequence 136-148 of the primary structure of human

serum albumin is important in the binding of bilirubin (50). Lysine and arginine residues were shown to favour the binding of bilirubin whereas glutamic acid reduced it markedly. Subsequently it was shown that lysine-containing pendants show positive cooperativity effects for the binding of bilirubin, while arginine-containing pendants eventually result in negative cooperativity behaviour (137).

The binding of bilirubin has also been shown to be stereoselective. Lightner et al. (138) used circular dichroism to show that human serum albumin can bind the positive chirality conformational enantiomer of bilirubin preferentially, though not exclusively.

Finally, it is important to note that the binding of bilirubin to HSA and BSA have been found to be different. Using fluorescence spectroscopy, Lee and Gillispie (139) showed that bilirubin is buried more deeply within the HSA molecule than it is with BSA. More recently, Y. Z. Hsieh, et al. obtained results that are in agreement with this observation (140).

1.4.1 A DESCRIPTION OF BINDING SITES AND MECHANISMS OF BINDING

Before looking more closely at the binding site of bilirubin to HSA, it is important to first describe, in general, the types of binding that can exist between a ligand and a substrate. In principle, the binding may occur in two ways: (i) competitively or (ii) noncompetitively. In the former case each ligand is competing for the same binding site and thus two ligands cannot bind simultaneously, while in the latter case both ligands can, indeed, bind at the same time. In addition, three types of noncompetitive binding are possible; the two ligands can bind cooperatively, anti-cooperatively or independently. Positive cooperativity occurs when the second ligand binds more strongly than the first because, for example, of a conformational change in the substrate due to the binding of the first ligand. The opposite effect is also possible, that is, the binding

of the first ligand adversely affects the binding of the second and thus its binding decreases. The last option describes binding of one ligand that does not affect the subsequent binding in any way.

The binding isotherm of bilirubin to albumin has been shown (141) to follow the equation:

$$r = k_1 b/(1 + k_1 b) + k_2 b/(1 + k_2 b) \qquad [1.1]$$

where: r = the average number of bilirubin molecules bound to each albumin;

b = the free bilirubin concentration;

k_1, k_2 = the site binding constants for the first and second sites, respectively.

Equation [1.1] is consistent with the binding of bilirubin to two **independent sites**. However, despite the fact that excellent fit of the data to the equation has been observed, it has been pointed out (14) that this does not prove that the binding actually takes place at two independent sites since this equation may be transformed into Adair's binding equation, an equation used for **stepwise binding**:

$$r = (k_1 b + 2k_1 k_2 b^2)/(1 + k_1 b + k_1 k_2 b^2) \qquad [1.2]$$

Equation [1.2] can also be used to express the binding in terms of stoichiometric binding constants, (K_1 and K_2) which do not infer any specific binding site.

The data fit the theory in equation [1.2] very well, but according to this mechanism only one binding site, with a binding constant k_1, is available in the absence of bilirubin. Only after the binding of one molecule of bilirubin occurs does a second binding site become available, with a binding constant, k_2.

It is also possible that bilirubin can bind to two **equal** sites on the albumin molecule. If the two equal sites bind anti-cooperatively it can be shown that the binding equation would again yield the same binding isotherms as would result from equations [1.1] and [1.2] (14). In view of the above arguments, it is possible that the concept of a primary high-affinity and one or a few secondary sites with lower affinity may be incorrect. Accordingly there are several two-site models which are applicable to the experimentally obtained isotherm data. It may also be possible to describe the mechanism with three or more sites (14).

The kinetics of binding have been studied by many investigators (142-144). Faerch and Jacobsen (145), working with HSA, at 5.5°C, were able to record the course of the primary binding step of bilirubin to HSA. The model used to describe the binding involves four consecutive steps, the first of which is a bimolecular combination of bilirubin and albumin, which occurs within 3 ms. The second and third steps involve intermediate complexes which lead to the final stable complex, a process which requires ca. 10 ms. The explanation of these results, based on the light absorption spectrum of the primary complex, is that both chromophores of bilirubin are bound to the HSA and that the relaxation processes leading to the temporary complexes are due to conformational changes in the bilirubin - HSA complex. This agrees well with recent reports which indicate that bilirubin at the primary binding site is buried within the HSA (140).

It is obvious that the exact site or sites of binding of bilirubin to albumin is not fully identified or agreed upon. Likewise, the mechanism of the binding is not completely understood. The situation has become more complicated with the study by some investigators of the binding of the photoproducts of bilirubin to HSA.

1.4.2 BINDING OF PHOTOPRODUCTS TO HSA

As early as 1976 Kapitulnik, et al. (146) determined that a product they called "pigment 430", or the lumirubins, was formed in patients undergoing phototherapy, and they studied the effect of this product on the binding of bilirubin (BR) to HSA in vitro. Their results indicate that at molar ratios of BR/HSA > 1 pigment 430 reduces the binding affinity of HSA for bilirubin. This implies that the pigment can only compete for the secondary binding site, since at lower molar ratios, where all the bilirubin would presumably be bound to the primary site, no loss of bilirubin binding due to the presence of pigment 430 could be detected. Nonetheless, the implications of these results are serious. If the photoproducts formed during phototherapy can compete with bilirubin for the binding site on HSA, then it follows that the production of these photoproducts will result in more bilirubin in the blood plasma. Consequently, the formation of photoproducts can have a deleterious effect on the infant.

Hutchinson and Mutopo (147) and Meisel, et al. (148) presented results showing that irradiation of bilirubin in the presence of HSA causes the product to be firmly bound to the HSA. Meisel, et al. calculated a binding constant for photobilirubin II (which, in all probability, is the same product as that considered by Kapitulnik, et al.) of 2.2 X 10^6 M^{-1}, although they were not sure that the photoproduct was competing for the same binding site as bilirubin. Interestingly, there is some evidence that it is actually the bound bilirubin which isomerizes leading to the obvious conclusion that the photoproduct is bound at the same primary binding site as the bilirubin. This will be considered more fully in Chapter 2. At about the same time Lamola, et al. (149) published results showing that the EZ/ZE isomers can bind to HSA at the primary binding site, with an affinity 2-3 times lower than that of bilirubin, $K > 10^6 M^{-1}$. However, they argued that this would be beneficial since the albumin could therefore also reduce the

possible toxic effects of the photoproducts. Of course, since it has not yet been determined that the photoproducts are toxic, the displacement of bilirubin from HSA may be detrimental, contrary to the argument of Lamola, et al.

1.5 THE PRESENT STUDY

The primary aim of the present study was to determine the effect of photoisomerization of bilirubin on its binding behaviour with albumin and substituted polyacrylamide resins. To this end the study of the effects of solvent and environment (including the presence or absence of oxygen, albumin, polypeptides and pH) on photoproduct formation and bilirubin destruction was done. Using methods such as absorbance difference spectroscopy, high performance liquid chromatography and stopped flow analysis, photoproducts of bilirubin were prepared and analyzed in organic solvents and aqueous solvents containing various amounts of human serum and bovine serum albumin. The information presented in Chapter 2, gives some insight as to how phototherapy works and what may be done to improve the method.

In Chapter 3 the binding of bilirubin onto cholestyramine and selected polymeric adsorbents (previously prepared and reported by this laboratory) is compared to the binding of bilirubin from the same solutions with simultaneous irradiation with a light source. This study was done to ascertain whether an enhancement in the rate of adsorption could be detected when photoproducts were being formed.

Finally, the results of the photoisomerization and binding studies led to questions regarding polarity effects, hydrophobic effects and electrostatic effects. To understand the importance of each of these interactions, a study was made of the adsorption of "bilirubin-like" compounds, and is found in Chapter 4. Contributions due to specific parts of the bilirubin molecule (for instance, the

carboxylic acid side chains) were isolated by using substituted indoles and pyrroles and examining the effects on the binding of these molecules onto cholestyramine. In this way we sought to elucidate the mechanism of binding of bilirubin onto the various resins.

Finally, the last section of the thesis describes the contributions to knowledge and suggestions for future work.

The Appendix contains the experimental data points as well as a paper published in the Canadian Journal of Chemistry which is presented, almost in its entirety, in Chapter 2.

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CHAPTER 2

SOLVENT EFFECTS ON THE PHOTOISOMERIZATION OF BILIRUBIN

2.1 INTRODUCTION

The photochemistry of bilirubin has been studied extensively over the past fifteen years (1-4). The original hypothesis was that the lowering in the plasma bilirubin level during phototherapy involved photooxidation of the pigment (5). However, it has subsequently been shown that photoisomerization is much faster and hence precedes photooxidation (6-8).

Initial evidence suggested that the photoisomerization of bilirubin leads to only one photoproduct, first named "photobilirubin" (8). Further work showed that there are in fact a number of photoproducts, including both structural and geometric isomers (9-12). Furthermore, the photoproduct formed, and in what proportion, seems to be a function of many parameters, including the nature of the solvent and the presence or absence of oxygen or of proteins, such as albumin or polypeptides.

The conformation of the bilirubin molecule in solution is highly solvent dependent. In aqueous solution bilirubin is folded into a configuration that has six intramolecular hydrogen bonds (13). This folded, "ridge tile" structure results in the molecule being highly hydrophobic and thus sparingly soluble in aqueous solution. The reported solubility of bilirubin in aqueous solution at physiological pH ranges from 0.007 to 100 μM, with the lower value being favoured according to Brodersen (14). The bilirubin molecule assumes this tight, intramolecularly bonded structure in water and in chloroform (15), but in solvents which interfere

with hydrogen bonding, e.g., dimethylsulfoxide (DMSO), the molecule "opens up" as some of the hydrogen bonds are weakened (9, 16).

Certain conclusions from studies of the photolysis of bilirubin in organic solvents have been extended to phototherapy conditions. Since the ultimate goal of phototherapy is to lower the bilirubin levels in the infant's blood plasma, it is imperative that effect of solvent on the mechanisms of the photoisomerization and the photodestruction of bilirubin be understood. Thus, we have studied the photoreaction in various organic and aqueous media, with and without human serum (HSA) and bovine serum (BSA) albumin and with and without specific polypeptides, and offer some explanation for the photoisomerization results with respect to the environment of the bilirubin.

2.2 EXPERIMENTAL

Solutions of bilirubin (BR) were prepared daily by dissolving the powder (from bovine gallstones, Sigma) in the appropriate solvent. The purity of the bilirubin was checked using HPLC (17). Chloroform (Spectrograde, Anachemia), triethylamine (Reagent grade, Anachemia) and dimethylsulfoxide (HPLC grade, Anachemia) were used as received, except in experiments for which the ethanol stabilizer in the chloroform was removed immediately prior to use by first distilling over P_2O_5 and then passing the distillate through a column of basic I alumina (Sigma)

Aqueous solutions were made by first dissolving the bilirubin in 0.10 M NaOH and adjusting the volume of KH_2PO_4/NaOH buffer to achieve a final pH of 7.8 $\pm$ 0.1. Prior to use the solvents were purged with dried nitrogen.

Aliquots of the aqueous solution were added to HSA/buffer solutions to obtain the desired ratio of [HSA]/[BR]. Human serum (Cutter Laboratories) and bovine serum albumin (Sigma, Fraction V) were used as received. For all

solutions the final concentration of bilirubin was 1.71×10^{-5} M. Studies were done at room temperature, except for those with the pure buffer solutions, which were kept in an ice bath prior to use.

Poly-l-lysine (Sigma, degree of polymerization, DP = 2700, 1150, 17) was used as received. Solutions of the polypeptide were made by dissolving 4-5 mg of the polymer in ca. 90 mls of distilled water and then adjusting the pH to 11.00 by the addition of 0.10 M NaOH. The final solution was then brought to 100.0 mls by the addition of distilled water. The bilirubin-polypeptide complex was prepared by adding equal amounts of both the bilirubin solution and the polypeptide solution to one flask, the former being added to the latter dropwise. The final lysyl residue to bilirubin (L/B) ratio was approximately 10 in each case, with [BR] = 1.4×10^{-5}. Final pH values were 11.00 unless otherwise indicated. For the solutions with lower pH values, 0.10 M HCl was added dropwise to the complex until the desired pH was reached.

The solutions (<1 ml), contained in 1.00 cm path length cells, were irradiated directly in the cell holder of the Hewlett Packard Model 8451A Diode Array Spectrophotometer by placing the light source (Cole-Parmer Fiber Optic Illuminator Model 9745-00, fitted with a blue filter with a bandpass of 410 - 470 nm) over the cell, i.e., by shining the light down through the cell opening (intensity = 0.5 $\mu W.cm^2$). Since the course of bilirubin photochemistry has been shown to depend on the excitation wavelength (18), the same light source was used throughout this study. The spectrophotometer was capable of recording a spectrum within 1 s *while* the irradiation light source was still on.

The HPLC apparatus and methods, described previously by McCarthy et al., (17), used reverse phase methods. The chromatograph consisted of a Waters Associates Model 590 pump, a Rheodyne 7125 injection valve and a Schoeffel Spectraflow (Kratos Analytical Instruments, Ramsay, New Jersey) Model SF770

variable wavelength detector. The column was a 15.0 x 0.46 cm blank packed in-house with 5 µm Spherisorb (Chromatographic Specialties Company, Town of Mount Royal, Quebec) and a guard column consisting of a 3.5 x 0.46 cm blank packed with a 30-38 µm octyldecyl silica (Whatman Inc., Clinton, NJ). The mobile phase contained 50% phosphate buffer (pH = 7.4), 25% acetonitrile, and 25% dimethylsulfoxide, and 1.5 g/l of tetrabutylammonium chloride (Sigma) as an ion-pairing agent. Prior to sample injection the sample was dissolved in the appropriate amount of phosphate buffer/acetonitrile/dimethylsulfoxide so that the solvent phase matched the mobile phase. All analyses were done at room temperature using a flow rate of 1.0 ml/min with the detector wavelength set at 455 nm.

The stopped flow experiments, made with a Model 1A Stopped Flow Apparatus (Cantech Scientific Ltd., Winnipeg, Canada), monitored the changes in absorbance, at a fixed wavelength, with a TDI photometer and stored the data with a TDI 1024C transient recorder. The data were transferred to an IBM PC that performed the desired calculations. For these experiments, an aqueous solution of bilirubin was placed into one syringe and an aqueous solution of HSA ([HSA]/[BR] = 10) in the second syringe. The syringe containing the bilirubin could be irradiated, using the light source described above, prior to mixing. The two syringes emptied into the observation cell, where the data points (208 points) were collected over a 2 s time span, beginning within 5 ms.

2.3 RESULTS AND DISCUSSION

2.3.1 PHOTOLYSIS IN PURE CHLOROFORM IN THE ABSENCE OF OXYGEN.

When a solution of bilirubin in pure $CHCl_3$ purged with N_2 is irradiated a characteristic absorbance difference (AD) spectrum is obtained (Fig. 2.1), as

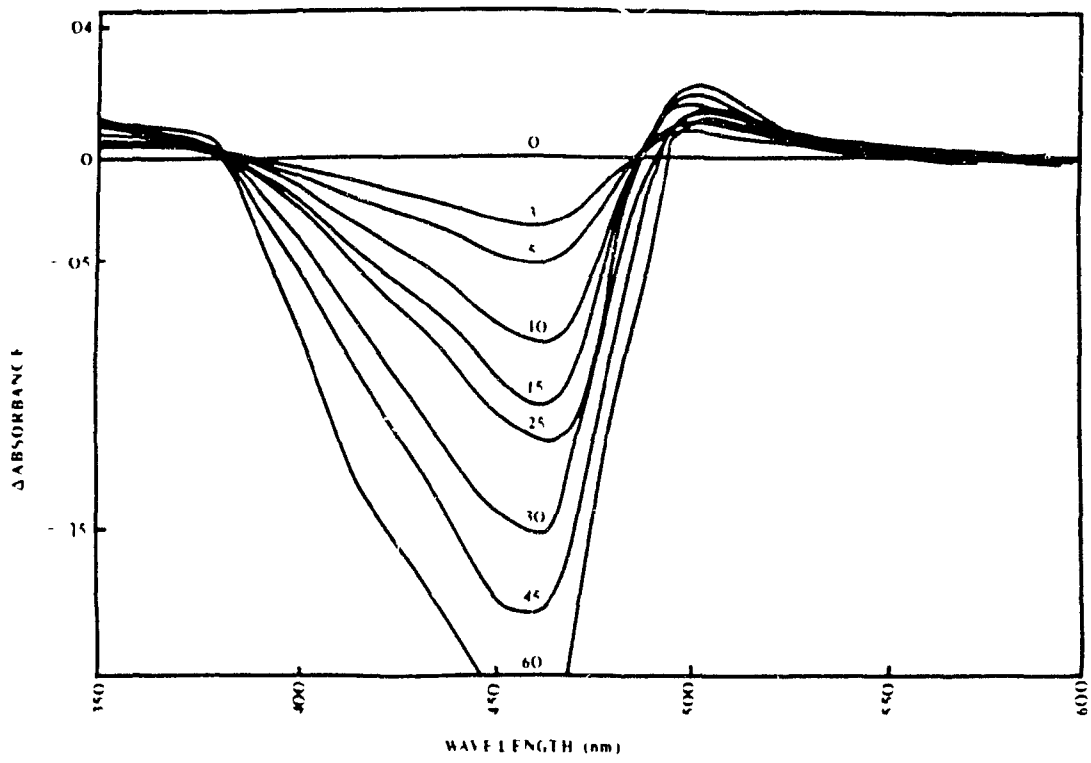

Figure 2.1 Absorbance difference spectra for a solution of bilirubin in pure $CHCl_3$ after various times of irradiation. (The numbers refer to the irradiation times, in minutes.)

reported previously (7,19). During the first 25 minutes of irradiation two isosbestic points are obtained. The isosbestic point at 490 nm is not maintained at irradiation times > 30 minutes, but that at 380 nm remains, suggesting two separate photoproducts. The production of the EZ/ZE isomers of bilirubin leads to the isosbestic point at 490 nm (8). The second isosbestic point results from the formation of a second photoproduct, previously identified as "lumirubins" (11), also referred to as "cyclobilirubin" (8), "unknown pigment" (10), and "pigment 430" (20), that has an absorbance maximum around 430 nm (9). The lumirubins, as discussed in Chapter 1, are configurational isomers of bilirubin formed by an endo-vinyl cyclization on one end ring of the molecule and can be in the EZ and ZE conformations, in the same way that the bilirubin molecule can exist in various conformations (10, 12, 21).

The slight gain peak at the low wavelength side of the spectra probably results from the production of small amounts of photooxidation products, as will be discussed later.

Based on structural arguments, by use of molecular models, it seems likely that cyclization to form lumirubins occurs **after** a rotation about the C4 - C5 bond of the bilirubin, i.e., by a reaction of the EZ/ZE isomers. This has been the subject of considerable dispute (10, 21) and is considered more fully later. Although an induction period for the formation of lumirubins would be expected for such a mechanism, it is not detected, possibly because the necessary buildup in the concentration of the EZ/ZE isomers is fairly rapid compared to the frequency at which the spectra were taken.

2.3.2 PHOTOLYSIS IN CHLOROFORM/ETHANOL IN THE ABSENCE OF OXYGEN

The photolysis of bilirubin in $CHCl_3$ containing 1% ethanol stabilizer is similar to that in pure chloroform (Fig. 2.1) with the exception that, at a given time of irradiation, the resulting **gain** peaks (Fig. 2.2A) are less than one half the size of those using pure $CHCl_3$. However, within experimental uncertainty, the loss peaks remain unchanged (Fig. 2.2B). It should be noted that even 1% stabilizer in the solvent corresponds to a molar ethanol concentration that is much greater than the bilirubin concentration.

The following experiment was made to elucidate the role of the ethanol: bilirubin in pure $CHCl_3$ purged with N_2 was irradiated for 30 minutes after which 10 µl of ethanol (1% of the total volume of the solution being irradiated) was added. The cell was gently shaken, the irradiating light was turned off, and additional spectra were taken. Possible side effects due to the addition of ethanol were accounted for by simultaneously adding an equal amount of ethanol to the reference solution. Upon addition of ethanol the height of the gain peak, due to the EZ/ZE isomers, immediately decreased substantially while the bilirubin loss peak remained essentially unchanged (Fig. 2.3). Concomitantly the isosbestic points disappeared. When irradiation of this solution was resumed the gain peak showed a further decrease while the loss peak continued to increase, and the isosbestic point at 380 nm was restored. These observations are consistent with the continued formation of lumirubins, since there is still a loss of bilirubin and an isosbestic point. Meanwhile the peak at 500 nm, corresponding to the EZ/ZE isomers, is decreasing slightly such that a new photostationary state is established. Interestingly, the absorbance difference at 500 nm reaches the same final value, 0.011, as in the $CHCl_3$/ethanol system discussed above.

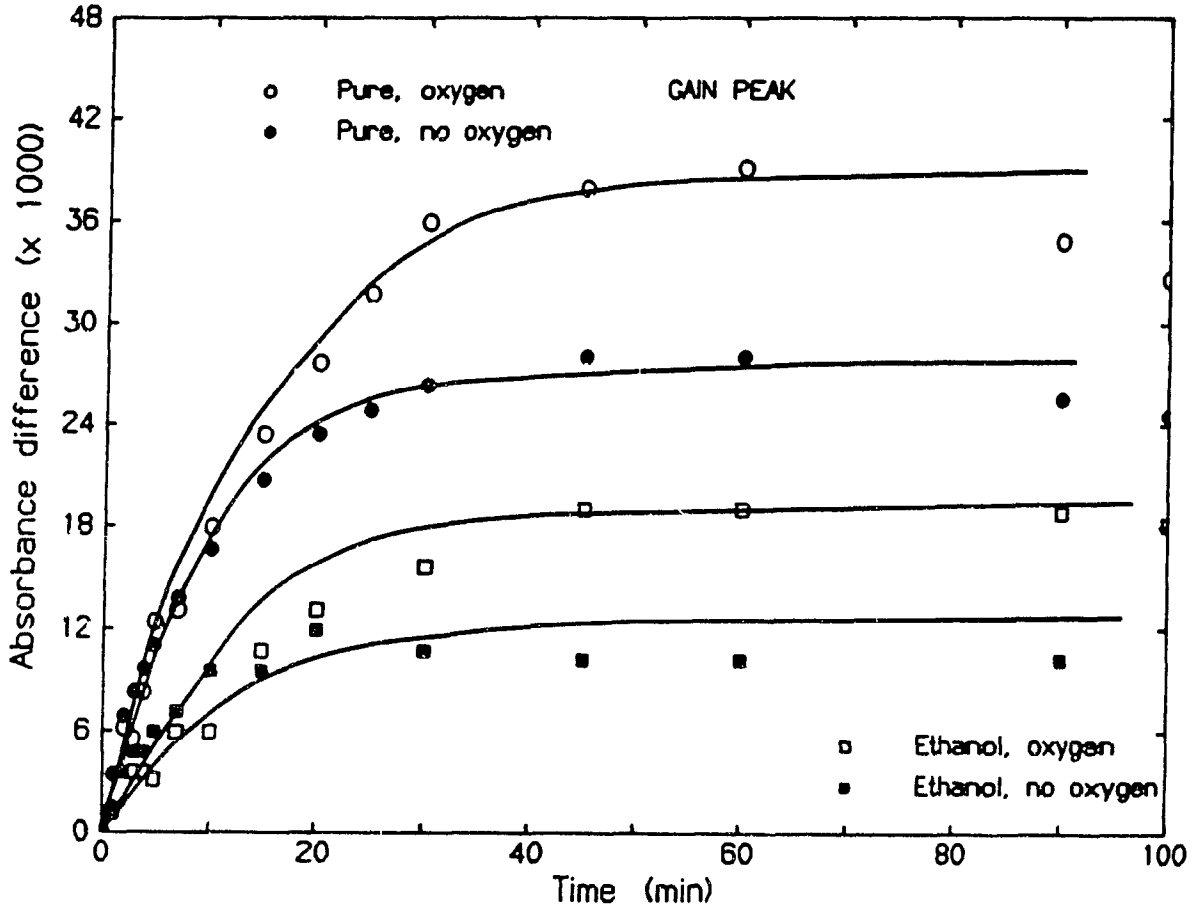

Figure 2.2 A The absorbance difference (x 10^3) of the gain peak (500 nm) for a photolyzed solution of bilirubin in pure $CHCl_3$ and $CHCl_3$/ethanol in the presence and absence of oxygen, as a function of irradiation time.

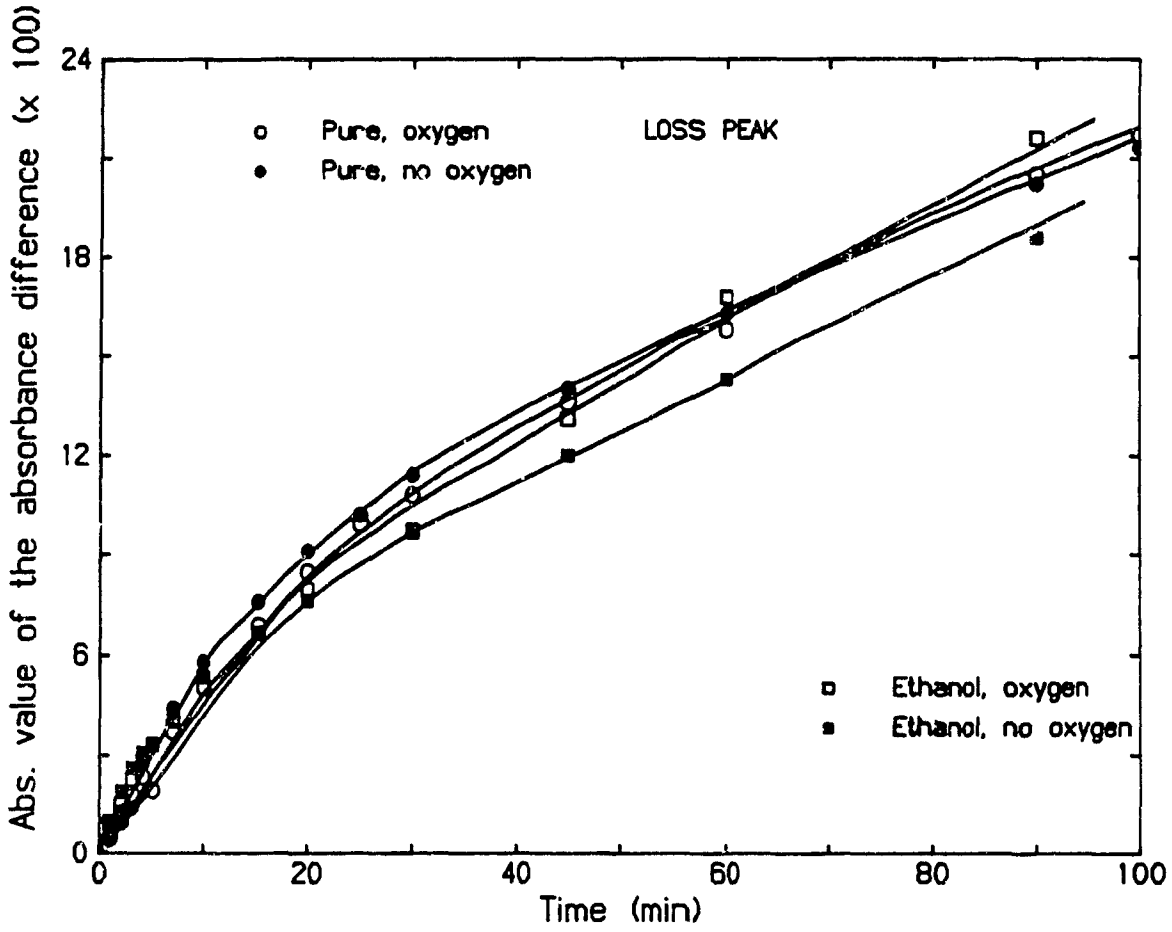

Figure 2.2 B The absolute value of the absorbance difference (x 100) of the loss peak (460 nm) for a photolyzed solution of bilirubin in pure $CHCl_3$ and $CHCl_3$/ethanol in the presence and absence of oxygen, as a function of irradiation time.

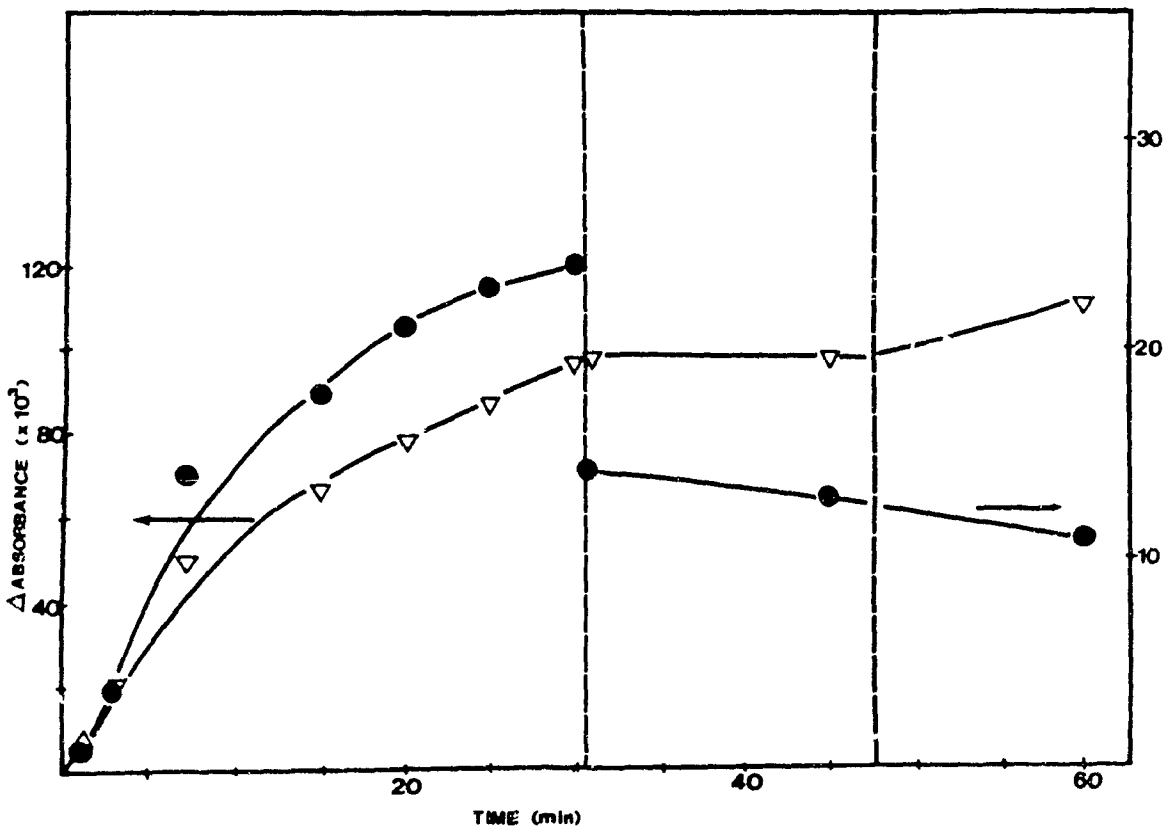

Figure 2.3 The absolute values of the absorbance difference of the gain peak (500 nm) and the loss peak (460 nm) for a photolyzed solution of bilirubin in pure $CHCl_3$ as a function of irradiation time. At t = 31 min, irradiation was discontinued and ethanol was added. At t = 47 min, photolysis was resumed: ●, gain peak; ▽, loss peak.

The explanation of these results must account for a rapid decrease in the gain peak due to added ethanol that is not accompanied by any change in the size of the loss peak. A possible explanantion is a reaction of ethanol with the EZ/ZE isomers. Photochemical addition of alcohols to the exo-vinyl group of the bilirubin has been reported previously by Garbagnati and Manitto (22). However, the data are not consistent with Manitto's overall mechanism which requires the reaction to be light induced and irreversible. Since the EZ/ZE isomers are not entirely consumed, despite a large molar excess of ethanol, an equilibrium process is indicated. Furthermore, since the reaction occurs after the light is turned off, it is a reaction with the photoproduct. A reversible reaction of photoproducts with ethanol is consistent with a decrease in the concentration of EZ/ZE isomers in $CHCl_3$/EtOH solutions relative to that in pure $CHCl_3$ as well as the immediate decrease in its concentration when ethanol is added after the irradiation.

Alternatively, the changes in the height of the gain peak upon addition of ethanol may indicate solvatochromism of the photoisomers. It is well known that bilirubin dimethyl ester in dilute solution exhibits solvatochromism (23), which has been attributed to the presence of two co-existing conformers with different orientations of the A/B and C/D pyrromethenone moieties with respect to each other (23). It seems likely that the EZ/ZE photoisomers can also co-exist in two orientations. Since these photoisomers have less intramolecular hydrogen bonding than bilirubin they are more polar and should have characteristics similar to the bilirubin dimethyl ester so that the relative proportions are likely to be solvent dependent. Thus, while little solvatochromism is expected for native ZZ bilirubin in weakly hydrogen bonding solvents, it may well be significant in the case of the EZ/ZE isomers.

2.3.3 PHOTOLYSIS IN CHLOROFORM/ETHANOL IN THE PRESENCE OF OXYGEN

The photolysis of bilirubin was also studied in the presence of oxygen. Certain previous studies indicate that oxygen favours the destruction of bilirubin but leaves the formation of photoproducts rather unaffected, (24), while others found that in $CHCl_3$ the bilirubin does not undergo photodestruction in the absence of oxygen (25). Figure 2.2A shows that both in pure $CHCl_3$ and in $CHCl_3$/ethanol the presence of oxygen does not significantly affect the rate of photodestruction of bilirubin. Thus, within the time span of these experiments, photooxidation does not seem to be an important factor for bilirubin in this solvent. However, the presence of oxygen increases the overall rate of production of EZ/ZE isomers by 30% in pure $CHCl_3$ and by 80% in $CHCl_3$/ethanol (Fig. 2.2B).

Since the loss peak is unaffected by the presence of oxygen, the **first** step in the photoreaction, i.e., the formation of the EZ/ZE isomers from bilirubin, must be independent of the presence of oxygen. However, since the presence of oxygen affects the formation of EZ/ZE isomers, oxygen must block, or inhibit, a **secondary** reaction which would otherwise decrease the concentration of EZ/ZE isomers. Such a secondary reaction, proposed previously in Section 2.3.2 to explain the decrease in the amount of EZ/ZE isomers in the presence of ethanol, is a reversible reaction of the EZ/ZE isomers with the ethanol. Based on the data obtained here for the photolysis of bilirubin in the presence of oxygen, it is likely that an additional reaction of the EZ/ZE isomers occurs that is inhibited by the presence of oxygen. The largest amount of EZ/ZE isomers is formed in the absence of ethanol (which decreases the amount of EZ/ZE isomers formed) and in the presence of oxygen (which inhibits a reaction which decreases the concentration of EZ/ZE isomers). The trend seen in Fig. 2.2B, i.e., that the

amount of EZ/ZE isomers formed is lowest in the $CHCl_3$/ethanol/no oxygen case, followed by the $CHCl_3$/ethanol/oxygen, pure $CHCl_3$/no oxygen and finally pure $CHCl_3$/oxygen, is thus explained in terms of secondary reactions of the EZ/ZE isomers.

2.3.4 PHOTOLYSIS IN DIMETHYL SULFOXIDE IN THE ABSENCE OF OXYGEN

A more dramatic effect on the photoisomerization due to environment can be seen when DMSO is used as solvent rather than $CHCl_3$ (Fig. 2.4). Similar results were obtained for both DMSO and $CHCl_3/Et_3N$. Comparison with Fig. 2.1 indicates a difference in shape on the short wavelength side of the loss peak. Specifically, the shoulder at ca 430 nm seen previously for long irradiation times with $CHCl_3$ is now more distinct such that the loss peak is double-humped. Furthermore, the two isosbestic points do not appear simultaneously, and the slight gain peak at 350 nm is not seen at short irradiation times. The isosbestic point at ca. 490 nm occurs only at short irradiation times (<7 min), while the isosbestic point at 380 nm develops only after longer irradiation times (>7 min).

These data are consistent with the two-step process presented above. The spectra for short times of irradiation indicate that the production of EZ/ZE isomers predominates (one isosbestic point). An induction period is now evident which could not be detected when bilirubin was irradiated in $CHCl_3$. Since an initial buildup of EZ/ZE isomers is required before the lumirubins are formed, it is now apparent that the latter result by a reaction of the former. As the time of irradiation is increased, the isosbestic point is no longer maintained, indicating that the EZ/ZE isomers have reached an equilibrium concentration, but another isosbestic point appears at shorter wavelength, indicating that the lumirubins continue to be formed. The increased distortion of the loss peak at 430 nm for

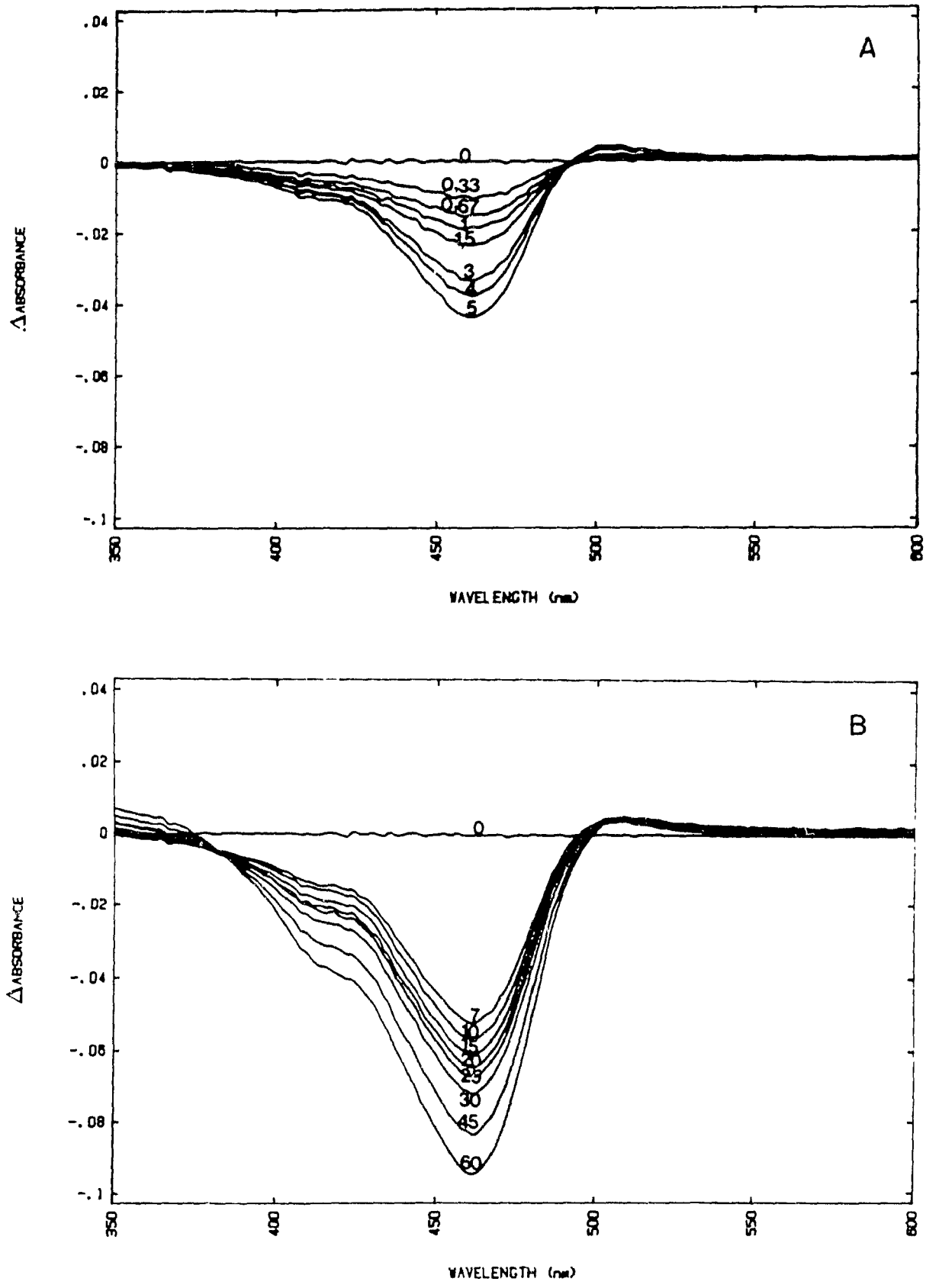

Figure 2.4 A/B Absorbance difference spectra for a solution of bilirubin in DMSO for various irradiation times. (The numbers refer to the irradiation times, in minutes.)

DMSO as compared to that with $CHCl_3$ is probably due to an increased concentration of this photoproduct relative to that of the EZ/ZE isomers. Ostrow and co-workers have shown that in DMSO lumirubins can be produced in sufficient quantities to be isolated (9).

The development of a double-humped loss peak on irradiation of aqueous solutions of bilirubin containing human serum albumin (HSA) has also been attributed, by Davies and Keohane (26), to the presence of a second synthesis peak. To verify that the double-humped loss peaks in the AD spectra result from a superimposed gain peak, experiments were made using HPLC methods described previously by McCarthy, et al (17). Chromatograms of the DMSO solution of bilirubin after irradiation confirmed the presence of more than one photoproduct In fact, McDonagh, et al. (21) have also reported that irradiation of bilirubin in a $CHCl_3/Et_3N$ system results in a second photoproduct that absorbs at 434 nm. This is consistent with the hypothesis that lumirubins can be formed in solvents which interfere strongly with the intramolecular hydrogen bonding of bilirubin

Thus, the mechanism for the formation of photoproducts in DMSO is similar to that in $CHCl_3$ but appears to differ only in the relative quantum yields for the formation of the various photoproducts. It seems that solvents like DMSO and Et_3N permit the EZ/ZE isomers to attain certain orientations which favour internal cyclization Similar behaviour is seen during photolysis of bilirubin in aqueous solutions containing HSA As will be shown later, the measureable amounts of photoisomers of bilirubin are detected in aqueous solution only when HSA is added. The formation of lumirubins from EZ/ZE isomers is consistent with the mechanism proposed previously by Onishi, et al. (10), rather than that of McDonagh, et al. (21), who propose that the cyclization process takes place directly from the bilirubin, and not from the EZ/ZE isomers.

2.3.5 PHOTOLYSIS IN DIMETHYL SULFOXIDE IN THE PRESENCE OF OXYGEN

The photolysis behaviour of bilirubin in oxygenated DMSO is very similar to that in oxygenated $CHCl_3$. The addition of oxygen does not affect the photodestruction peak at 460 nm, as seen in Fig. 2.5A. However, as was the case with $CHCl_3$, the presence of oxygen increases the formation of photoproducts with both the lumirubins and the EZ/ZE isomers increasing by a factor of ca. 2 (Fig. 2.5B). Thus, as described in Section 2.3.3, the presence of oxygen must inhibit a secondary reaction which would otherwise decrease the amount of photoproducts formed.

2.3.6 PHOTOLYSIS IN DIMETHYL SULFOXIDE/WATER

To obtain a better understanding of the effect of changes in the polarity, a solution of bilirubin in DMSO/aqueous buffer (90.10) was irradiated. The AD spectra obtained during the first 5 minutes of irradiation are analogous to those for the $CHCl_3$ system in that there is only one loss peak with two isosbestic points (Fig. 2.6). As for photolysis in $CHCl_3$, with increasing time the isosbestic point at 490 nm disappears. Simultaneously, a shoulder begins to form on the loss peak, indicating that the production of EZ/ZE isomers has reached a photostationary state but lumirubins are still being formed.

Thus, the relative importance of the different pathways for bilirubin photoisomerization, photodegradation and photooxidation is highly dependent on the environment of the bilirubin molecule. The AD spectra resulting from irradiation of bilirubin in DMSO are considerably different from those for bilirubin in $CHCl_3$. Yet, if 10% aqueous phase is added to the DMSO, the spectra become very similar. The addition of water to DMSO apparently causes

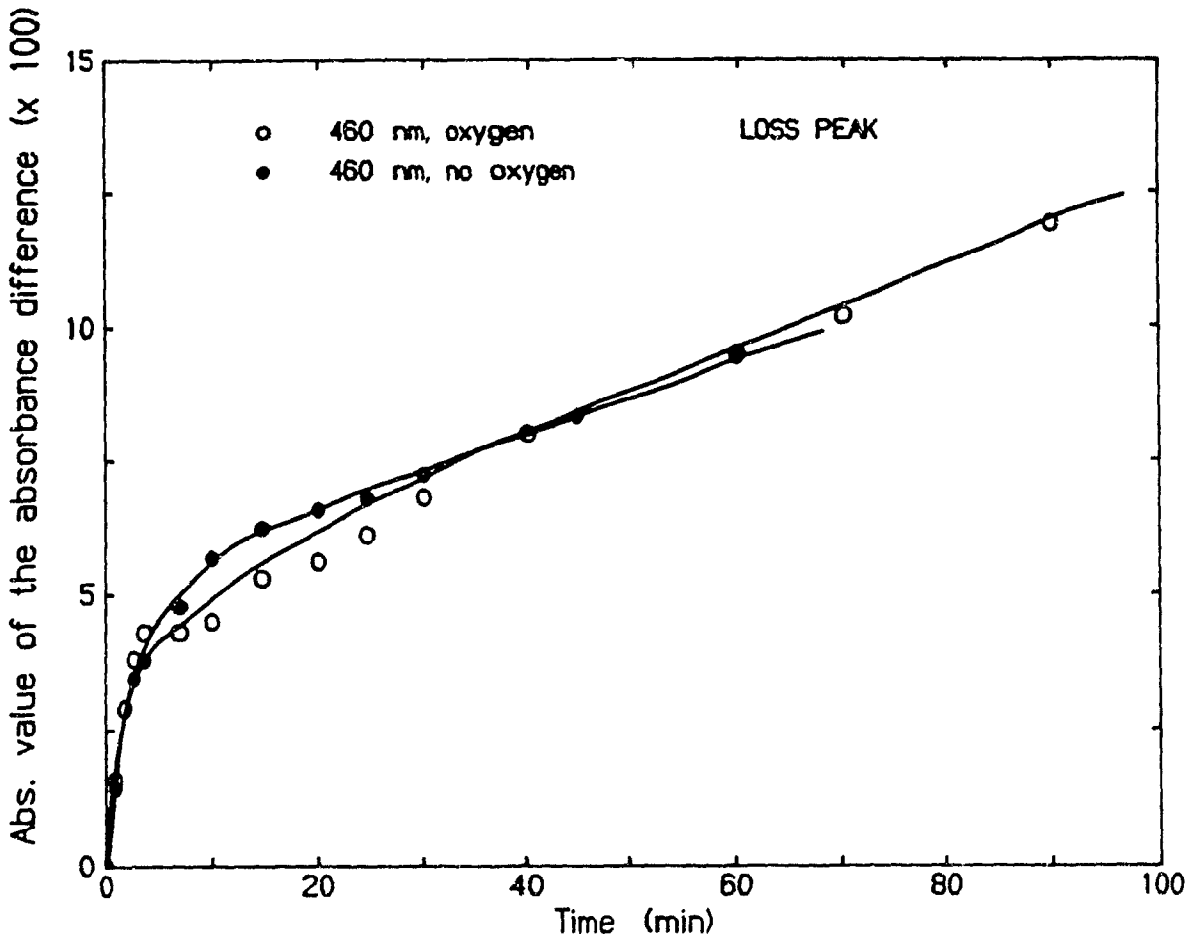

Figure 2.5 A The absolute value of the absorbance difference (x 100) of loss peak (460 nm) for a photolyzed solution of bilirubin in DMSO with and without oxygen, as a function of irradiation time.

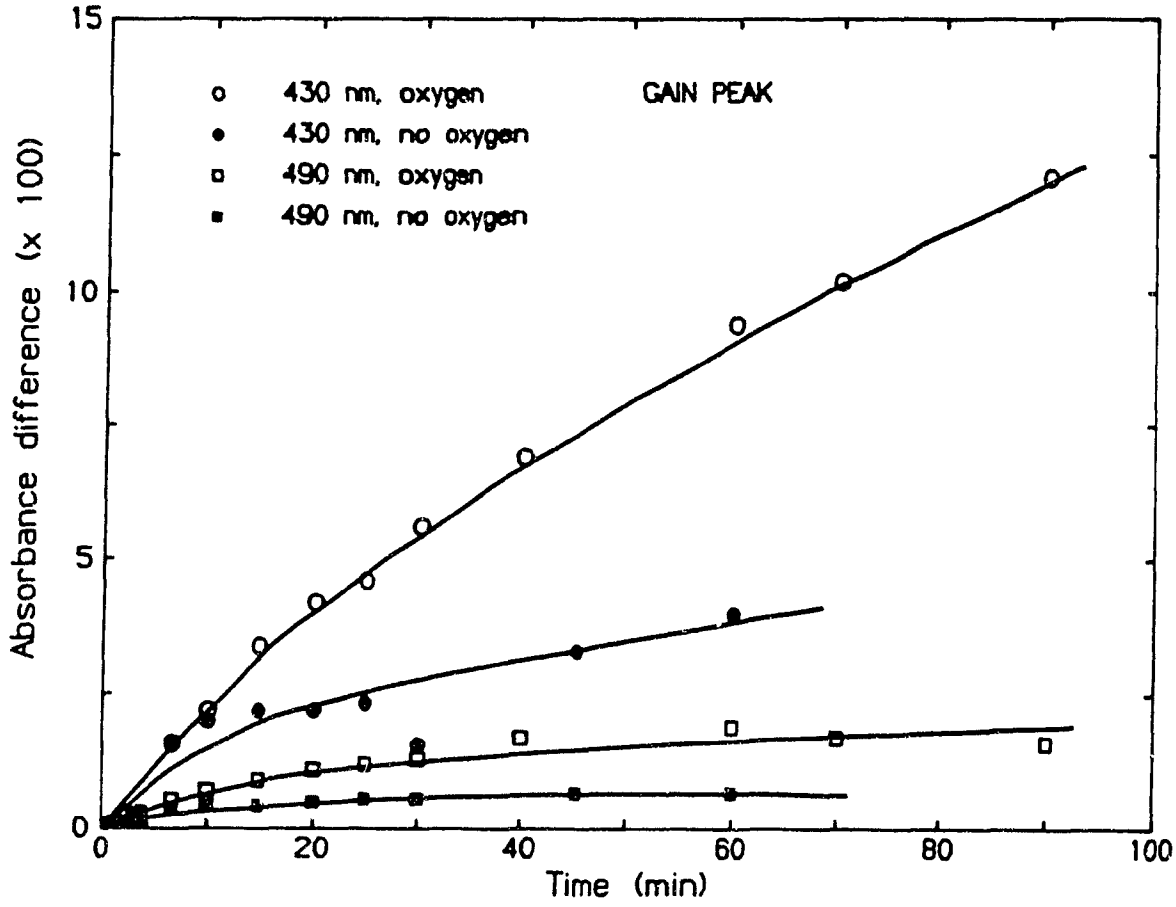

Figure 2.5 b The absorbance difference (x 100) of the synthesis peaks (490 nm, 430 nm) for a photolyzed solution of bilirubin in DMSO with and without oxygen, as a function of irradiation time.

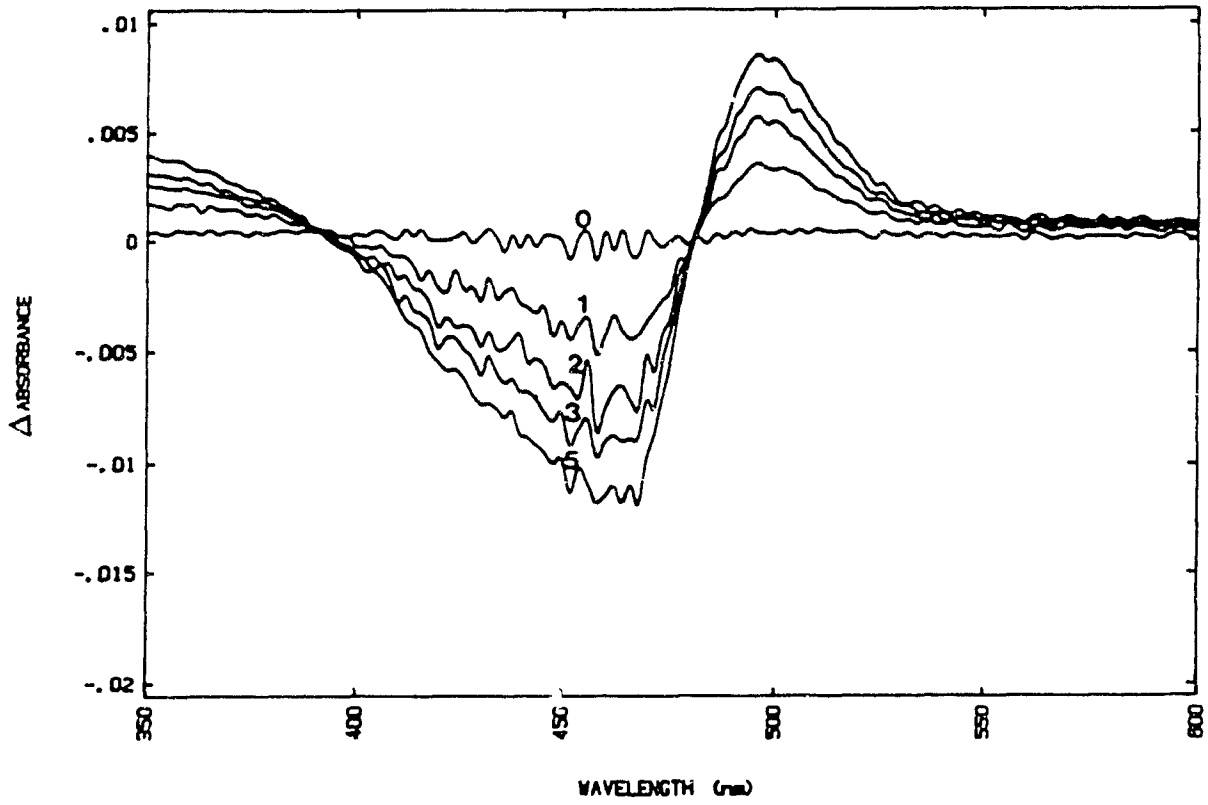

Figure 2.6 Absorbance difference spectra for a solution of bilirubin in DMSO/buffer (90:10) for various irradiation times. (The numbers refer to the irradiation times, in minutes.)

bilirubin to adopt a tightly closed, hydrophobic structure, similar to that in $CHCl_3$, rather than the more open structure thought to be present in DMSO. The resulting photoprocess in DMSO/buffer becomes more similar to that in the $CHCl_3$/EtOH system than that in pure DMSO. In fact, not only are the shapes of the AD spectra very similar, but the absorbance difference is 0.011 in the $CHCl_3$/EtOH system and 0.010 in the DMSO/H_2O.

2.3.7 PHOTOLYSIS OF AQUEOUS SOLUTIONS CONTAINING ALBUMIN

The dramatic changes resulting from the addition of water to DMSO suggested that photolysis of bilirubin in aqueous medium should differ considerably from photolysis in organic solvents. Indeed, as reported previously (27), attempts to identify photoproducts after irradiation of bilirubin in aqueous phosphate buffer (pH = 7.8) were unsuccessful. However, as will be discussed later, small amounts of photoproducts were seen in aqueous solutions at pH = 11.0.

Photoproduct formation is readily appparent after the irradiation of the aqueous bilirubin solution containing small amounts of HSA. When a solution of HSA with a molar albumin concentration seven times that of the bilirubin is irradiated, the resulting AD spectra (Fig. 2.7) show three peaks: two loss peaks at 408 nm and 448 nm, and one large gain peak at 492 nm. It is also interesting to note that the absorbance difference at 350 nm is essentially zero, giving no evidence of photooxidation products.

In contrast, replacing HSA with BSA results in only one gain peak at ca. 508 nm and a corresponding loss peak at ca 460 nm (Fig. 2.8).

The AD spectra for the photolysis of bilirubin in aqueous solution containing HSA show characteristics similar to those for irradiated DMSO

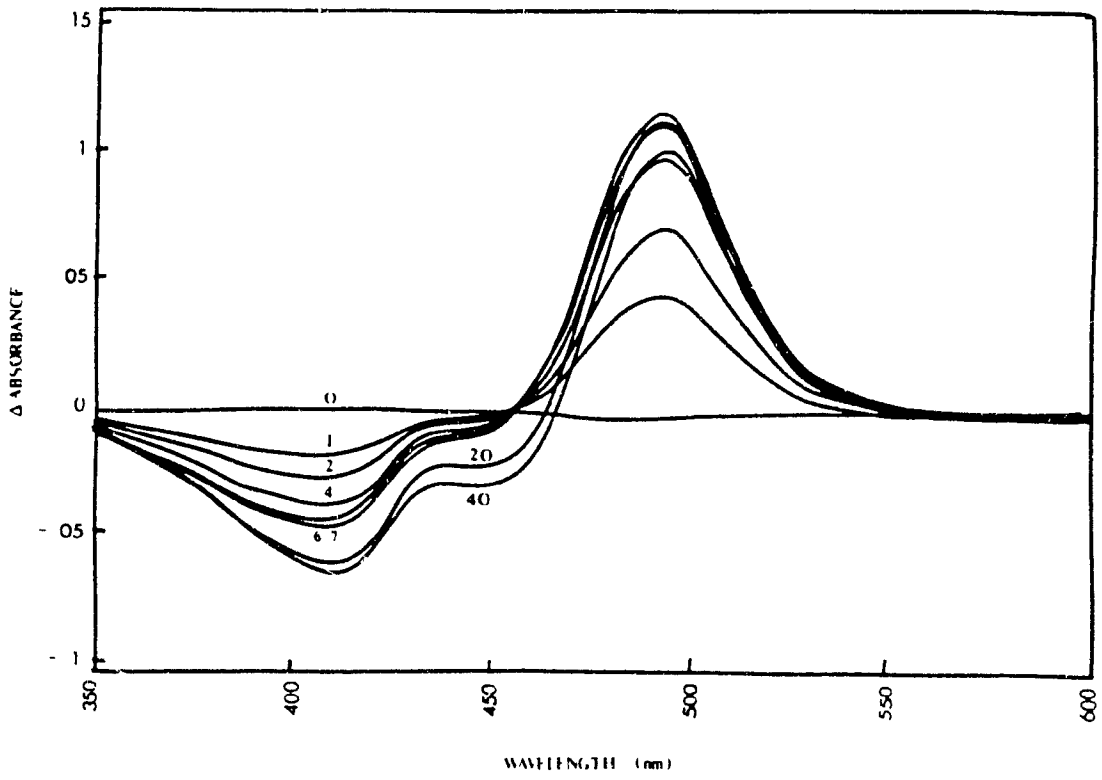

Figure 2.7 Absorbance difference spectra for an aqueous buffer solution of bilirubin and human serum albumin for various irradiation times. (The numbers refer to irradiation times, in minutes.)

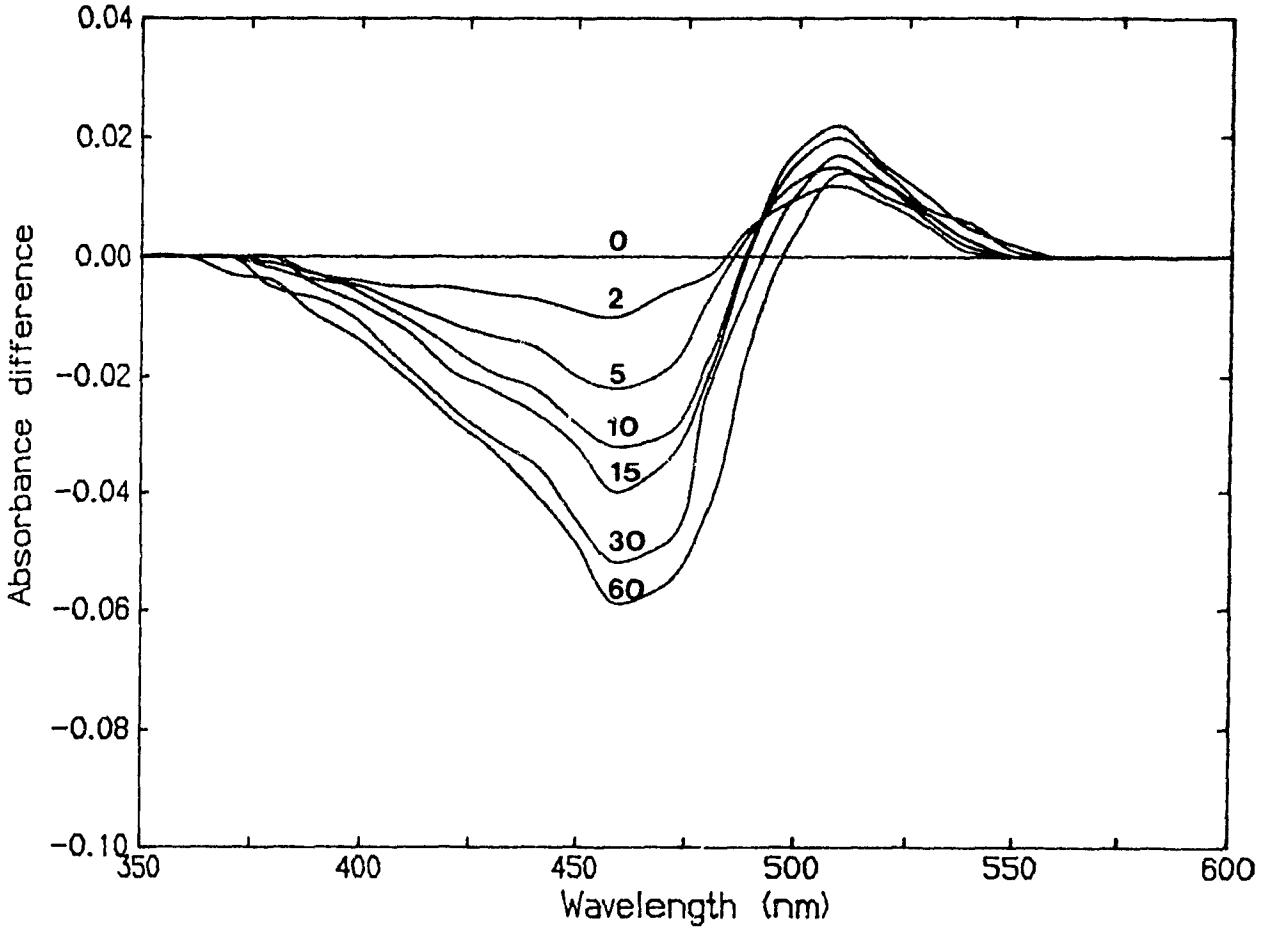

Figure 2.8 Absorbance difference spectra for an aqueous buffer solution of bilirubin and bovine serum albumin for various irradiation times. (The numbers refer to irradiation times, in minutes.)

solutions of bilirubin. That is, at short irradiation times there is only one isosbestic point, at 460 nm, indicating that initially there is only one product being formed, the EZ/ZE isomers. As the time of irradiation is increased, this isosbestic point disappears, indicating a disruption in the equilibrium, BR <----> EZ/ZE isomers, while a second isosbestic point (360 nm) appears, indicating that a second product is being formed. Thus, the mechanism for photoproduct formation in HSA appears to be the same as in DMSO.

When the height of the gain peak (492 nm) is plotted as a function of the time of irradiation in the presence of HSA a sharp increase is seen initially, followed by the appearance of a photostationary state (Fig. 2.9). Similar behaviour is seen, but with an increase in initial slope by a factor of 3, so that the photostationary state is reached more rapidly, when the intensity of the the irradiating light is increased approximately threefold. Thus, a direct relationship is seen between the intensity of the irradiating light and the initial rate of formation of photoproduct. The occurrence of a photostationary state indicates that the formation of EZ/ZE isomers from bilirubin is reversible. Furthermore, since the AD spectrum of irradiated bilirubin remains unchanged when it is left in the dark, the reverse process must also be light induced, as proposed by McDonagh, et al. (21).

As mentioned above, it has been suggested that the double loss peak is in fact due to the development of a synthesis peak (26). Evidence of this can be seen in the HPLC chromatogram (Fig. 2.10) which clearly shows two photoproducts of differing polarity appearing before the bilirubin peak.

Additional evidence is obtained from a plot of the absorbance difference peak height at 492, 408, and 448 nm, after irradiating for 7.0 minutes, as a function of log {[HSA]/[BR]}, at a fixed bilirubin concentration of 1.71×10^{-5} M (Fig. 2.11). The loss peak at 408 nm shows the same trend as the gain peak at 492

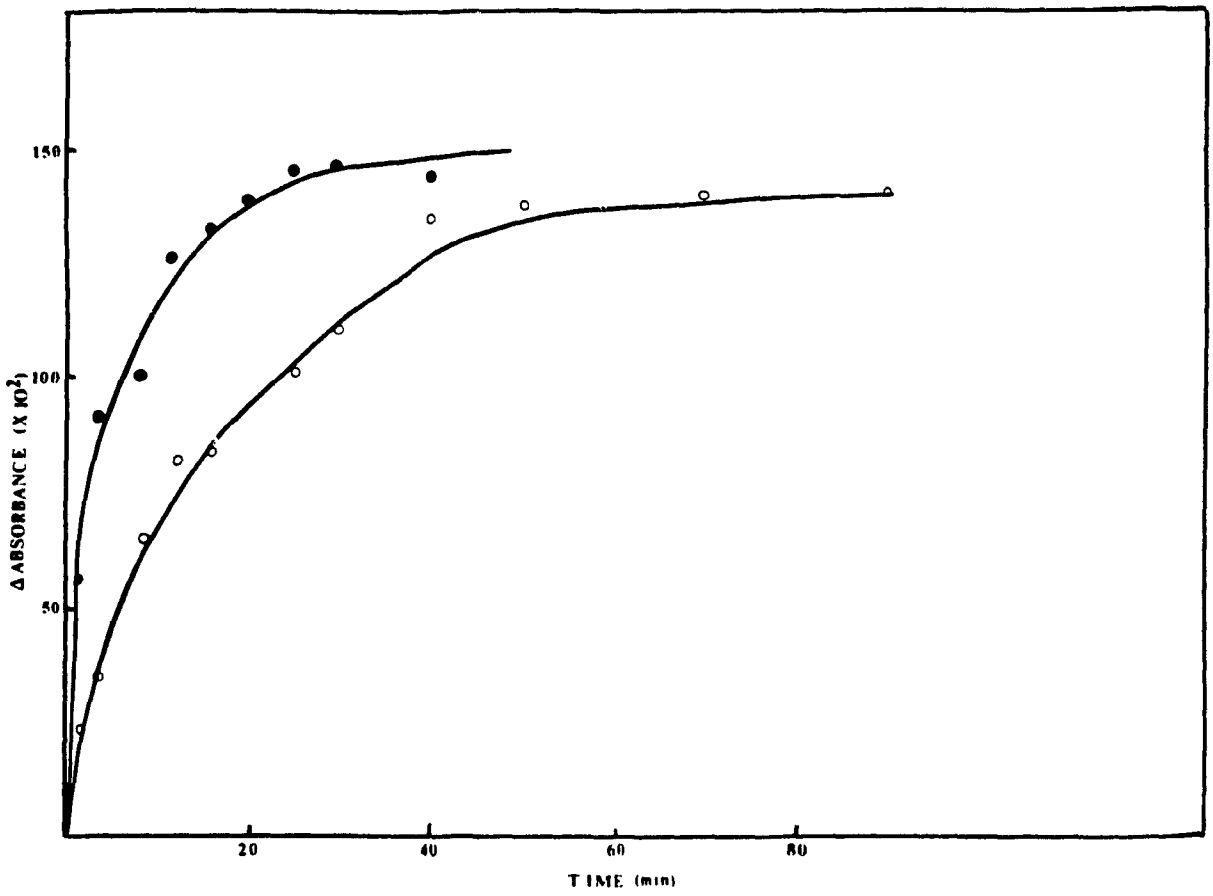

Figure 2.9 The absolute values of the absorbance difference of the gain peak (490 nm) for a photolyzed aqueous buffer solution of bilirubin and human serum albumin at different light intensities: ●, high intensity; ○, low intensity.

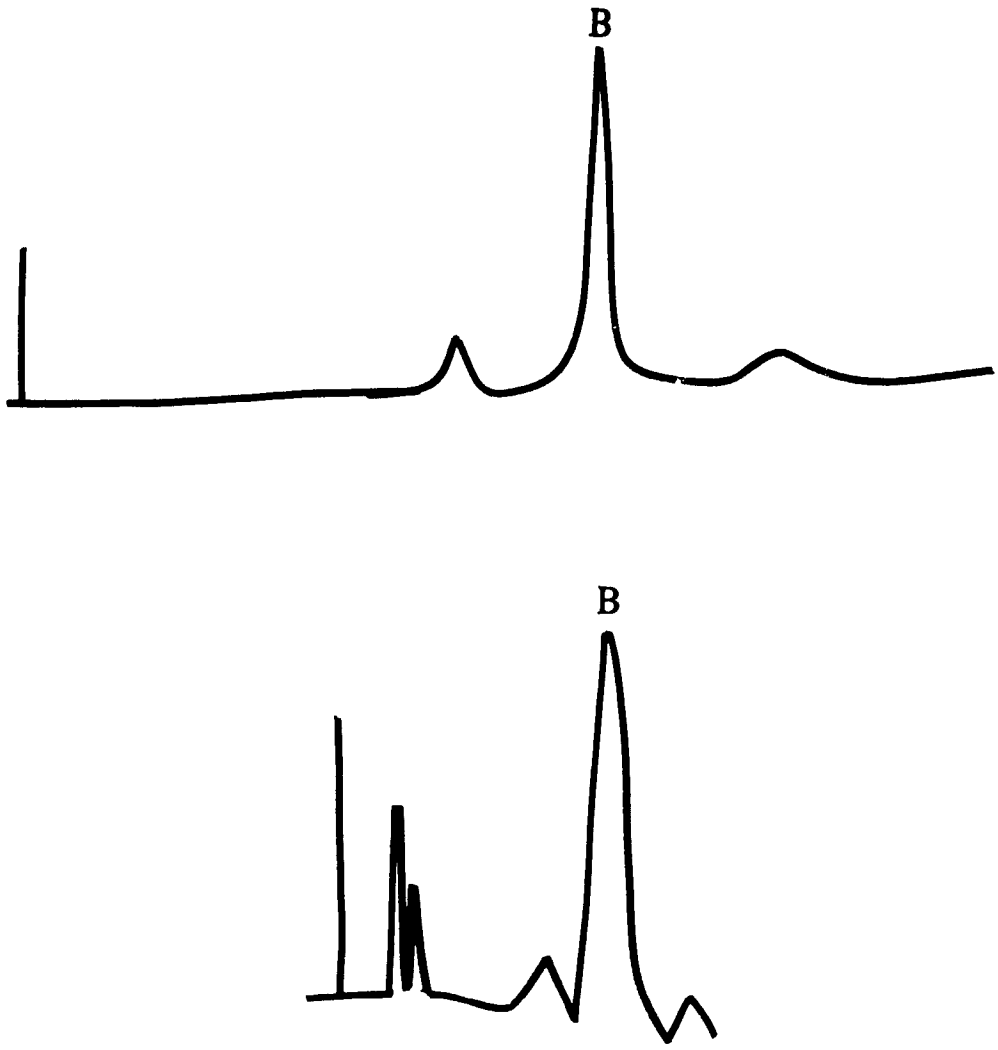

Figure 2.10 HPLC chromatograms of an unirradiated aqueous bilirubin solution (top) and an irradiated aqueous bilirubin/human serum albumin solution (bottom) showing two photoproducts. The first photoproduct peak corresponds to the more polar (lumirubins) photoisomers while the second photoproduct peak corresponds to the EZ/ZE isomers. (B = Bilirubin IX-α peak; the peaks on either side of this peak are the III-α and the XIII-α isomers).

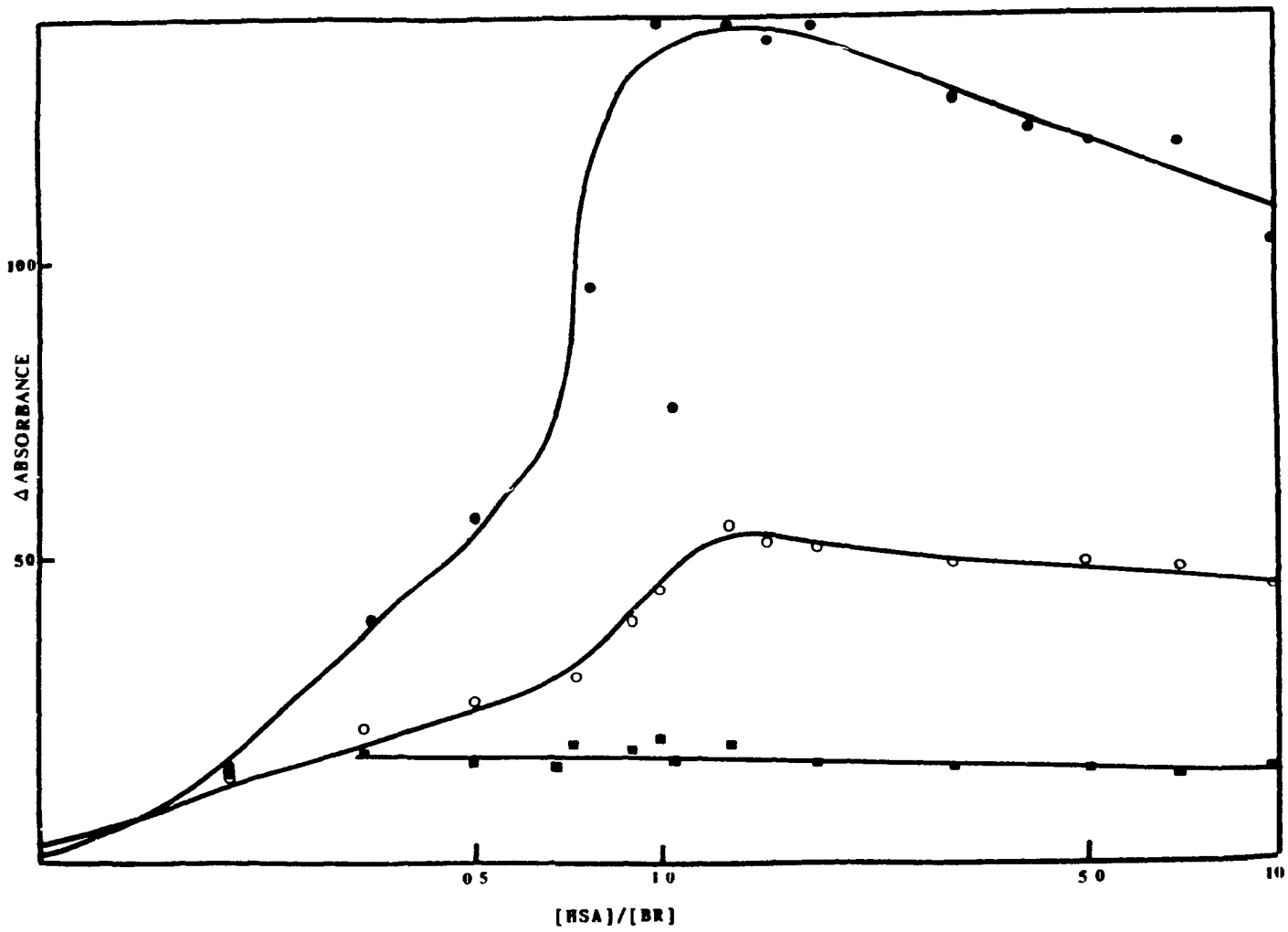

Figure 2.11 The absolute values of the absorbance difference, multiplied by 100, of the gain peak (490 nm) and the two loss peaks (448 nm and 408 nm) for a photolyzed aqueous buffer solution of bilirubin and human serum albumin as a function of Log [HSA]/[BR], at 7.0 min of irradiation: ●, 492 nm; ○, 408 nm; ■, 448 nm.

nm, while the loss peak at 448 nm behaves differently. (The values have been corrected for the absorbance of HSA at the illuminating wavelength). The magnitude of the loss peak at 448 nm is, within experimental error, independent of [HSA]/[BR] whereas the other two peaks go through a maximum at a ratio of ca. 1.5/1.0 and then decrease to a plateau. The limit appears to be related to the binding of the bilirubin and/or its photoproducts to the HSA. As the HSA concentration increases, more photoproduct is produced until a [HSA]/[BR] of 1.5 is reached. At higher concentrations of HSA the amount of photoproduct decreases, perhaps because the binding of bilirubin decreases due to protein aggregation. Such a decrease in binding affinity at high concentrations of HSA has been reported previously (28). When both HSA and bilirubin concentrations are decreased by a factor of 10, a plateau without a "hump" results (Fig. 2.12).

The apparent requirement of albumin in aqueous solution of bilirubin to bring about the production of a measurable amount of photobilirubin suggests either that: (a) photoisomerization requires that the albumin bind the bilirubin to facilitate isomerization, or (b) unbound bilirubin can undergo photoisomerization but rapid reversion occurs in the absence of albumin. If the latter were true it should be possible to "trap" photoproducts formed by the irradiation of an aqueous solution of bilirubin by adding albumin to bind the photoproducts immediately after irradiation.

This was attempted using the method of stopped flow analysis. The bilirubin buffer solution was irradiated for a given period of time, mixed with a solution of HSA and the absorbance at the desired wavelength was recorded immediately. A small amount of photoproduct was formed, as evidenced by the increase in absorbance at 490 nm with time of irradiation (Fig. 2.13). As expected, a concomitant decrease in absorbance was noted at 410 nm while, within experimental error, there was no change in absorbance at 460 nm, which

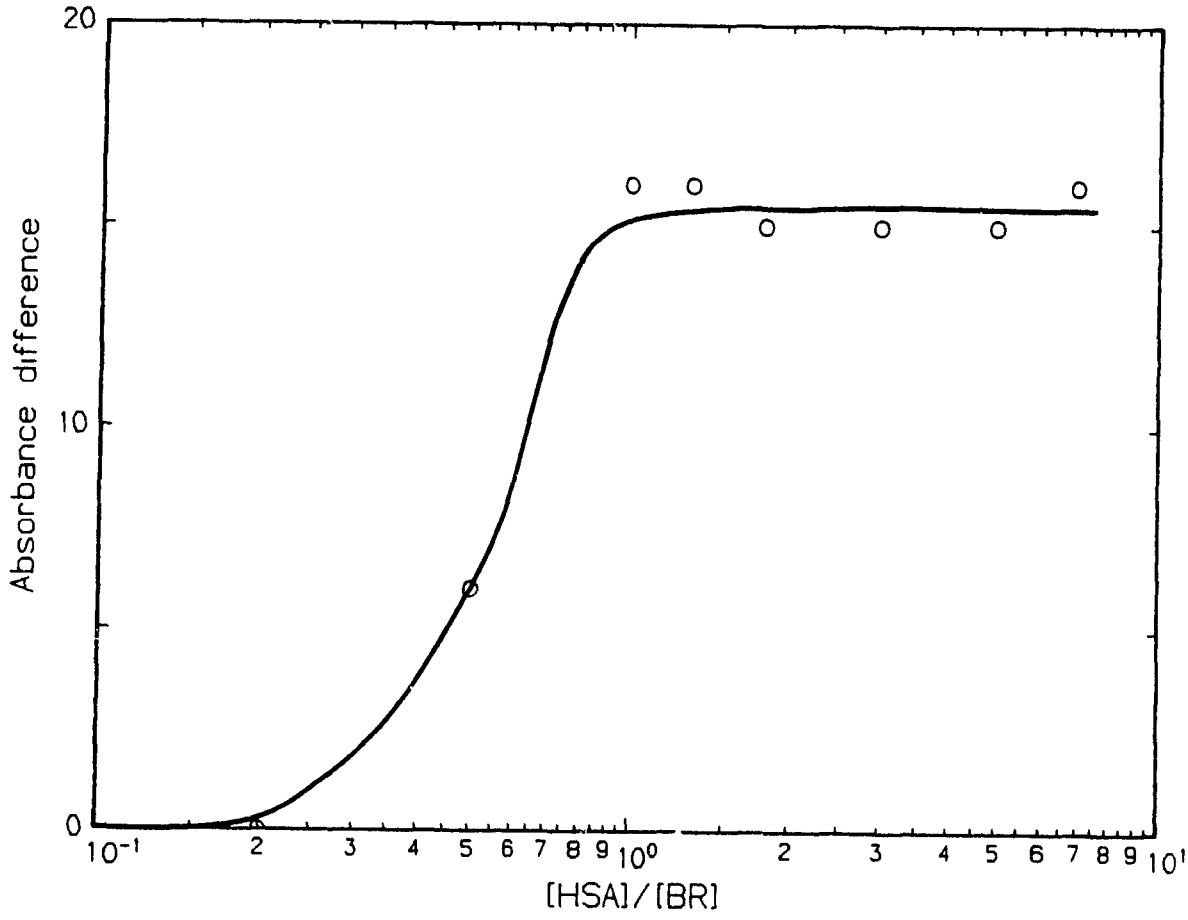

Figure 2.12 The absorbance difference of the gain peak (490 nm) for a photolyzed aqueous buffer solution of bilirubin and human serum albumin as a function of Log [HSA]/[BR], with the concentrations of each species equal to 1/10 that in Figure 2.11, at 7.0 min of irradiation.

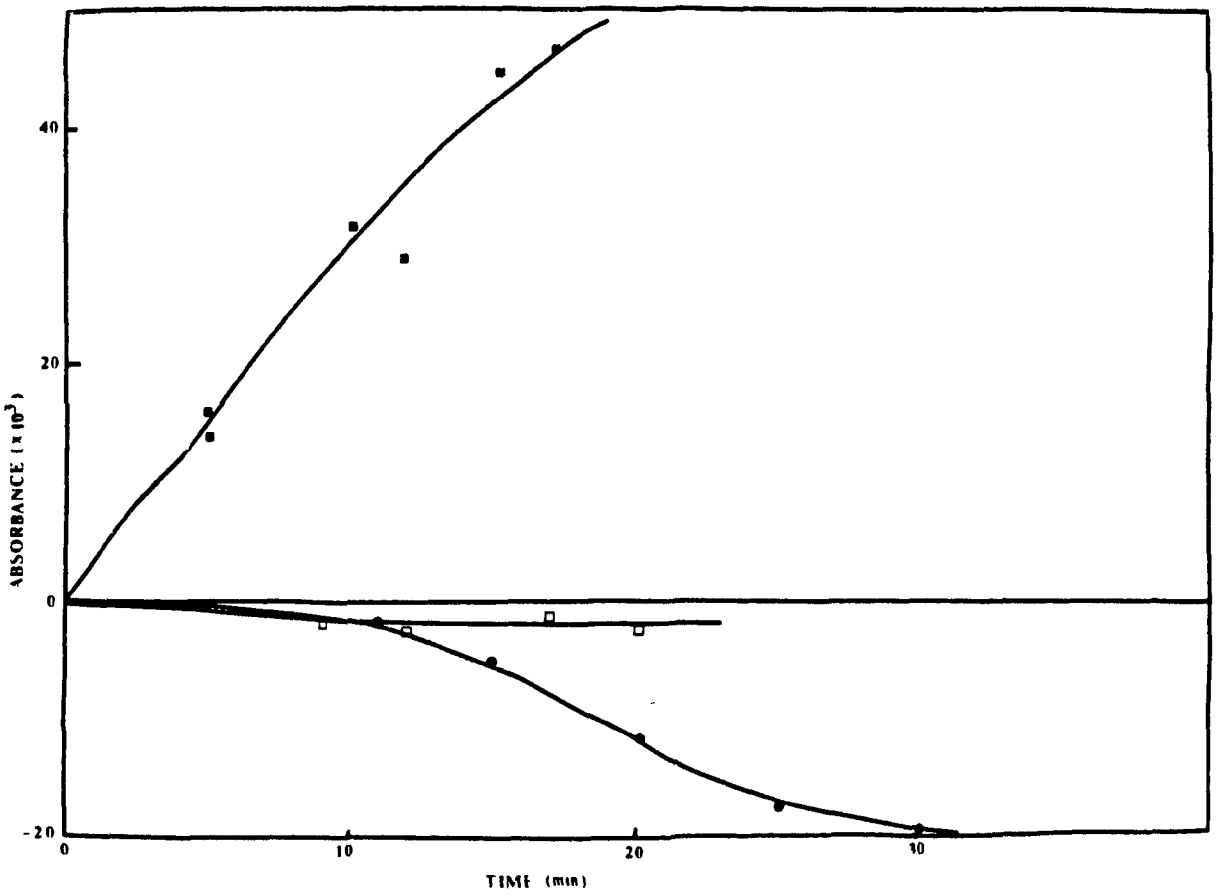

Figure 2.13 The absolute values of the absorbance difference at three separate wavelengths (490 nm, 460 nm, and 410 nm) as a function of the irradiation time for a photolyzed aqueous solution of bilirubin after mixing with human serum albumin: ■, 490 nm; ❑, 460 nm; ●, 410 nm.

corresponds to an isosbestic point in the AD spectrum of an irradiated soltuion of bilirubin/HSA. In contrast, no photoproduct formation was detected in an analogous experiment when an irradiated aqueous solution of bilirubin was mixed with buffer, indicating that the presence of HSA is indeed necessary to protect the photoproduct. By comparison, there was approximately a threefold increase in photoproduct formation when the irradiated solution contained both HSA and bilirubin. Thus, it would appear that it is possible for bilirubin to isomerize to form the EZ/ZE isomers in aqueous solution, but that under these conditions the reversion is too fast to permit their detection. However, when albumin is added to the solution immediately following the irradiation, the EZ/ZE isomers are bound to the albumin before they revert to the ZZ form.

The fact that more photoproduct is formed when albumin is present during the irradiation than when it is added just after the irradiation follows from Fig. 2.11 which shows an increase in photoproduct formation with increasing [HSA]/[BR]. Below the [HSA]/[BR] ratio of 1/1 relatively little photoproduct is formed. Since the first molecule of bilirubin binds strongly to HSA, with a binding constant of 10^6 - 10^7 (29), the fraction of free bilirubin is very small at the ratio of [HSA]/[BR] of 1/1, suggesting that it is the irradiation of **bound** bilirubin that gives rise to the increased formation of photoproducts. Lee and Gillispie (30) and Hsieh, et al. (31) have shown that the bilirubin molecule is buried deeply within the HSA. Consequently, HSA can provide a protective environment where the photoproduct is more readily formed. It has been suggested (29) that the HSA molecule maintains the bilirubin in an environment that changes the energies of the transition state leading to the formation of photoproduct. The idea that bilirubin is protected by albumin is also discussed by Rubaltelli and Jori (32). However, as indicated above, photoproducts can also be produced, albeit at lower efficiency, in the absence of albumin.

2.3.8 PHOTOLYSIS IN THE PRESENCE OF POLY-L-LYSINE

On comparing the relative yields of photoproduct in aqueous solutions containing HSA and BSA it was apparent that the different types of binding of the biliribin to these proteins leads to the differences in the photoisomerization mechanism, as suggested previously by Onishi, et al. (33). The photoprocess behaviour of bilirubin in the presence of BSA is not very different from its behaviour in organic solvent. The unique spectra obtained for HSA lead to the question of whether it is possible to bind the bilirubin to some other protein which would yield similar results. To this end, bilirubin was irradiated in aqueous solutions of poly-l-lysine at pH = 11.00 since it has been shown by CD spectroscopy (34) that at a [polypeptide]/[BR] (L/B) ratio of 10, all of the bilirubin is indeed bound. Furthermore, the polylysine is predominantly in the α-helix conformation at this pH.

Irradiation of a bilirubin-polylysine solution (DP = 1150, L/B = 10, pH = 11.00) for one hour results in an absorbance difference spectrum which shows a very large loss peak at 450 nm with a tight isosbestic point at 388 nm, and a corresponding gain peak at 312 nm (Fig. 2.14). According to the work of Bouvier, et al. (34), under these conditions it must be the bound bilirubin which is being photolyzed. The loss of bilirubin, calculated as a percentage of the total bilirubin in the original solution prior to irradiation, plotted as a function of the time of irradiation in Fig. 2.15 shows that ca. 50% of the chromophore is destroyed after one hour of photolysis. By comparison, for irradiation of the BR/HSA, BR/$CHCl_3$ or BR/DMSO systems, with an absorbance at peak maximum of the bilirubin of ca. 1.0 in the original solution, the maximum decrease in the loss peak was never more than 20% over the same time period.

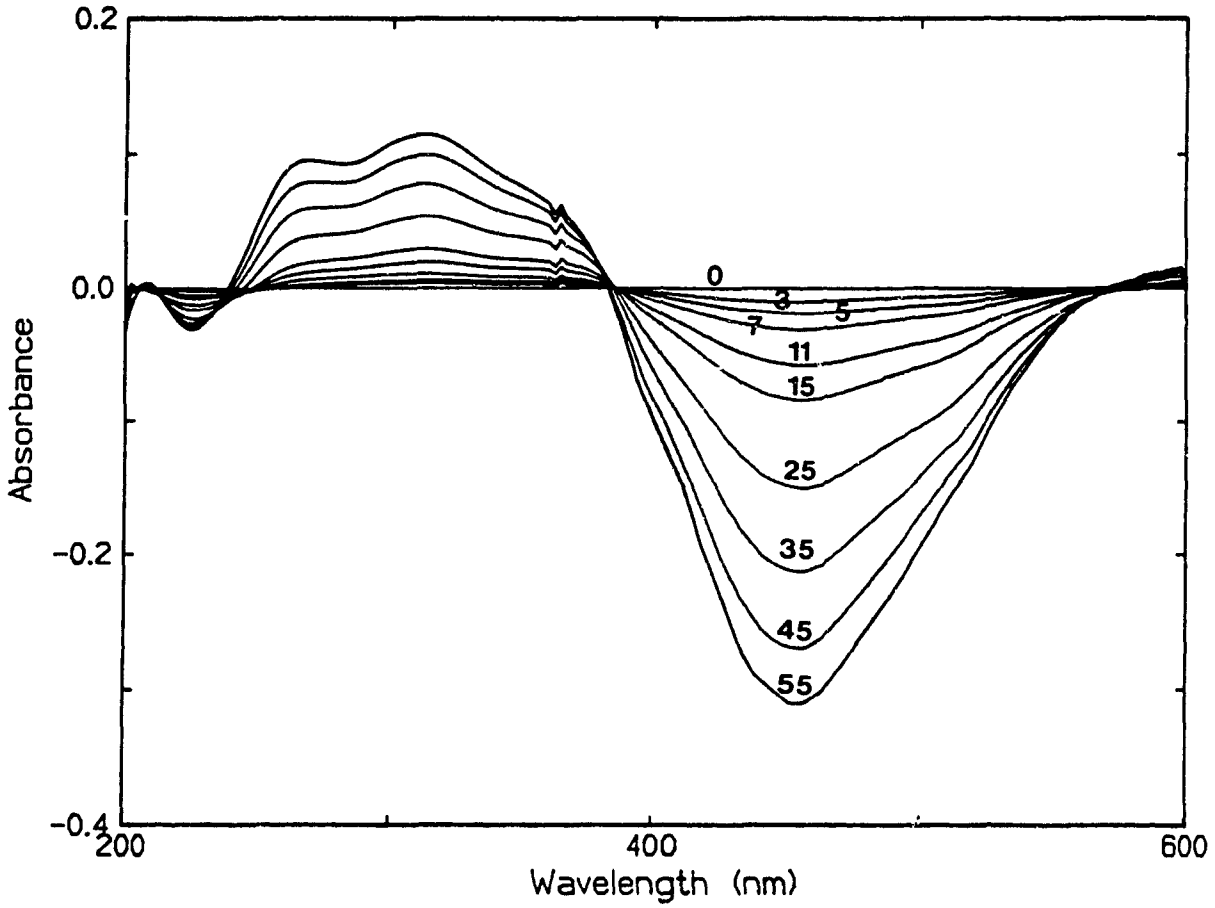

Figure 2.14 Absorbance difference spectra for a solution of bilirubin and polylysine (DP = 1150, pH = 11.0) after various times of irradiation. (The numbers refer to the irradiation times, in min.).

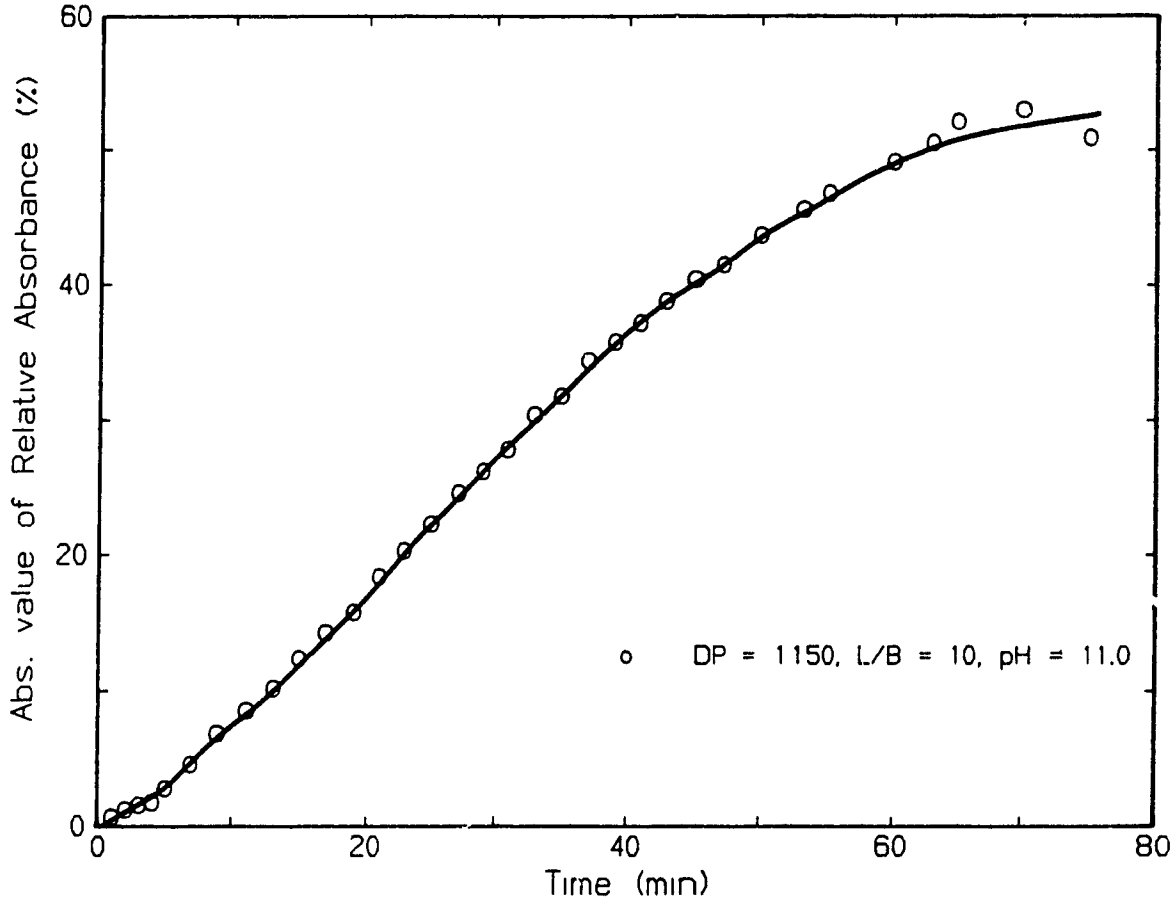

Figure 2.15 The percentage loss of bilirubin (448 nm) as a function of time of irradiation for a photolyzed solution of bilirubin and polylysine (DP = 1150, pH = 11.0, L/B = 10).

In all of the other systems studied, particularly those where the bilirubin was bound to serum albumin, there was clear evidence of the formation of photoisomers. However, such is not the case in the irradiation of the BR/PL solution. There is no evidence (Fig. 2.14) of a gain peak at either 490 nm (EZ/ZE isomers) or at 430 nm (the lumirubins). However, the isosbestic point implies that at least one product is being formed directly from the bilirubin, absorbing around 300 nm. In fact, the shape of the gain peak suggests that there could be more than one product. Based on evidence presented below, it is likely that these products are closely related to photooxidation products. (Although the solutions were degassed prior to use, completely anaerobic conditions are not certain.)

Evidence that the products are photooxidation products can be seen by a comparison of the AD spectra obtained for irradiation of bilirubin solutions in $CHCl_3$, with and without O_2, (Fig. 2.16A/B). There is a very large gain peak below 350 nm in the irradiated solution containing O_2, not seen in the absence of O_2. In addition, Lightner, et al. (35) showed that substituted pyrromethenones, bilirubin-model compounds, are photooxidized to give products similar to those obtained from bilirubin, absorbing at 315 nm. Thus, it would appear that the products formed upon irradiation of a bilirubin-polylysine solution are photooxidation products, not photoisomers.

Since photoisomerization and photooxidation are the two predominant mechanisms for the formation of new products on irradiation of a bilirubin solution, the question arises as to why in this case the photooxidation predominates. It has been shown by Sloper and Truscott (36) that, in most of the previously studied conditions, photoisomerization precedes photooxidation since $\phi_{ISOM} >> \phi_{OX}$ (where ϕ is the quantum yield). However, they also found that the ratio, ϕ_{ISOM}/ϕ_{OX} is solvent dependent, and can be as high as 130 for aqueous HSA and as low as 20 for $CHCl_3$. Based on the data obtained for bilirubin it appears

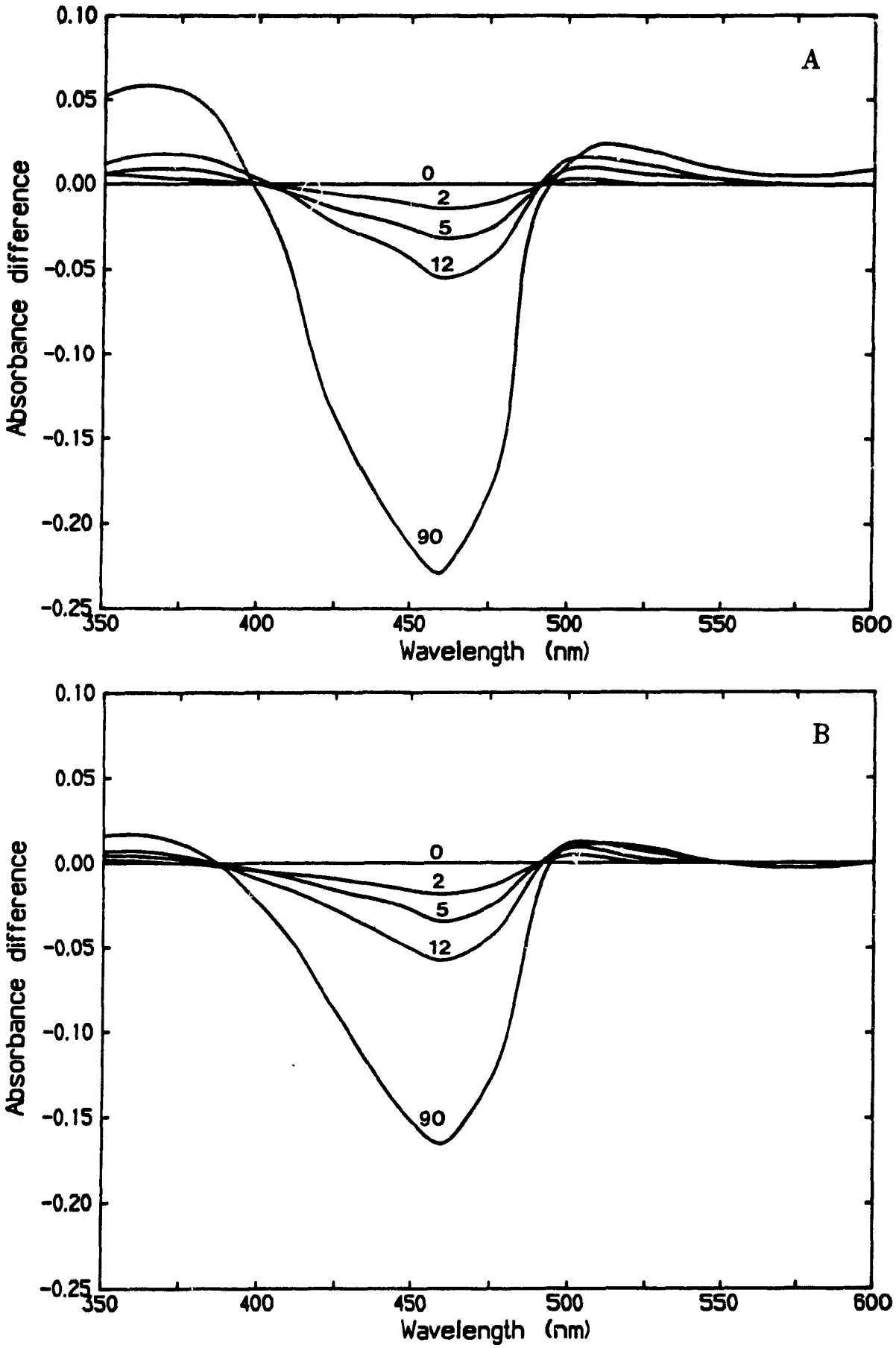

Figure 2.16A/B Absorbance difference spectra for the irradiation of a solution of bilirubin in $CHCl_3$/EtOH with (A) and without (B) oxygen, showing a large gain at ca. 350 nm in the oxygenated sample.

that in aqueous polylysine at pH = 11.00, $\phi_{ISOM} < \phi_{OX}$, and thus photooxidation precedes photoisomerization.

Data from Lightner, et al. (36) suggest that the photooxidation proceeds via an electron transfer radical mechanism, according to equation [2.1]:

$$BR\ (S_1 \text{ or } T_1) + {}^3O_2 \text{ <---> } [BR^{+\cdot}\ O_2^{-\cdot}] \text{--->BR photox. pdts} \qquad [2.1]$$

The photoisomerization reaction is thought to proceed via an excited singlet state with twisting about the C=C bond:

$$ZZ\ S_1 \text{--->} EZ/ZE\ S_1 \text{--->} EZ/ZE\ S_0 \qquad [2.2]$$

The binding of bilirubin to the polypeptide causes [2.1] to occur in preference to [2.2], either by increasing the triplet yield (assuming [2.1] goes via T_1) or by stabilizing the radical complex. There is some indication that bilirubin molecules stack when bound to poly-l-lysine (34) and it is possible that it is this stacking which leads to the stabilization. Such stacking may lead to a self-sensitized photooxidation, a mechanism which has been postulated previously (36). In this way the initially excited bilirubin reacts with a ground state bilirubin, and by electron transfer, leads to $[BR^{+\cdot} + BR^{-\cdot}]$ which in turn can lead to photooxidation products.

To verify that it is in fact the bound bilirubin involved in the photoreaction, the experiment was repeated with polylysine DP = 17, which does not bind bilirubin (34). In this case (Fig. 2.17) photodestruction of bilirubin as indicated by the loss peak at 450 nm is ca. 20%, which is comparable to that obtained previously in organic media. The gain peak below ca. 350 nm, indicating photooxidation products, is very small (ca. 5%). In addition, there is

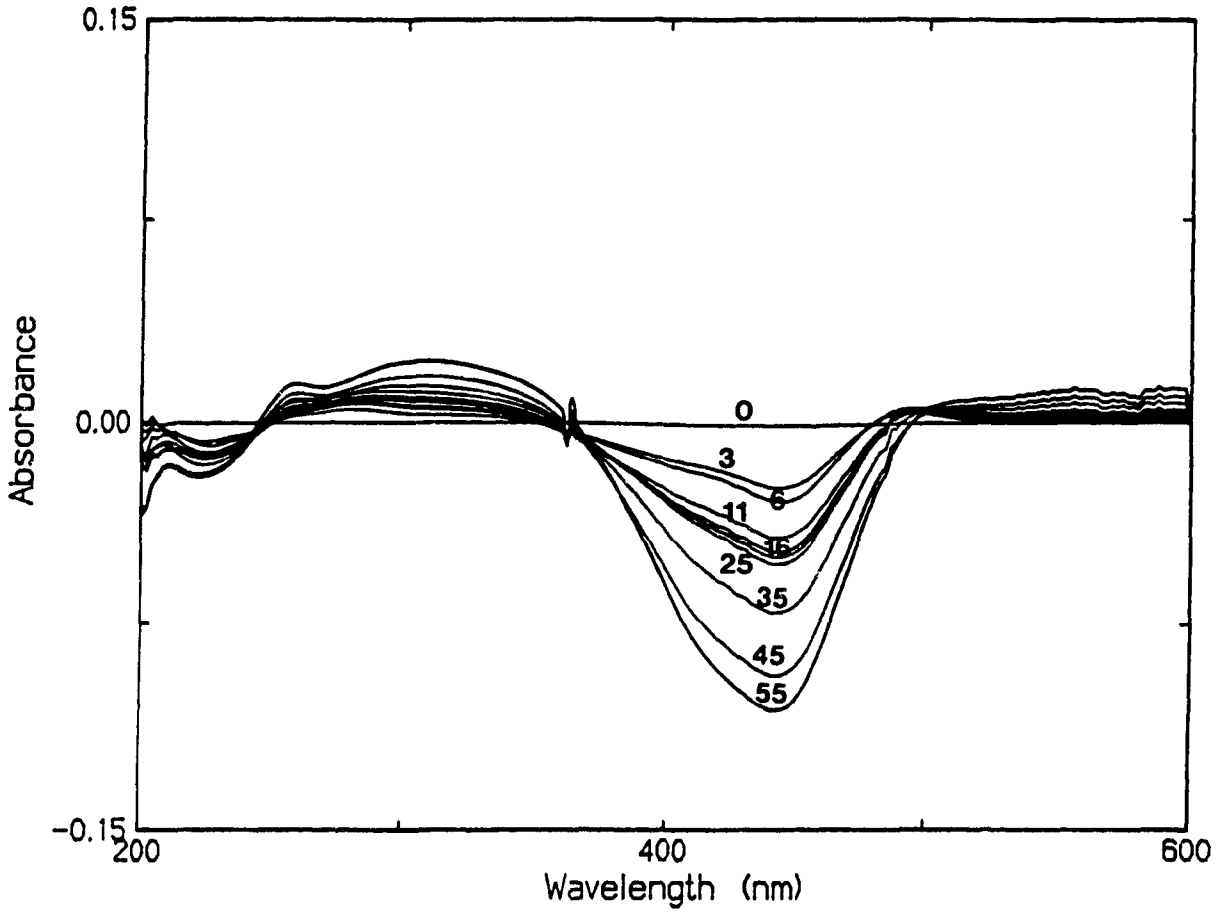

Figure 2.17 Absorbance difference spectra for a solution of bilirubin and polylysine (DP = 17, pH = 11.0) after various times of irradiation. (The numbers refer to the irradiation times, in min.).

some indication that very small amounts of the EZ/ZE isomers are produced, as evidenced by the small gain peak at 490 nm. These data indicate that in the presence of poly-l-lysine with a low degree of polymerization, (which does bind bilirubin well) the photoisomerization reaction predominates over the photooxidation reaction, as is the case in organic media. When the polylysine is completely removed from the solution (Fig. 2.18) the destruction of bilirubin remains about 20% over the period of irradiation and the gain in the lower wavelength end remains very low, ca. 1.5%. Thus, when bilirubin is not bound to polylysine the mechanism for its relaxation after irradiation is similar to that in organic solvent or in the presence of serum albumins, i.e., $\phi_{ISOM} > \phi_{OX}$.

Lowering the pH from 11.0 to 7.9 for the original BR/PL (DP = 1150) solution yields similar results for the destruction of bilirubin, i.e., the loss remains at about 50%; however the shape of the gain peak is different (Fig. 2.19). The change in pH directly affects the polylysine (34), increasing the percentage of β-sheet relative to the α-helix. The change in conformation of the polylysine does not change the destruction of the bilirubin, but it does affect the relative amounts of the different photooxidation products formed, as evidenced by the different shape in the low wavelength area of the AD spectra.

Bilirubin thus binds to polylysine very differently than it does to HSA or BSA. In the albumins the binding is such that photoisomerization is readily achieved. In the case of HSA it was shown that the photoisomerization involves protein bound bilirubin and that prolonged irradiation leads to other photoproducts. This type of binding appears to be unique to the BR/HSA system. Likwise, the binding of bilirubin to polylysine is unique in that it is the only system studied so far where photoisomerization is not detected. Rather, photooxidation is the preferred mechanism of relaxation for the excited bilirubin molecule.

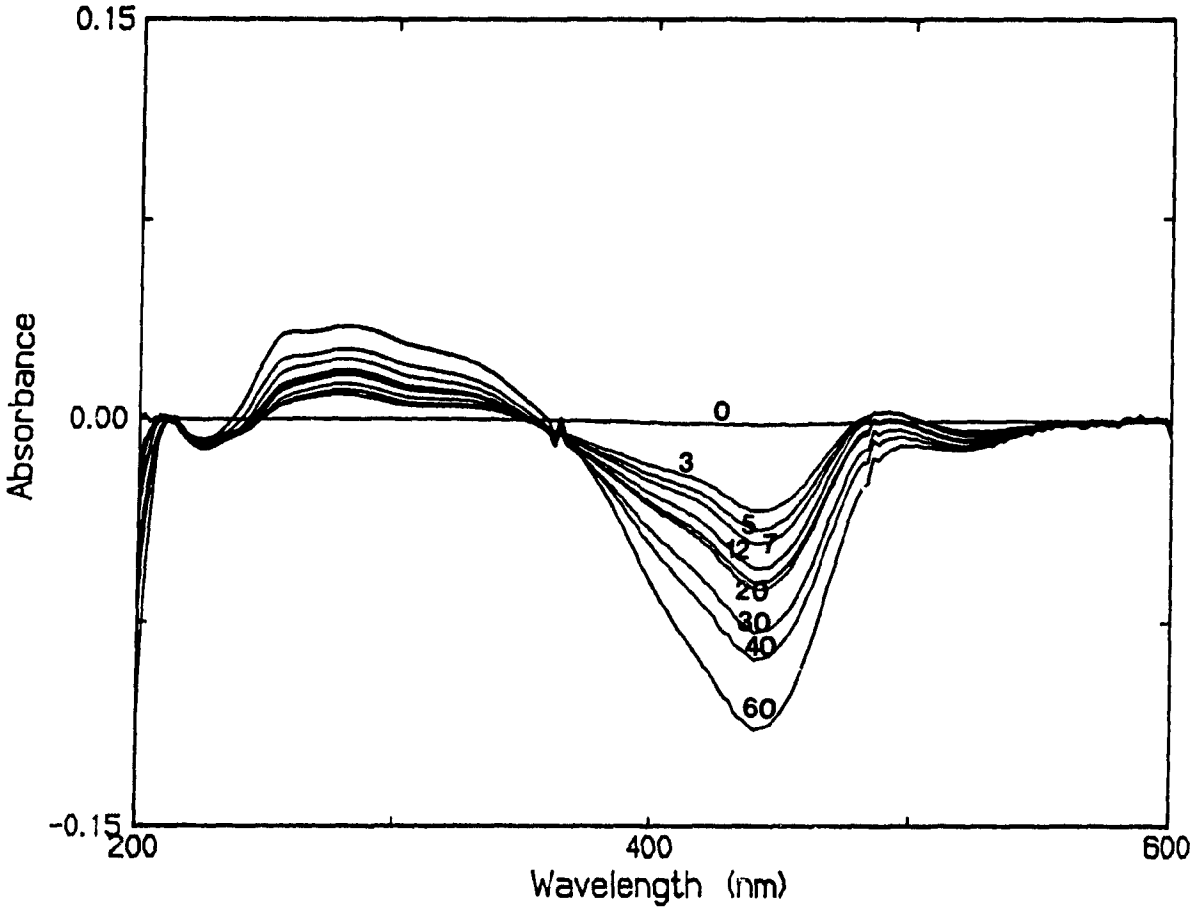

Figure 2.18 Absorbance difference spectra for a solution of bilirubin in pure water after various times of irradiation. (The numbers refer to the irradiation times, in min.).

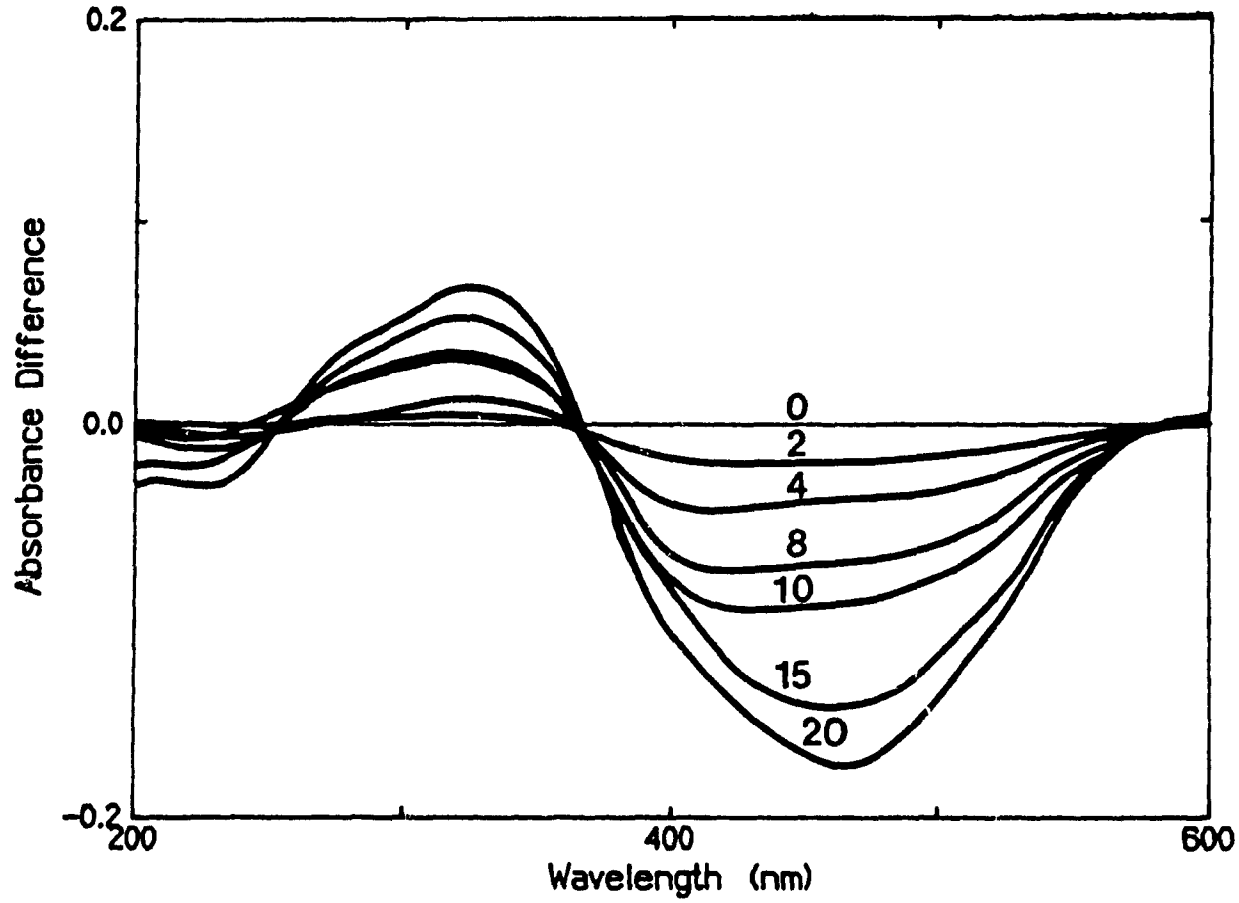

Figure 2.19 Absorbance difference spectra for a solution of bilirubin and polylysine (DP = 1150, pH = 7.9) after various times of irradiation. (The numbers refer to the irradiation times, in min.).

2.4 SUMMARY

It is apparent that the photolysis of bilirubin is a very complex process, involving various photoprocesses including photoisomerization, photodestruction and photooxidation. Although photoisomerizaion reactions predominate at short irradiation times under anaerobic conditions, the relative yields of isomers is highly dependent on the environment of the bilirubin at the time of irradiation. In solvents like water or ethanol, which interfere weakly with the intramolecular hydrogen bonds of bilirubin, formation of lumirubins from the EZ/ZE isomers is less than in stronger interfering solvents like DMSO and Et_3N. The presence of oxygen in organic media does not affect the overall destruction of bilirubin, but it does lead to higher yields of photoproducts. Although small amounts of photoisomers can be "trapped" in irradiated aqueous buffer solutions of bilirubin, substantially greater amounts are formed in the presence of HSA. Indeed, photoisomer production in the presence of HSA is similar to that in DMSO solutions. Irradiation of bilirubin bound to polylysine does not lead to photoisomerization, but, rather, it leads directly to photooxidation products, possibly due to the way the bilirubin is bound to the protein.

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CHAPTER 3

PHOTOENHANCED ADSORPTION OF BILIRUBIN BY CHOLESTYRAMINE AND SELECTED RESINS WITH PEPTIDE PENDANTS

3.1 INTRODUCTION

The adsorption of bilirubin by cholestyramine and various substituted resins has been studied previously in this laboratory (1- 4). D. S. Henning (2, 3) and M. Bouvier (4) have demonstrated the importance of a positively charged resin for the adsorption process, indicating that it is the interaction of the carboxyl groups of bilirubin and the charge on the resin which are the important parts of the binding process. This was confirmed in work done by X.X. Zhu (5, 6), using NMR techniques, to show the importance of the electrostatic interaction as well as secondary interactions, such as hydrophobic and hydrogen bonding interactions. (A detailed description of hydrophobic interactions will be presented in Chapter 4.) Substituted polyacrylamide resins containing arginine (R group = $^{+}(NH_2)_2CNH(CH_2)_3$) and lysine (R group= $^{+}(NH_3)(CH_2)_4$) have been shown to be better adsorbents for bilirubin than cholestyramine (2, 3).

In aqueous solution at physiological pH, bilirubin exists as a dianion in its ZZ form (7). This permits extensive intramolecular hydrogen bonding, and as a consequence, the COO^- groups are buried within the molecule. It is expected that this would diminish its ability to bind to the resin, since the COO^- groups are consequently less accessible. However, as was discussed in the previous chapter, irradiation of bilirubin in organic solvents and, to a lesser extent, aqueous solution, leads to various amounts of photoproducts, depending on the

environment. One such isomerization is the rotation about the C-5 or C-15 double bond, leading to a mixture of EZ/ZE isomers. In the previous chapter it has been shown that, in aqueous solution, these isomers are formed in only small amounts, and that they revert to the ZZ form very quickly unless human serum albumin (HSA) is added, in which case the photoproducts can be "trapped". It was concluded that the isomers must be bound to the HSA.

It has also been shown in Chapter 2, and elsewhere (8), that in DMSO the intramolecular hydrogen bonds of bilirubin are broken, and the resulting photoisomers formed are different than in aqueous solution. As a result, a much higher proportion of lumirubins are formed from the EZ/ZE isomers.

Both the EZ/ZE isomers and the lumirubins result from a rotation about one of the double bonds, and thus the carboxyl group is rotated outward from the interior of the molecule. Hence, it is more accessible to the adsorbent, as shown by an increased polarity (8, 9). If the adsorbent is charged, and if the electrostatic interaction between the bilirubin and the adsorbent is of primary importance, then it follows that a more accessible carboxyl group should have a favourable effect on the adsorption process. This chapter presents a study of the effect of photoisomerization of bilirubin in aqueous solution and dimethylsulfoxide on the adsorption by cholestyramine, trimethylglycine-Ala_3-Support, Ala-Arg_5-Ala_3-Support, Lys_5-Ala_3-Support, and Arg-Ala_4-Support, where Support represents a cross-linked polyacrylamide resin.

The practical importance of a possible "photoenhanced adsorption" is obvious in light of current concern regarding the safety of phototherapy. It would permit a reduction in the time spent under the lamps by the infant, which could only be beneficial. A combination of simultaneous treatment with the resin and with phototherapy could achieve this result.

3.2 EXPERIMENTAL

3.2.1 BILIRUBIN SOLUTIONS

Solutions were prepared daily by dissolving the bilirubin (25 mg) (checked for purity by HPLC analysis, as described in Chapter 2) in approximately 2 mls of 0.10 M NaOH and then diluting to 250 mls with aqueous KH_2PO_4/NaOH buffer, degassed with nitrogen, to give a final pH of 7.8 $\pm$ 0.1 and [BR] = 1.71 x 10^{-4} M. These solutions were kept in the dark and in an ice bath until needed. For solutions in dimethylsulfoxide, (BDH Chemicals, Toronto) the powder was dissolved directly in degassed solvent.

3.2.2 ADSORPTION AND PHOTOADSORPTION PROCEDURE

The procedure for the adsorption experiments was as follows: The resin (ca. 10 mg) was added through the top of the specially designed adsorption flask (Fig. 3.1) containing 25 ml of bilirubin solution which was then sealed tightly. The solution was stirred mechanically and, at specific time intervals, small aliquots (ca. 0.5 mls) were withdrawn and analyzed spectrophotometrically using a Beckman Model 25 double beam spectrophotometer, with the buffer solution in the reference cell. Changes in the absorbance maximum, at 438 nm for aqueous solutions and at 456 nm for DMSO solutions, were monitored. For isotherm data, only one aliquot was withdrawn, usually after 60 minutes of stirring.

For adsorption experiments with irradiation, identical procedures were followed with the exception that the fiber optics illuminator, described previously in Chapter 2, was immersed directly into the solution through an opening in the front of the adsorption flask. The light was turned on as soon as the resin was added, except when pre-irradiation studies were being done, in which case the light was turned on ten minutes prior to the addition of the resin. When DMSO

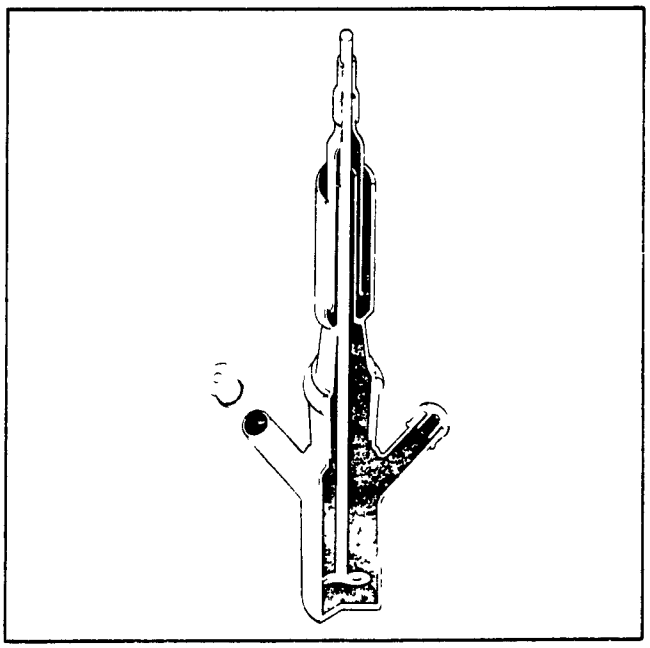

Figure 3.1 Adsorption flask used for the adsorption and photoadsorption experiments. Note that a third neck (not shown) was added for the photoadsorption experiments into which the end of the fibre optic illuminator was placed.

was used as a solvent the bilirubin solution was placed in the adsorption flask and irradiated for 25 minutes, whereupon a sample was withdrawn and placed in both the sample and reference sides of the spectrophotometer, which was then set to a reading of zero. The resin was then added to the flask, corresponding to time = 0, and the adsorption was followed as usual. The "reference" side of the spectrophotometer was used for the sample to get positive readings. The light source was kept on throughout the experiment, except in some pre-irradiation studies, or where otherwise noted.

Control experiments were done on both the aqueous and organic solvents containing bilirubin to ensure that decreases in the absorbance were due to adsorption by the resin and not due to loss of bilirubin chromophore by a photoprocess. This was done by repeating the photo-adsorption experiment in the absence of resin.

To verify that it was indeed possible to form photoproducts using this apparatus, an aqueous solution of bilirubin and albumin was placed in the adsorption flask and irradiated. Absorbance difference spectra, recorded as described in Chapter 2, confirmed photoisomerization of bilirubin.

3.2.3 PREPARATION OF CHOLESTYRAMINE

A washing procedure for cholestyramine, reported previously from this laboratory (1), involved washing the cholestyramine overnight with methanol, then twice for twenty minutes with 1.0 M HCl, twice with 0.01 M HCl, and finally twice with 0.05 M KH_2PO_4/NaOH buffer, pH = 7.8 $\pm$ 0.1. The resin was then filtered and dried under vacuum at 100°C and stored in a dessicator until use.

In this study a refined procedure was used to wash the cholestyramine because it was found that an impurity, quite possibly an amine, with absorbance

at 254 nm still remained in the resin which was interfering with some of the absorbance measurements and possibly also affecting the adsorption behaviour. In this procedure the resin was added to 50% (v/v) acetic acid/water, which was refluxed for approximately a week. The solvent was changed periodically, until a check of the absorbance at ca. 254 nm indicated purity.

3.2.4 SYNTHESIS OF TRIMETHYLGLYCINE-ALA$_3$-SUPPORT

This resin was prepared using standard solid phase peptide synthesis techniques with a Vega Biotechnologies Coupler Model 250C, interfaced to an Apple Computer. All solvents used were freshly distilled prior to use.

The polypeptide resin (3.0 g) (Polyamide Peptide Resin 1, Chemalog, poly(dimethylacrylamide-co-N-acryl-1,6-diaminohexane · HCl), 11% cross-linked) (Fig 3.2) was washed in the reaction flask with dichloromethane (CH_2Cl_2, dried over molecular sieves), then washed with 40% N,N diisopropylethylamine (DEA, 20 mls) in CH_2Cl_2. After a positive ninhydrin test, the anhydride of t-Boc alanine (Vega Biotechnologies) was prepared by dissolving the amino acid (1.0 g) in CH_2Cl_2 (10 mls) and adding N-N'-dicyclonexylcarbodiimide (DCC) at 0°C (5.7 mls from a 10% solution in CH_2Cl_2). The suspension was filtered to remove the urea and the filtrate containing the activated t-Boc Ala was added to the reaction flask on the synthesizer containing the neutralized resin and shaken overnight. The resin was then washed with CH_2Cl_2, dimethylformamide (DMF) and DEA. After a ninhydrin test showed that the coupling was complete, by the absence of free NH_2, the Ala just attached was deprotected with trifluoroacetic acid (TFA), and the resin was washed with dioxane (50% in CH_2Cl_2), neutralized with DEA

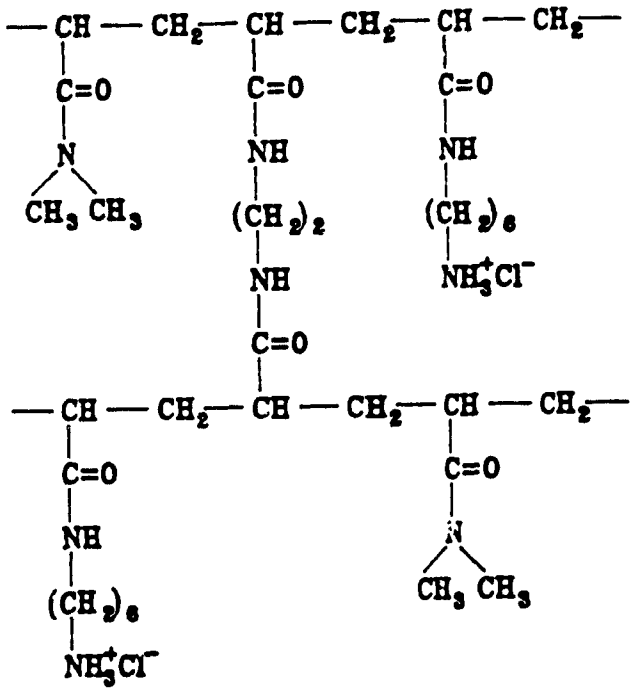

Figure 3.2 Structure of the polyacryalmide resin, dimethylacrylamide-co-N-acryl-1,6-diaminohexane · HCl, used in the synthesis of the polypeptide resins.

and the above procedure was repeated until the resin had three Ala's attached with the last one in the neutralized form.

The anhydride of dimethylglycine·HCl (Aldrich) was prepared by dissolving it in DMSO (BDH Chemicals, Toronto, used as received) in a glove bag under N_2, and then adding 0.7 mls of DEA dropwise. DCC in DMF was then added (5.7 mls) and stirred a few minutes. This solution was added to the reaction flask containing the trialanine, coupled and washed as above, to give DMG-Ala_3-Support.

The DMG on the resin (1.5 g) was methylated, by reacting twice with CH_3I (4.5 mls) in just enough DMF to wet the resin and allow stirring (18 mls). The solution was stirred ca. 18 hours in the dark, then filtered. The resin was washed three times with ethanol and finally diethylether, and then was dried.

To determine the percentage substitution the resin (20 mg) was titrated potentiometrically with $AgNO_3$. A value of 35% methylation was obtained, based on 100% coupling of the DMG, giving a substitution based on trimethylglycine (TMG) units of 7.6×10^{-5} moles/g (Moles I^-/g of resin).

The synthesis of the other resins, Ala-Arg_5-Ala_3-support, Lys_5-Ala_3-Support, and Arg-Ala_4-Support, prepared in similar fashion by D.S. Henning, has been described previously (2).

3.3 RESULTS AND DISCUSSION

3.3.1 PHOTOENHANCED ADSORPTION OF BILIRUBIN FROM AQUEOUS BUFFER SOLUTION, pH = 7.8, BY CHOLESTYRAMINE.

The adsorption of bilirubin is reported in terms of X, the number of moles of bilirubin adsorbed per equivalent site on cholestyramine, as a function of time. The value for X (moles/eq) is calculated as follows:

$$X\ (\text{moles/eq}) = \frac{((A_0 - A_t)/A_0) \times [BR] \times \text{volume}}{(\text{mass resin})(\text{substitution})}$$

where: A_o = absorbance of original solution;

A_t = absorbance of solution at time t;

[BR] = initial concentration of bilirubin, in moles/l;

substitution = 3.3×10^{-3} moles/eq;

mass resin = mass of resin used, in grams;

volume = volume of solution, in liters.

The results for the kinetics of adsorption of bilirubin by the adsorbents tested here are shown in Fig. 3.3 (a) - (e).

The effect of photolysis during adsorption of bilirubin by cholestyramine is given in Fig. 3.3 (a). Each point represents the average value of six data points from six separate experiments and the error bars correspond to the standard deviations. Although the difference in the kinetics of adsorption between irradiated and unirradiated is small, the increase in rate due to photolysis is nonetheless significant, as seen from the standard deviations.

The results of control experiments for the irradiation of bilirubin in aqueous solution in the absence of any resin are shown in Fig. 3.4 where the absorbance at 438 nm was followed as a function of time of irradiation. Clearly there is no significant change in the peak maximum, showing that the light has no

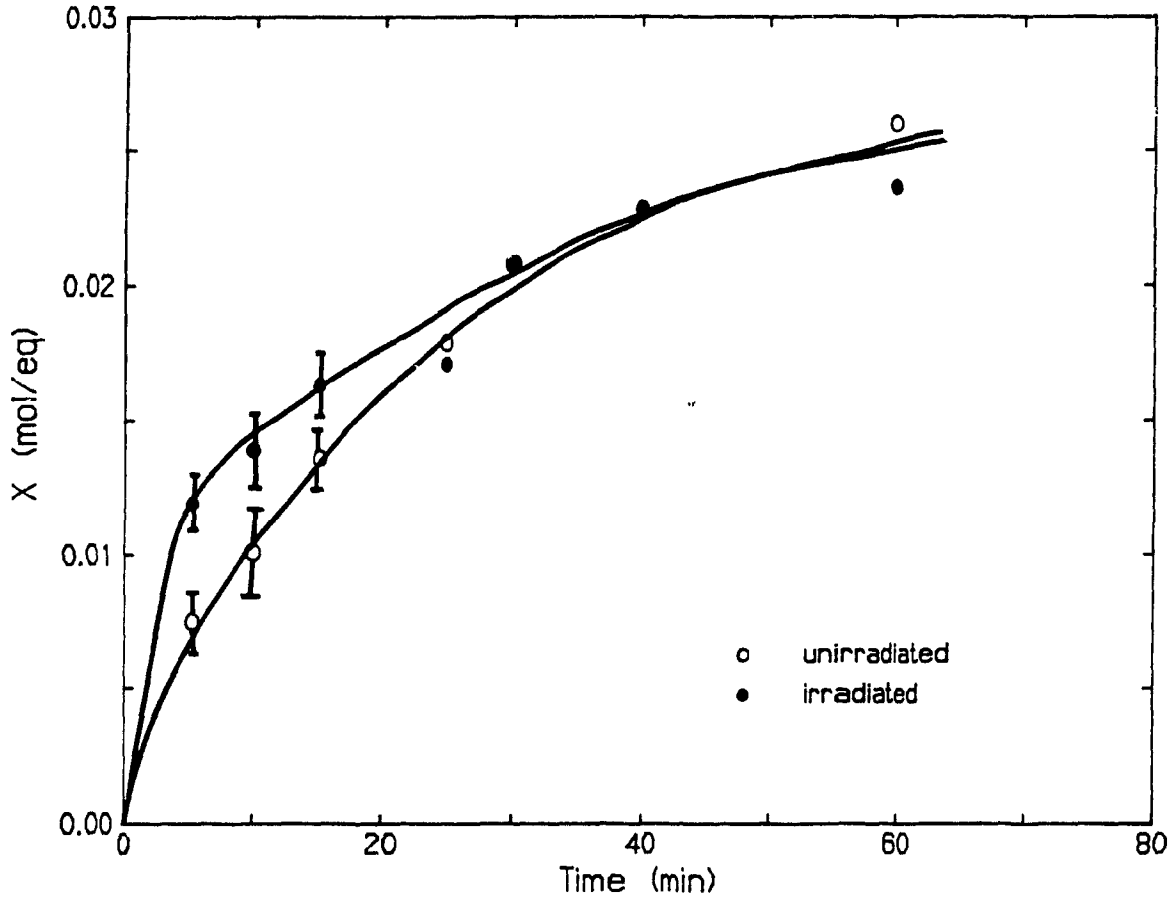

Figure 3.3 A The amount of bilirubin (in moles/eq) adsorbed onto cholestyramine from aqueous solution as a function of time for both irradiated and unirradiated solutions.

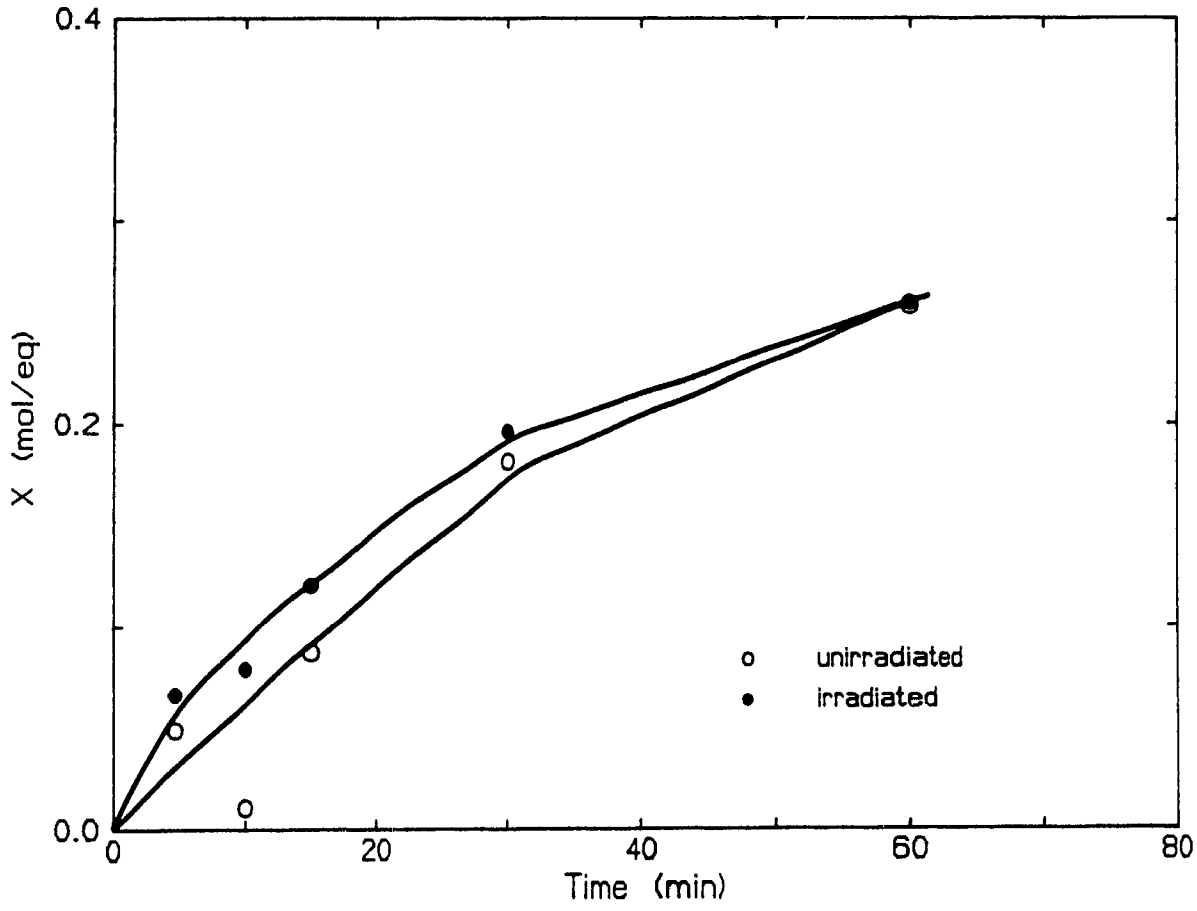

Figure 3.3 B The amount of bilirubin (in moles/eq) adsorbed onto TMG-Ala_3-Support from aqueous solution as a function of time for both irradiated and unirradiated solutions.

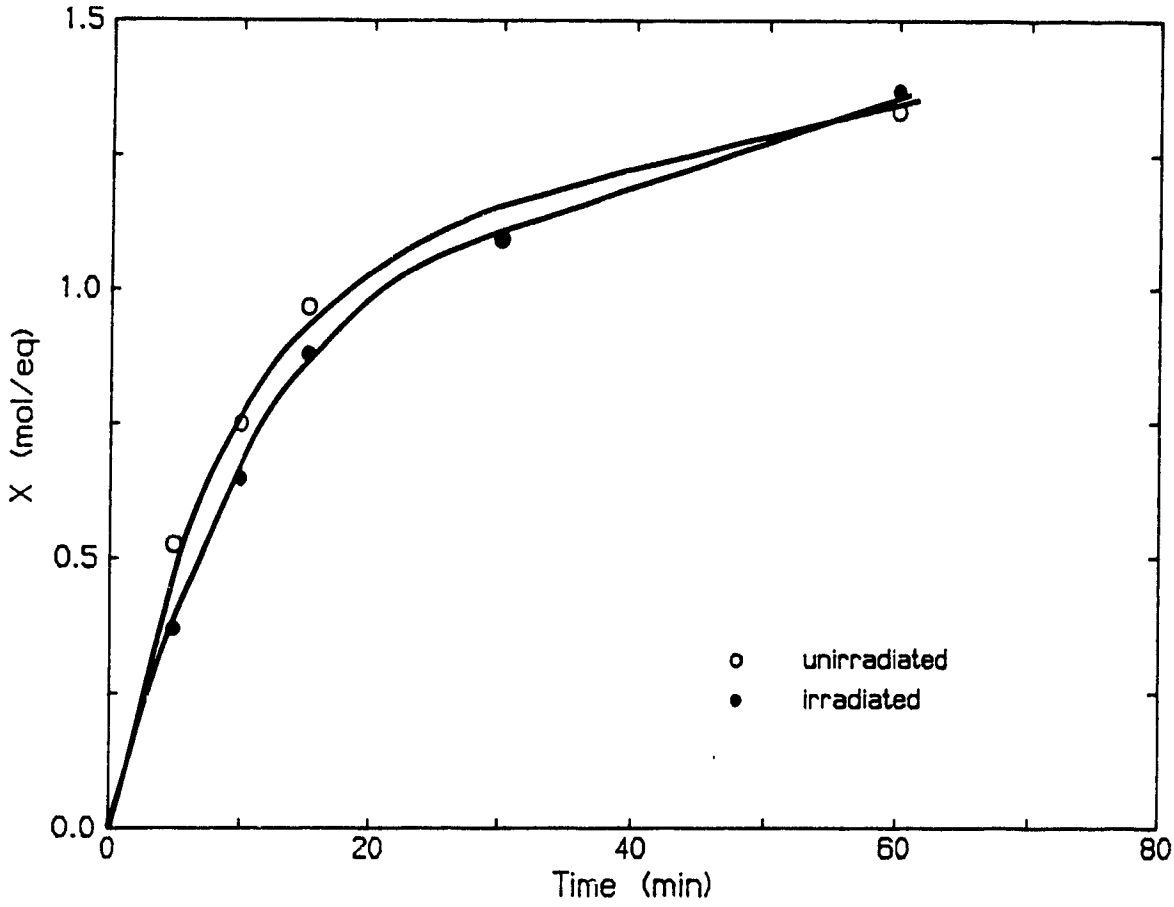

Figure 3.3 C The amount of bilirubin (in moles/eq) adsorbed onto Ala-Arg_5-Ala_3-Support from aqueous solution as a function of time for both irradiated and unirradiated solutions.

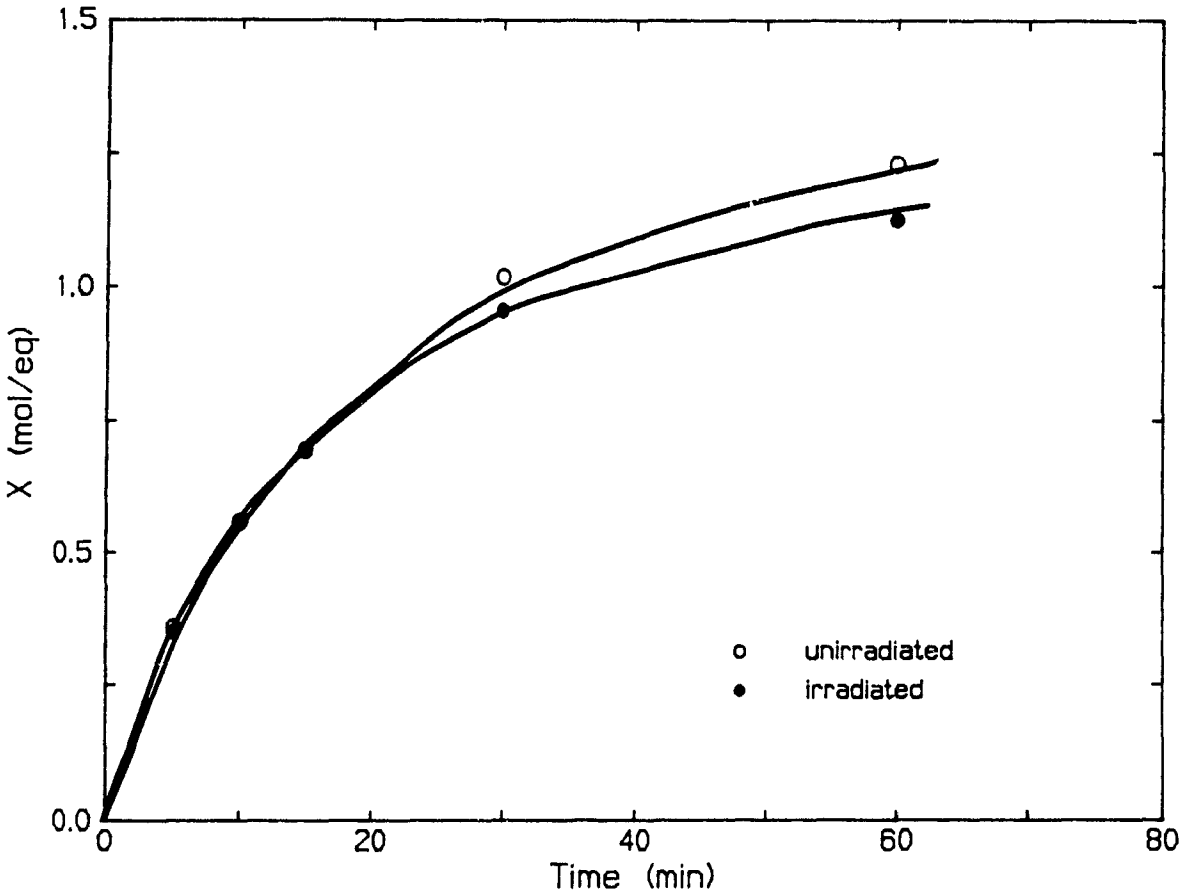

Figure 3.3 D The amount of bilirubin (in moles/eq) adsorbed onto Lys_5-Ala_3-Support from aqueous solution as a function of time for both irradiated and unirradiated solutions.

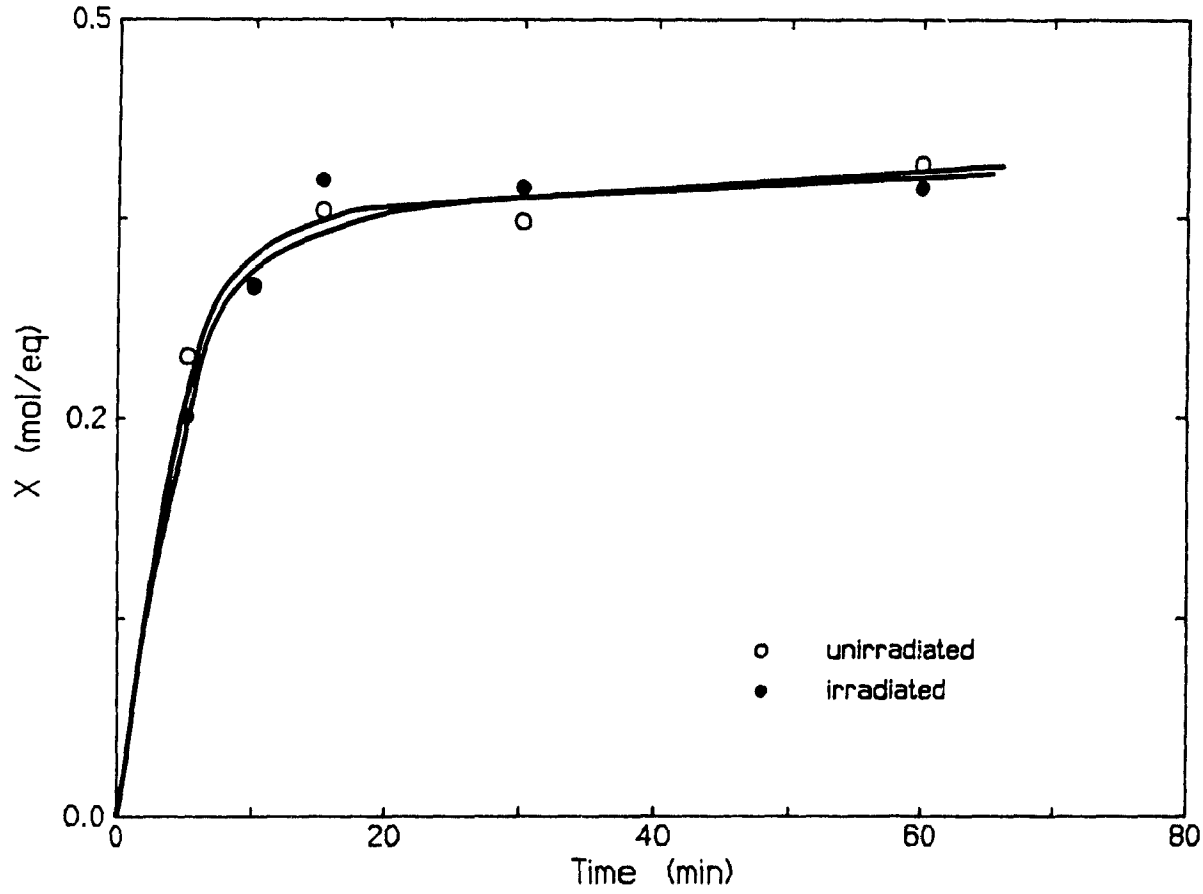

Figure 3.3 E The amount of bilirubin (in moles/eq) adsorbed onto ARG--Ala_4-Support from aqueous solution as a function of time for both irradiated and unirradiated solutions.

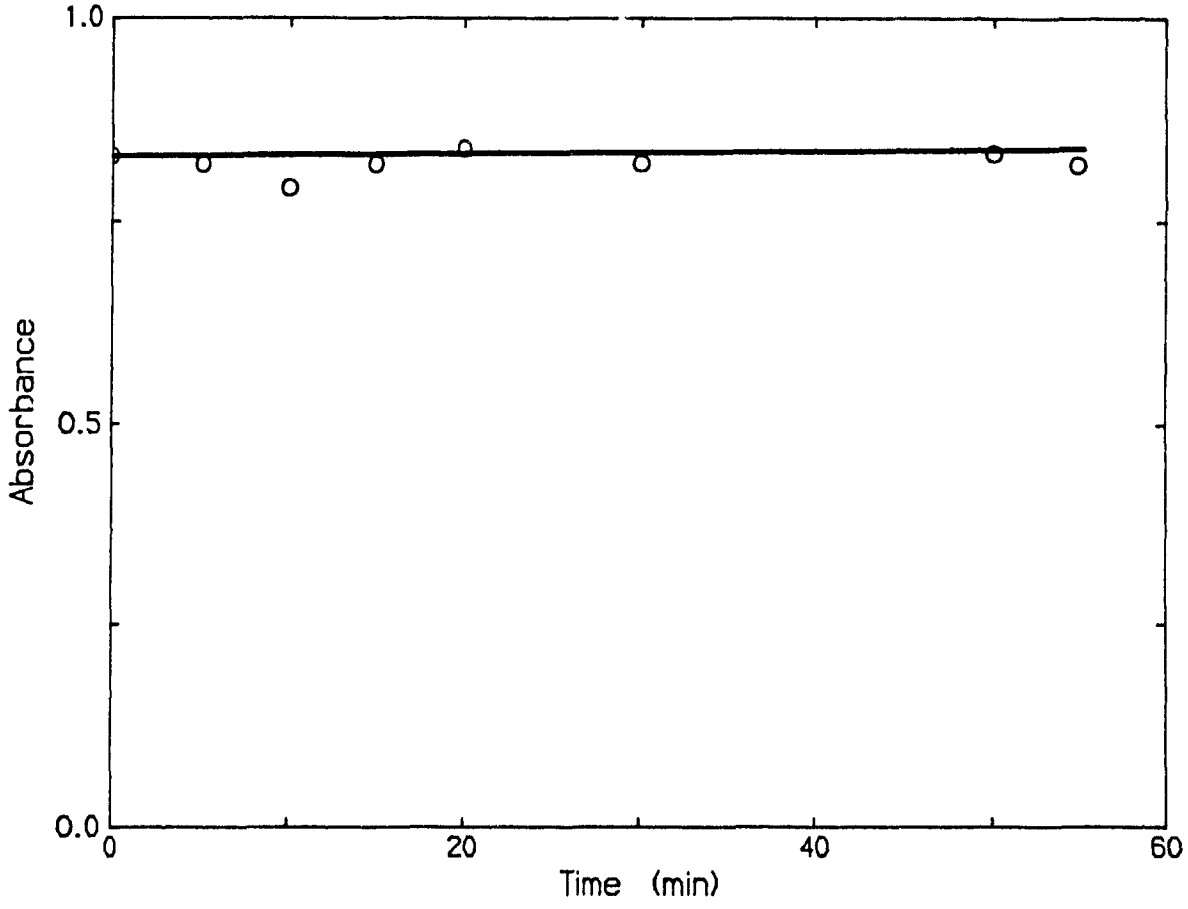

Figure 3.4 Control experiment showing the change in absorbance at 438 nm of an aqueous bilirubin solution irradiated in the absence of any resin.

effect on the measurements. Thus, the enhanced rate of adsorption by the resin must be due to the formation of photoisomers.

Another control experiment was used to show that photoisomers are produced in this experimental apparatus. A solution of BR/HSA was irradiated in the adsorption flask and photoisomerization was monitored in the usual manner, to give an absorbance difference spectrum. The results in Fig. 3.5 show clearly that photoproducts are formed with irradiation in this apparatus.

The results in Fig. 3.3 (a) are reproducible and indicate that, for cholestyramine, there is an increased rate of adsorption of bilirubin from aqueous solution. This difference is only manifested at the early stages of the adsorption. After about twenty minutes there is, in fact, no difference in the amount adsorbed between the irradiated and non-irradiated solutions. Thus, the capacity of the resin, at a fixed equilibrium concentration of bilirubin, is unaffected by photolysis.

Fig. 3.3 (b) illustrates the effect of photolysis on the rate of adsorption by TMG-Ala_3-Support. Although there is some indication of a photoenhancement in the rate of adsorption at short irradiation times, it is not as pronounced as in the case of cholestyramine. The difference in the adsorption behaviour of the irradiated and the unirradiated solution is very small and is, in fact, very close to experimental error.

The rates of adsorption of bilirubin by the other polyacrylamide resins, shown in Figs 3.3 (c) - (e), give no evidence for enhancement as a result of the formation of photoisomers. Within experimental error, the rates are identical with and without irradiation.

The difference in adsorption characteristics of the polyacrylamide resins and of cholestyramine and TMG-Ala_3-Support must be attributed to adsorbent

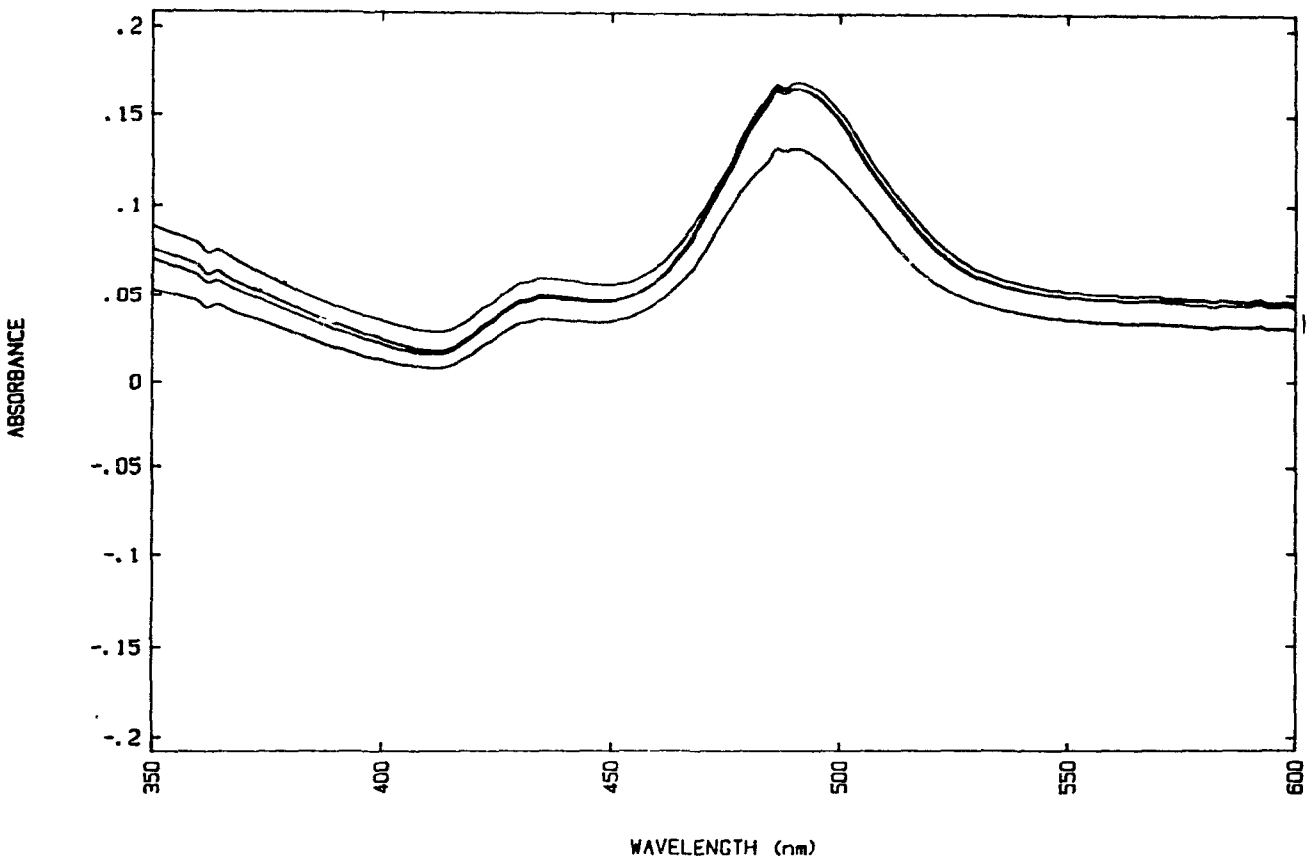

Figure 3.5 Absorbance difference spectra for a solution of bilirubin and human serum albumin irradiated in the adsorption flask to verify that photoproducts could be formed using this set up.

characteristics that affect the rate of adsorption. In the absence of protein, e.g., HSA, the amount of photoproduct formed in aqueous solution is exceedingly small, as described in Chapter 2, due to the rapid reversion of the isomers. Thus, the photoenhancement in the rate of adsorption requires efficient "trapping" of photoproducts. For this to occur two requirements must be met: (i) The rate of adsorption of photoproducts must be greater than the rate of adsorption of bilirubin; and (ii) the rate of adsorption of both the photoproducts and the bilirubin must be approximately equal to the rate of formation of photoproducts. If these criteria are not met it will not be possible to detect enhancement in the rate.

Consideration of the structure of the photoproducts suggest that the first requirement can be met. The photoproducts formed in these experiments result from rotation about the double bonds at the 4Z and 15Z positions of the bilirubin molecule to yield the EZ/ZE isomers, as described in Chapter 2. As a consequence, the intramolecular hydrogen bonds are disrupted and at least one (two if the EE isomer is formed) of the COO^- groups becomes more accessible as it is rotated outwards, away from the interior of the molecule. Since it is the ionized carboxyl groups that are the major area of the molecule responsible for binding, the more accessible they are the more readily the photoproduct can bind. It is for this reason that with a cationic adsorbent the rate of adsorption of the photoproduct is expected to be greater than the rate of adsorption of bilirubin, which probably requires disruption of intramolecular hydrogen bonds as part of the process.

The overall adsorption process may be outlined as follows:

$$BR + resin \xrightarrow{k_1} BR\text{-}resin \qquad [3.1]$$

$$BR + h\nu \xrightarrow{k_2} EZ/ZE \text{ isomers} \qquad [3.2]$$

$$EZ/ZE \text{ isomers} + resin \xrightarrow{k_3} EZ/ZE \text{ isomers-resin} \qquad [3.3]$$

As discussed above, (requirement (ii)), the rate of equation [3.2], i.e., the rate of formation of photoproducts, must be greater than the rate of adsorption. The **relative** rates of adsorption for the process may be expressed as $k_1[BR]/k_3[Photoproducts]$. Since, at steady state, [BR] >> [Photoproducts] and is independent of the resin used in the adsorption, the relative rate of adsorption can be expressed as proportional to the ratio of k_1/k_3. Thus, detection of the photoenhancement depends upon the ratio of the rate constants of the adsorption steps in equations [3.1] and [3.3] above. The detection of the photoenhanced adsorption is dependent upon $k_3 > k_1$, as described by requirement (i).

To a first approximation, initial rates of adsorption of bilirubin at equal initial concentrations can be estimated, assuming a constant stirring rate, from the initial slopes of the adsorption kinetics curves of Figs. 3.3(a) - (e). The results, given in Table 3.1, are expressed in terms of the amount of bilirubin adsorbed per **gram** of resin, per unit time, since this eliminates complications due to the very different functionality of the different resins.

TABLE 3.1

Estimated Initial Rates of Adsorption of Bilirubin by Cholestyramine and Select Resins from Aqueous Solution, pH = 7.8.

Resin	Initial Rate (moles/g/min) x 10^5
Cholestyramine	0.50
TMG-Ala_3-Support	0.074
Ala-Arg_5-Ala_3-Support	2.0
Lys_5-Ala_3-Support	1.4
Arg-Ala_4-Support	1.2

The differences in the initial rates of adsorption between the polyacrylamide resins and the cholestyramine probably reflect the differences in their respective backbones. Cholestyramine, with its polystyrene backbone, is hydrophobic and does not swell appreciably in aqueous solution, whereas the polyacrylamide resins swell considerably. This difference in swellability of the adsorbents leads to differences in **accessibility** of the sites, i.e., a larger fraction of sites on the polyacrylamide resin are available more quickly to the adsorbate. If the binding sites are more accessible, it follows that the initial rate of adsorption is greater for these resins as well, or, conversely, that the initial rate of adsorption of the bilirubin and/or photoproducts by cholestyramine is slower, as the data indicate.

The initial rate of adsorption of the TMG-Ala_3-Support is small relative to that of the other polyacrylamide resins, which is consistent with a photoenhanced adsorption with this resin as per requirement (ii) above (Fig 3.3b). However, it is not as clear a case as the cholestyramine. This suggests that the TMG-Ala_3-Support does not preferentially bind the photoproducts, i.e., requirement (i) is not

met. Whether this also applies to the other polyacrylamide resins can not be ascertained, since the adsorption process for these resins is too fast, i.e., even requirement (ii) is not met. However, there seems little doubt that the TMG-Ala_3-Support does not selectively bind photoproducts instead of bilirubin.

Since the final capacity of the resin is unchanged by irradiation of bilirubin, it is likely that the EZ/ZE isomers and bilirubin compete for the same binding sites. The resin is saturated at about the same level whether or not the solution is irradiated.

3.3.2 ADSORPTION AND PHOTOADSORPTION OF BILIRUBIN ONTO TMG-ALA_3-SUPPORT FROM DIMETHYLSULFOXIDE

The photoproduct formation from irradiation of bilirubin in dimethylsulfoxide (DMSO) is very different than most solvents due to its ability to disrupt the intramolecular hydrogen bonds of bilirubin. As shown in Chapter 2, the lumirubins are formed in much higher concentrations in this solvent than in any of the others used in this study, with the possible exception of aqueous solutions containing human serum albumin. It might be expected, therefore, that the effect of photolysis of bilirubin on the adsorption processes in this solvent would be different than for bilirubin in aqueous buffer.

A control experiment, described in section 3.3.1, was repeated for the bilirubin solutions in DMSO. Unlike the aqueous solution experiments, these showed a slight decrease in the absorbance peak upon irradiation in the absence of resin. Thus, based on the curve seen in Fig. 3.6, a correction was used in all calculations. Although the corrections in the absorbance are very small, these changes, as a function of time, were always taken into account.

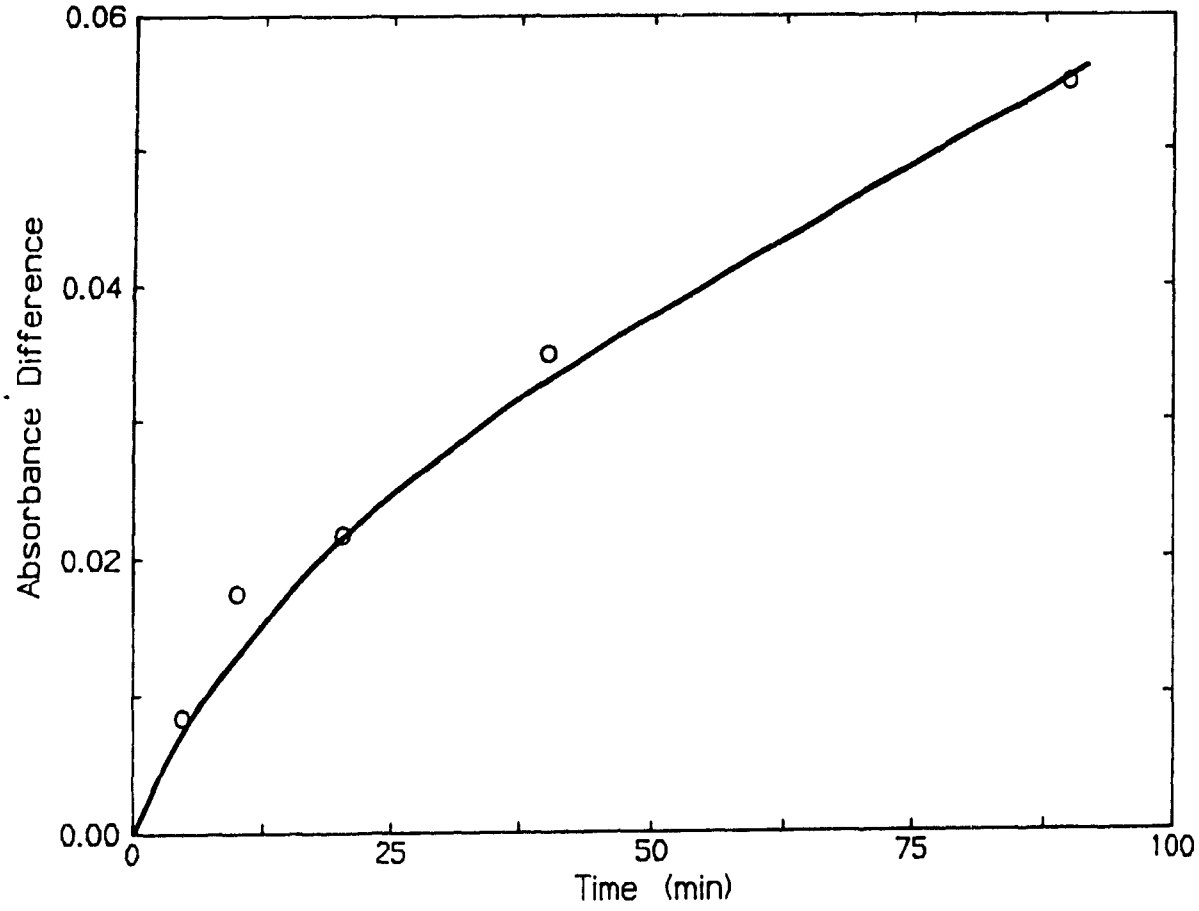

Figure 3.6 The loss of bilirubin (456 nm) as a function of irradiation time in the absence of any resin. This curve was used as a correction curve in all calculations. The percentage loss of total bilirubin at specific values of irradiation times are: 20 min, 2.50% loss; 45 min, 4.14% loss; 90 min, 6.08% loss.

The kinetics of adsorption of bilirubin in DMSO by TMG-Ala_3-Support are markedly changed by photolysis, but more significantly, the adsorption capacity is also dramatically higher for the adsorption of bilirubin in the presence of light (Fig. 3.7). The kinetics of adsorption show an induction period, consistent with an initial buildup of lumirubins before an increased rate of adsorption takes place. This is seen at the beginning of the kinetics curve where the slope is initially small but increases with time, and therefore concentration of lumirubins, increases. Since only minimal amounts of bilirubin are adsorbed without irradiation, it appears that only the lumirubins are adsorbed by the resin. Furthermore, the resin shows saturation with the lumirubins as shown by the data in Table 3.2 where, in separate experiments, the amount of resin was varied but the capacity for photoproduct, on a per equivalent basis, remains the same.

TABLE 3.2

The Amount of Bilirubin Adsorbed from DMSO as a Function of the Amount of TMG-Ala_3-Support Added (C_{eq} = 9.5 mg/dl)

Mass of Resin (mg)	Capacity (moles/eq)
9.45	0.16
11.2	0.14
17.4	0.17
20.5	0.16
27.6	0.17

It was shown above that, in aqueous solution, the initial **rate** of adsorption of bilirubin is increased by irradiation, but that the capacity of the resin is unchanged. However, the adsorption maximum for the TMG-Ala_3-Support

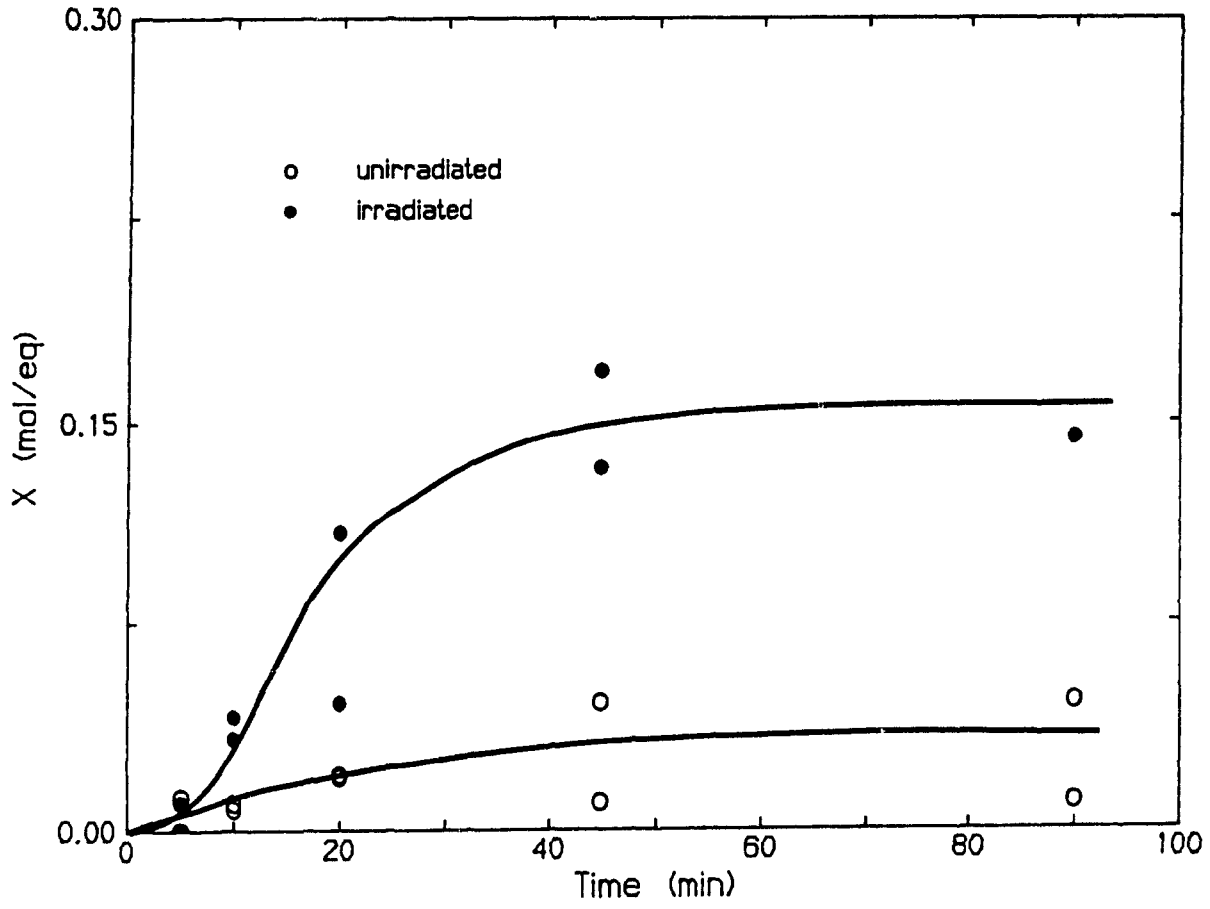

Figure 3.7 The amount of bilirubin (in moles/eq) adsorbed onto TMG-Ala_3-Support from DMSO as a function of time for both irradiated and unirradiated solutions.

(and, as will be seen later, for cholestyramine) in DMSO is very much larger for the irradiated solution. Thus, it is clear that in **aqueous** solution both bilirubin and itsphotoproducts (EZ/ZE isomers in this case) are adsorbed by the resin, most probably at the same binding site However, in DMSO solution the photoproducts (lumirubins in this case) are selectively bound. A comparison of the adsorption characteristics of TMG-Ala_3-Support in the two solvents can be seen diagramatically:

	Aqueous solution Adsorbing species	DMSO solution Adsorbing species
	Bilirubin EZ/ZE isomers	Lumirubins
Total capacity:	0.24 mol/eq	0.17 mol/eq

An explanation for the results can be given from a consideration of the solubility. In aqueous solution neither the bilirubin nor its photoproducts are very soluble, and hence binding occurs readily. The overall binding involves secondary interactions due to hydrophobic effects arising from the special arrangement and thus loss of entropy of the water molecules (Chapter 4). In DMSO there are no such complications due to the structuring of water. However, strong interactions between DMSO and bilirubin disrupt the intramolecular hydrogen bonds of bilirubin. This leads to **inter**molecular hydrogen bonding between bilirubin and DMSO, which explains the higher solubility of bilirubin in this solvent. Consequently, there is less driving force for bilirubin to bind to the

resin and very little, if any, adsorption is seen (Fig. 3.7). On the other hand, the lumirubins do adsorb. The driving force for binding may be attributed to a decrease of solubility in DMSO as a result of the photoisomerization of bilirubin to form the lumirubins. In fact, Stoll, et al. (9) have isolated lumirubins from irradiated bilirubin in DMSO by an extraction into $CHCl_3$, leaving the bilirubin in DMSO. Thus, the bilirubin is more soluble in DMSO than are the lumirubins, which were extracted into $CHCl_3$. The decreased solubility of the lumirubins relative to bilirubin is consistent with the binding behaviour observed in Fig. 3.7. Since there is strong evidence (10, 11) that lumirubins do not revert back to bilirubin, unlike the EZ/ZE isomers in aqueous solution, the effect of the increased adsorption due to irradiation is large.

The temperature dependence of adsorption isotherms (Fig. 3.8) can provide information regarding thermodynamic aspects of the binding mechanism. The free energy of binding can be expressed as:

$$\Delta G^\circ = -RT \ln k = \Delta H^\circ - T\Delta S^\circ \qquad [3.4]$$

where R is the universal gas constant, T is temperature, k is the binding constant, ΔH is the enthalpy change and ΔS is the entropy change. For simple systems a plot of ln k as a function of the reciprocal temperature yields a straight line with a slope equal to $-\Delta H^\circ/R$ and an intercept of $\Delta S^\circ/R$. The adsorption of lumirubins from DMSO decreases as the temperature in increased, as shown by the isotherms in Fig. 3.8. This leads to a negative ΔH of binding, i.e., the binding is exothermic. An exothermic enthalpy change is consistent with an ionic interaction, or salt linkage, which is expected in this case, and a minimal role of solvation effects.

The overall binding of an irradiated solution of bilirubin in DMSO by TMG-Ala_3-Support can, therefore, be described as follows: The binding involves only the lumirubins formed from the irradiation, since they are less soluble in the

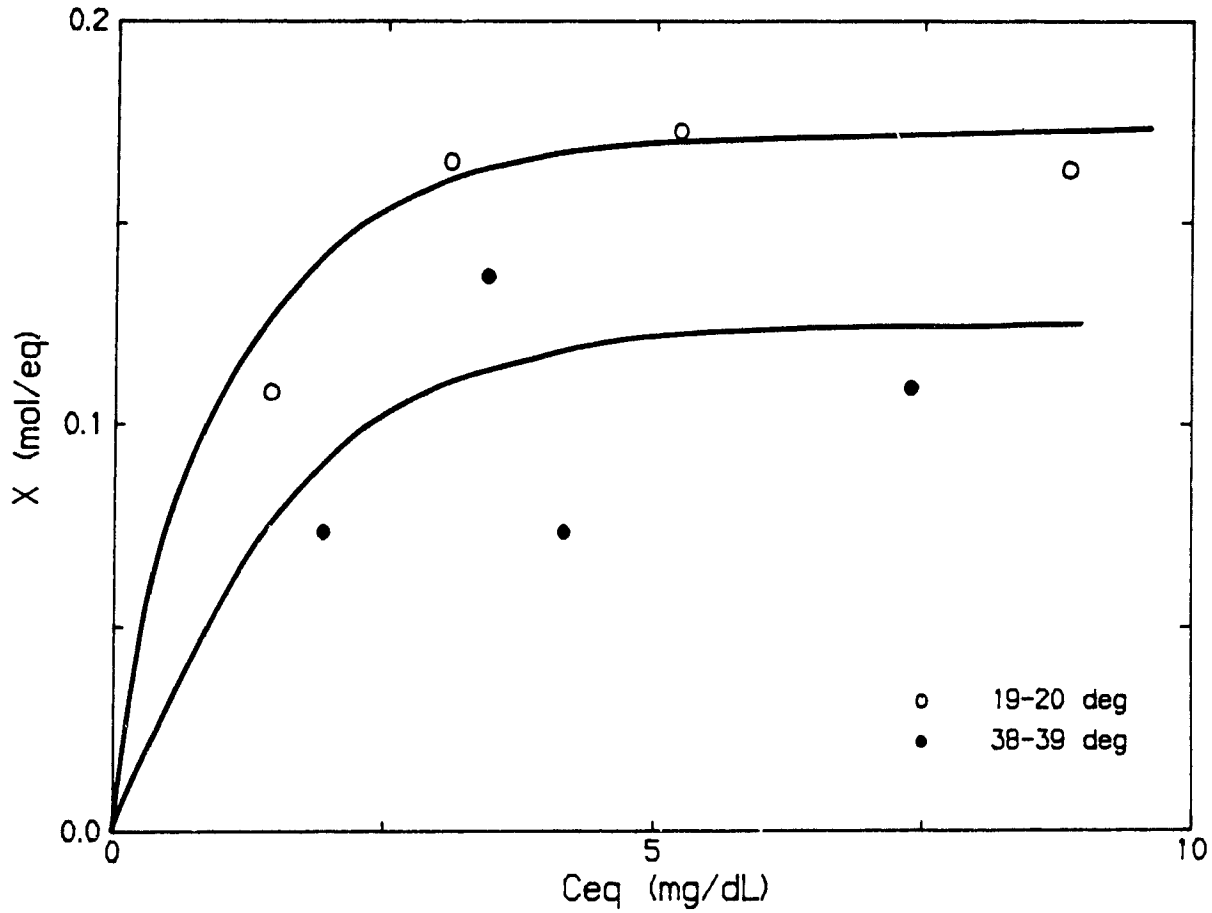

Figure 3.8 Adsorption isotherms for the photoadsorption of bilirubin in DMSO onto TMG-Ala_3-Support.

DMSO than the bilirubin. The lower solubility of the lumirubins provides a driving force for binding; the interaction being between the COO^- of the lumirubins and the quaternary amine of the resin.

3.3.3 ADSORPTION AND PHOTOADSORPTION OF BILIRUBIN ONTO CHOLESTYRAMINE FROM DIMETHYLSULFOXIDE

When cholestyramine is used as the resin and the solution of bilirubin in DMSO is irradiated for twenty minutes prior to the addition of the resin, adsorption occurs as shown in Fig. 3.9 A. The maximum amount of bilirubin, or its photoproducts, adsorbed is now much smaller than in the previous case. Furthermore, an initial buildup of lumirubins is still needed before there is significant adsorption, (at t = 5 min and 10 min there is no adsorption) as seen by the induction period in the first ten minutes. As shown in Chapter 2, the lumirubins are not formed immediately in DMSO, but rather a buildup of the EZ/ZE isomers in required. The lumirubins are then formed from these isomers.

A comparison of the time required to form the lumirubins in this apparatus as compared to that used for the experiments described in Chapter 2 can be made by determining the loss in the bilirubin peak required before lumirubin formation is seen. Using those data, it can be seen that ca. 4% of the bilirubin chromophore was photoisomerized before the lumirubins formation could be detected. Estimations based on the curve in Fig. 3.6 indicate that it would require ca. 40 minutes to photoisomerize the same amount of bilirubin in this apparatus. Thus, the induction period of almost 15 minutes of stirring, which represents 35 minutes of irradiation since the light was not turned off during adsorption, coincides well with the time required to form the lumirubins.

To verify that an initial buildup of lumirubins is, in fact, what is happening in this adsorption, two experiments were done. First, a solution of

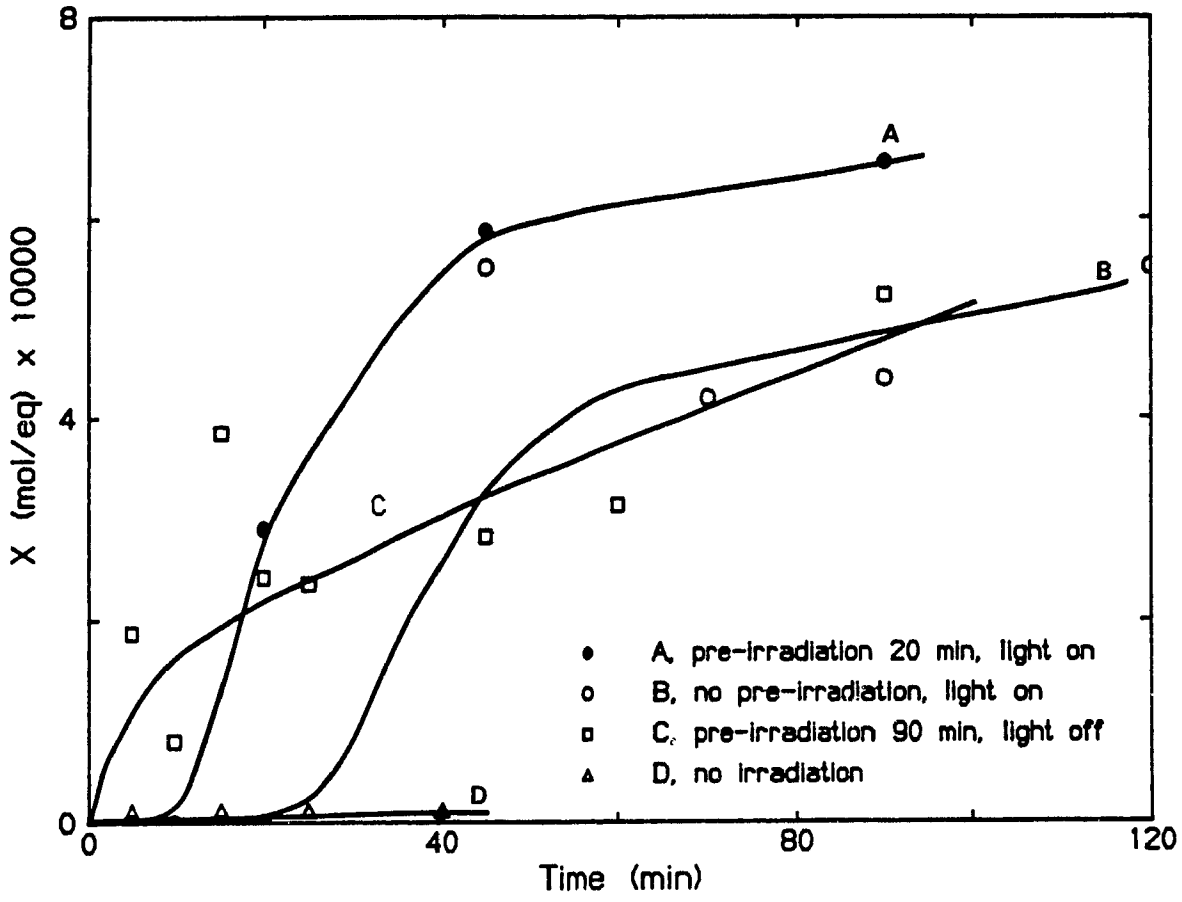

Figure 3.9 The amount of bilirubin (in moles/eq x 10^4) adsorbed onto cholestyramine from a DMSO solution as a function of time for unirradiated and pre-irradiated solutions.

bilirubin in DMSO was **not** pre-irradiated; curve B of Fig. 3.9 shows that the induction period is increased when the light was turned on only after the resin has been added. Secondly, a similar solution was pre-irradiated for 90 minutes (curve C of Fig. 3.9) and the light was turned off when the resin was added. Hence, no correction factor was needed here since the spectrophotometer was set to zero using the solution after the irradiation. In case B, the induction period increased to greater than twenty minutes but by 40 minutes the lumirubin concentration is beginning to build up, and concomitantly the adsorption increases as well. In fact, the maximum adsorption is very close to that in curve A, i.e., with pre-irradiation, (using an experimental error of ca. 10%. Note that the scale is very small and the absorbance differences obtained experimentally are very small, increasing the error). In case C there is sufficient lumirubin formed prior to the addition of the resin and hence no induction period is seen. The adsorption kinetics are thus not dependent on any buildup of adsorbate and the curve increases to a maximum very close to that of curves A and B.

The mechanism for the photoadsorption of a solution of bilirubin in DMSO onto cholestyramine can be represented as follows:

$$\text{BR} \overset{h\nu}{\longleftrightarrow} \text{EZ/ZE isomers} \longrightarrow \text{LR} \overset{\text{bind}}{\longleftrightarrow} \text{LR-CA}$$

When there is a sufficiently high concentration of lumirubins, the binding of the isomer to the resin begins. If there is no pre-irradiation and, hence, no lumirubins in solution, there is no adsorption.

The differences in the adsorption capacities of cholestyramine and the TMG-Ala_3-Support in DMSO may be due to the swellability of the respective resins. The polyacrylamide resin swells more in DMSO and therefore more of its sites are accessible, whereas the cholestyramine does not swell and hence is saturated at a lower level than expected.

3.4 SUMMARY

In summary, the photoenhanced adsorption of bilirubin is seen with some resins and not with others. To detect an enhancement in the rate of adsorption upon photolysis two requirements must be met: (i) The rate of adsorption of the photoproducts must be greater than the rate of adsorption of bilirubin, i.e., there must be selectivity for the adsorption of photoproducts, and (ii) The rate of adsorption of both the photoproducts and the bilirubin must not exceed the rate of formation of photoproducts.

In aqueous solution, both requirements are met for cholestyramine and hence photoenhanced adsorption is detected. However, for the adsorption by TMG-Ala_3-Support of photolyzed bilirubin in aqueous solution it is not apparent that this resin selectively binds the photoproducts instead of bilirubin. Thus, requirement (i) is not met and enhancement in the rate is not definite, despite the fact that requirement (ii) is met, i.e., the initial rate of adsorption of bilirubin and/or photoproducts is very slow. The remaining polyacrylamide resins do not exhibit an enhanced rate of adsorption for a photolyzed aqueous solution of bilirubin since the initial rate of adsorption is high, hence requirement (ii) is not met.

In DMSO solution, bilirubin is very soluble and therefore is not driven to bind to the resin. However, the lumirubins are less soluble and do indeed bind to both cholestyramine and TMG-Ala_3-Support from DMSO.

The clinical importance of these results lies in the relative concentrations of the respective photoproducts formed in vivo. There is evidence that the lumirubins do form in low concentrations, and this would, in fact, limit the effect of phototherapy. If the lumirubins do have strong binding tendencies, they can

bind to HSA thus reducing the total amount of bilirubin bound to the protein and releasing more of the toxic substance back into the plasma. One possible way to avoid too much of the photoproducts being bound to the protein would be to limit the time of phototherapy so that the photoproducts do not have a chance to buildup. In fact, this is already being done in some hospitals where intermittent phototherapy (12, 13) is being used.

Care must be taken in extending the data for photolysis in vitro to the in vivo situation. In vitro experiments involve a sample of exact bilirubin concentration and the irradiation of the entire sample, whereas irradiation via phototherapy involves the irradiation of the bilirubin in the tissue of the skin, which is always being replenished by bilirubin in the plasma. Thus, there is less of a chance of a buildup in photoproduct concentration in vivo. Nonetheless, care should still be exercised in terms of the length of time that the infant should be exposed to the irradiation.

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CHAPTER 4

THE BINDING OF INDOLE AND PYRROLE DERIVATIVES BY CHOLESTYRAMINE AND LYS_5-ALA_3-SUPPORT

4.1 INTRODUCTION

The binding of bilirubin to cholestyramine and the polymeric resins discussed in the previous chapters and in other papers from this laboratory (1-3) show that the the primary interaction in the binding process is electrostatic. The interaction involves the COO^- groups of bilirubin and the basic R groups of the amino acid pendants on the polymer resins or the positive charge of the quaternary amine of the cholestyramine. However, much of the data collected has, in fact, shown that there are other important interactions which enhance the adsorption (4).

The most important of these interactions is the hydrophobic effect which arises when a hydrophobe (a hydrocarbon-like molecule) is dissolved in water. The hydrophobic molecule cannot form many, or sometimes any, hydrogen bonds with the water molecules. Thus, on dissolution of the hydrophobe in water there is no compensation for the energy required to break the existing hydrogen bonds between the water molecules; therefore, these bonds are retained, or are broken and then reformed. The end result is that the inclusion of a hydrophobe into water causes an ordering of the water molecules around the hydrophobe (formation of a "cage" or "iceberg") while the water molecules retain the intermolecular hydrogen bonds. This ordering process of the water leads to a decrease in the overall entropy and it is this loss of entropy that leads to an unfavourable free energy

change for the process. Thus, the correct interpretation of the hydrophobic effect is to assign the predominant role to the properties of water.

Although the theory for the hydrophobic effect is well accepted, there is still discussion regarding the structuring of water around the solute. Specifically, there are two theoretical explanations for the changes in the thermodynamic functions that describe the process in which a solute is dissolved in water (5). Both approaches attempt to describe the dissolution of a hydrophobe by reducing the overall process into a series of hypothetical steps. The essential difference between the two theories lies in the picture of the way the water-structuring process takes place in solution.

According to the approach used by Shinoda (6,7), the first step that occurs when water and a hydrophobic liquid are mixed is a hypothetical state of complete randomness. In this state $T\Delta S = 0$, i.e., it is assumed that the mixing is ideal. However, the placement of a hydrophobic group into an aqueous solution is an unfavourable process and hence the excess free energy change for this first step must be positive. The corresponding change in excess enthalpy must also be positive, since the hydrogen bonds between water molecules are broken, or at least distorted. According to Shinoda this random state is followed by a two-step process that leads to the structuring of water. The first step involves "iceberg" formation as the water molecules rearrange themselves around the solute to produce a more structured situation than existed previously. The accompanying entropy change is negative, as is the enthalpy change, since hydrogen bonds are being formed. The excess free energy is also negative, but very small, since the entropy contribution, $T\Delta S$, is unfavourable while the enthalpy is favourable. The net result, however, is a negative ΔG°, which thus favours iceberg formation. Finally, there is additional ordering in the solution leading to the final state, which

is accompanied by a very large negative entropy change which leads to an overall positive free energy.

A more traditional approach, adopted by Kauzmann (8) and Scheraga (9), proposes that the first step is the dispersion of the hydrophobic molecule directly into cages that already exist in pure water, which is followed by iceberg formation. The essential difference lies in the fact that since the cages are already formed, the enthalpy change for the first step is zero, and since the state is completely random, the entropy change must also be zero. Thus, the change in the free energy is zero as well. In the second hypothetical step iceberg formation takes place, giving a negative enthalpy change and a large negative entropy change, with the free energy thus positive and unfavourable. The final stage in the process yields the same results as Shinoda, i.e., a negative enthalpy, very negative entropy and positive free energy for the overall process.

Although the two approaches are similar, they lead to very different predictions of the temperature dependence of the excess heat capacities of the solution. This is due to the difference in the set of thermodynamic functions for the intermediate state of water, i.e., the free energy for iceberg formation is negative and favourable due to enthalpy-entropy compensation in Shinoda's approach, but positive and unfavourable for Scheraga's. Thus, in Shinoda's approach the stabilizing influence of iceberg formation decreases with increasing temperature whereas the opposite is predicted by the traditional treatment.

Both treatments ascribe hydrophobic interactions to the formation of icebergs around the hydrophobe, leading to a more ordered state. This will be shown to be of importance in the binding of small molecules to cholestyramine in terms of an increased adsorption due to hydrophobic effects. There is much evidence that hydrophobic effects are important in the in vitro binding of bile acids and bile acid salts by cholestyramine (10-13).

To obtain a better understanding of the mechanism by which cholestyramine and the polymeric resins bind bilirubin, a study was made of the adsorption from aqueous solution of "bilirubin-like" compounds. Since bilirubin is a tetrapyrrole with two hydrophilic (carboxylic acids) pockets, indole and pyrrole carboxylic acids and derivatives were chosen as the models for the adsorption of bilirubin. The small molecules chosen differ in size and dissociation constants of the acids as well as in their aqueous solubilities.

4.2 EXPERIMENTAL

Aqueous solutions were prepared by dissolving the compounds in warm, deionized water and adjusting the final pH to 7.5 $\pm$ 0.5 by the addition of 0.10 M NaOH. Acidic solutions were prepared by addition of concentrated HCl. Cholestyramine (4.0 - 8.0 mg), previously washed with water/acetic acid, as described in Chapter 3, was added to a flask containing 50.0 mls of solution of appropriate concentration and the flask was shaken for two hours, after which the adsorbent was removed by filtration. For studies done at 0°C and 40°C the adsorption flasks were placed in an ice bath, and a water bath, respectively, and stirred with a stirring bar.

The concentrations before and after adsorption were determined by high performance liquid chromatography (HPLC). The chromatograph consisted of an Eldex dual piston pump (Model 1016/AA), a Rheodyne injector (Model 7125), a C6 reverse phase column and a Waters differential refractometer detector (Model 410). The mobile phase was a methanol/aqueous acetic acid (0.10 M) solution in a volume ratio of 60/40 for the indoles and 55/45 for the pyrroles. The data were collected by a Varian data system (Model DS604). All compounds were found to be greater than 95% pure, based on HPLC data, and consequently were used as received from Aldrich.

The functionality of the cholestyramine was determined by a potentiometric titration of the chloride counterion with silver nitrate.

Aqueous solubilities were determined by shaking a flask containing excess solute in water (pH adjusted to 7.5 $\pm$ 0.5 by the addition of a small amount of 0.1 M NaOH) in a thermostatted water bath for 2-3 days. The attainment of the equilibrium solubility was verified by repeated measurement of the concentration over a period of two days. The concentrations were determined by UV spectroscopy using a Hewlett Packard Model 8451A Photodiode Spectrophotometer.

Heats of fusion were determined on a Perkin Elmer Model 2C Differential Calorimeter using indium and tin as the calibration standards.

4.3 RESULTS AND DISCUSSION

4.3.1 EFFECT OF IONIZATION

Previous studies of the binding of bilirubin by polymeric adsorbents with peptide pendants containing the basic amino acids lysine and arginine give strong indication that ionic interactions are of predominant importance (2, 3). The isotherm for the binding of bilirubin by cholestyramine showed similar behaviour (14). This is as expected since the functional groups of cholestyramine are quaternary amines, predominantly in the ionized form at pH 7-8.

The role of ionic interactions in binding by cholestyramine is clearly demonstrated in a comparison of the adsorption behaviour of indole-3-COOH with that of indole-3-CHO (Fig. 4.1). While the "plateau" value for the adsorption of the acid, under conditions of full ionization (pH = 7.5), approaches one molecule per functional site, adsorption of the aldehyde does not exceed 5% of available sites. This is confirmed by a study of the effect of pH. At pH 7.5 the

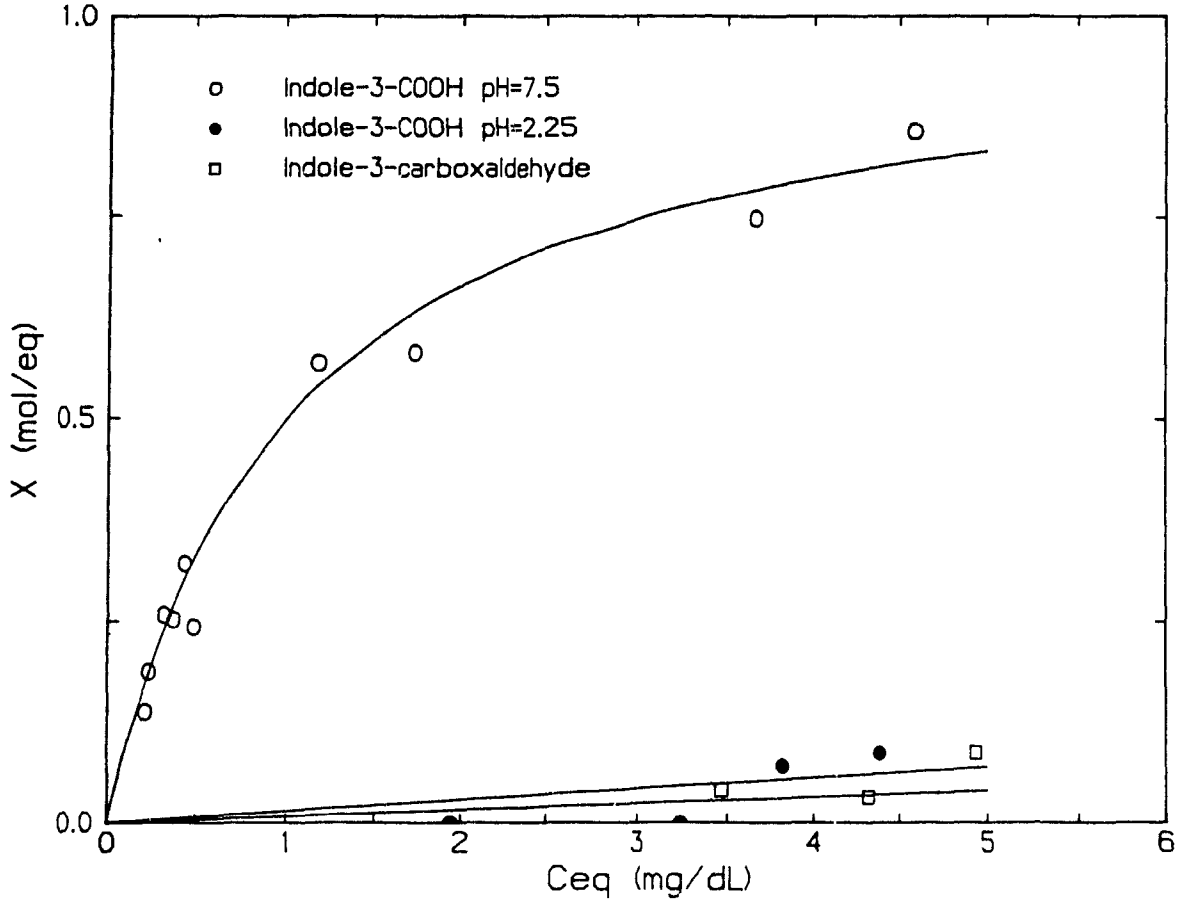

Figure 4.1 Binding isotherms for indole-3-COOH (at different pH values) and indole-3-carboxaldehyde, onto cholestyramine at 20°C, showing the importance of the acidic proton. The curves represent the best fit of points by the Scatchard equation based on a one site model.

binding of indole-3-COOH by cholestyramine occurs to 85% of available sites but at a pH of 2.25 the coverage decreases to ca. 10% (Fig. 4.1). At the lower pH, the dissociation of indole-3-COOH (pK_a = 3.25 (15)) to the ionic form is <10%. Consequently, the acid becomes equivalent to the aldehyde, since neither possesses a charged group to interact with the cholestyramine.

In fact, the isotherms for a series of indole carboxylic acids show that the adsorption maximum (number of molecules adsorbed per functional site), under conditions of complete ionization, decreases slightly as the pK_a increases, i.e., the strength of the acid decreases (Fig. 4.2). Thus, the importance of the acidity in the adsorbate is evident.

The effect of pk_a on adsorption was studied further with similar types of acids. Pyrrole-2-COOH (pK_a = 4.45) and phenyl acetic acid (pK_a = 4.28) were tested and, contrary to predictions based on pK_a's, these acids are less efficiently adsorbed than the indoles (Table 4.1 and Fig. 4.3). Specifically, although indole-3-acetic acid is a much weaker acid than phenyl acetic acid, yet the maximum extent of adsorption of the former is much higher.

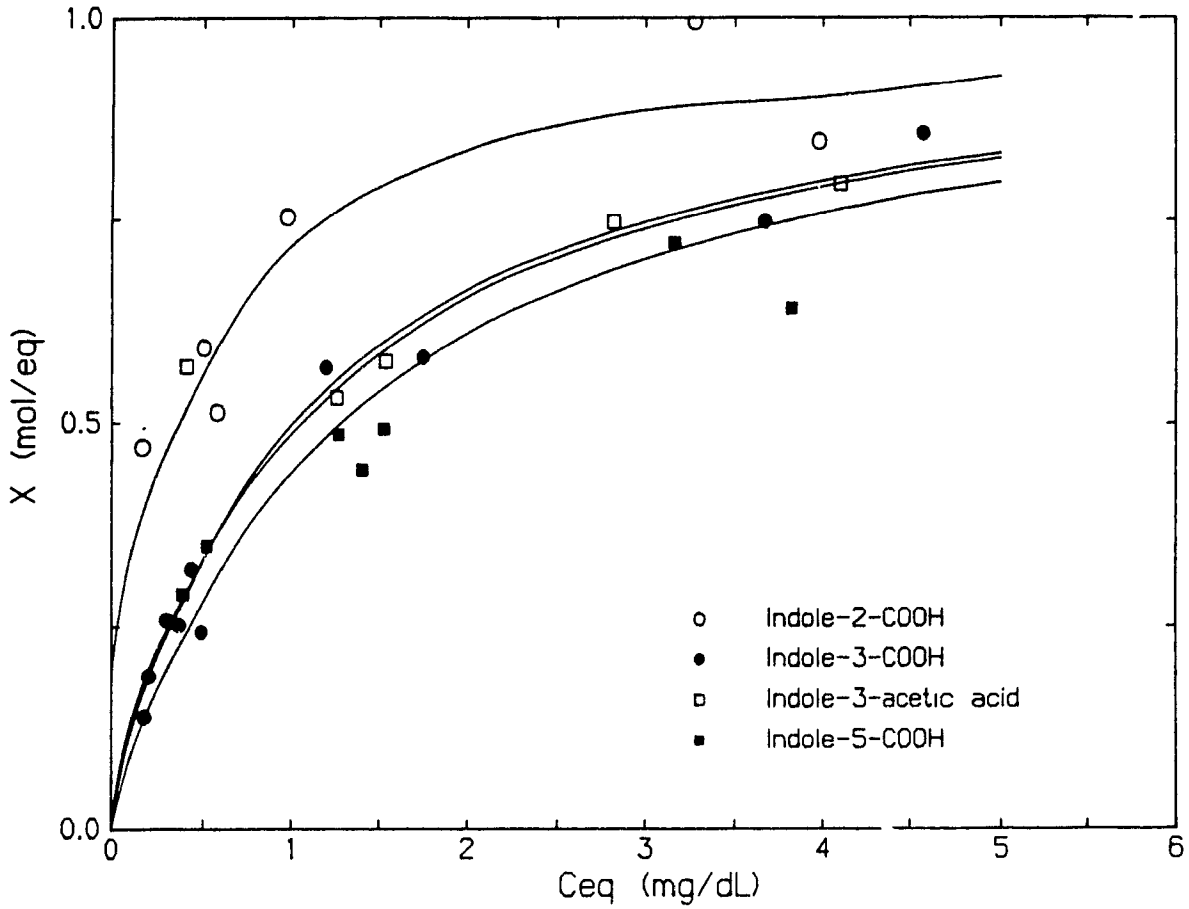

Figure 4.2 Binding isotherms for some substituted indole compounds with various pK_a values (see text) onto cholestyramine at 20°C, pH = 7.5. The curves represent the best fit of points to the Scatchard equation based on a one site model. Note that the curves are drawn in the same order as they are listed in the figure.

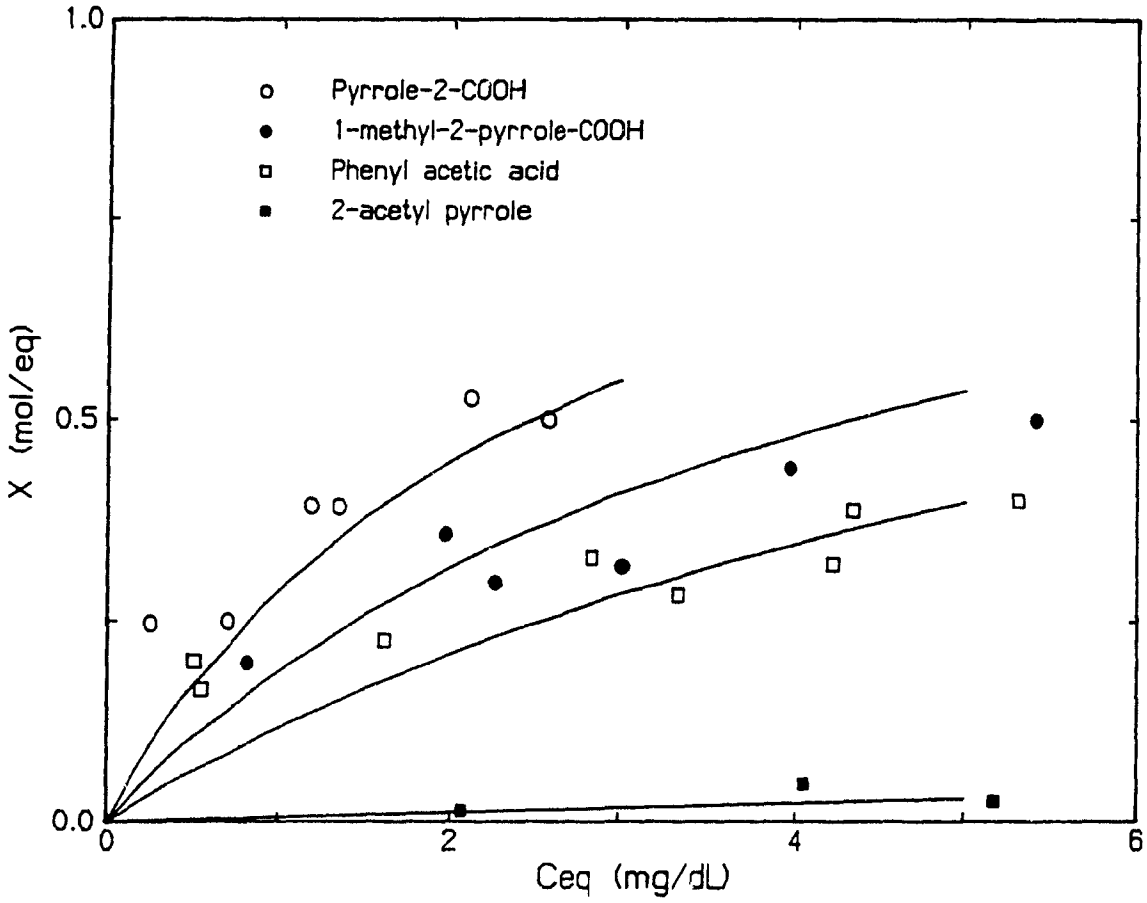

Figure 4.3 Binding isotherms for some substituted pyrroles and phenyl acetic acid onto cholestyramine at 20°C and pH = 7.5. The curves represent the best fit of points to the Scatchard equation based on a one site model.

TABLE 4.1

The pK_a's for Indole and Pyrrole Derivatives and Extents of Adsorption by Cholestyramine.

Molecule	Capacity[a] (mol/eq)	pK_a	Reference
Indole-2-carboxylic acid	0.93	1.75	(13)
Indole-3-carboxylic acid	0.83	3.25	(13)
Indole-3-acetic acid	0.81	4.75	(16)
Indole-5-carboxylic acid	0.77	4.83	(17)
Pyrrole-2-carboxylic acid	0.55	4.45	(14)
1-methyl-2-pyrrole carboxylic acid	0.53	4.4	Estimate
Phenyl acetic acid	0.39	4.28	(18)
Indole-3-carboxaldehyde	0.043	--	
2-acetyl pyrrole	0.029	--	

[a] The capacities were calculated using a Scatchard plot and the binding constants calculated in Table 4.2.

The data in Figs. 4.1 - 4.3 were used to calculate binding constants for all the molecules tested based on the model X=KA/(1+KA), where X is the capacity of the resin (the y scale in the isotherm), A is the equilibrium concentration in moles/l, and K is the binding constant. In all cases Scatchard plots yield a straight line, indicating that the binding sites are equivalent. Furthermore, it implies that the binding of one molecule is independent of the binding of any other molecules. The experimental points were fitted by computer, in a non-linear regression plot, and the calculated binding constants are listed in Table 4.2. The quality of the fit to the data may be seen in the isotherms where the points are the actual data points and the smooth curve is from the calculated binding constants.

TABLE 4.2

Calculated Binding Constants for the Interaction of the Pyrrole and Indole Derivatives With Cholestyramine (20°C, pH = 7.5).

Molecule	K(l/mole X 10^{-4})
Indole-2-carboxylic acid	4.1
Indole-2-carboxylic acid (pH=2.8)	0.059
Indole-3-carboxylic acid	1.7
Indole-3-carboxylic acid (pH=2.25)	0.024
Indole-3-acetic acid	1.7
Indole-5-carboxylic acid	1.3
Pyrrole-2-carboxylic acid	0.45
1-methyl-2-pyrrole carboxylic acid	0.29
Phenyl acetic acid	0.18
Indole-3-carboxaldehyde	0.012
2-acetyl pyrrole	0.0066

Note that the binding constant for indole-3-acetic acid is a factor of ten times greater than that for phenyl acetic acid, yet the former is less acidic. Clearly, although the acidity and, thus, the electrostatic interaction is very important in binding, this is apparently not the only effect contributing to the adsorption, i.e., a secondary interaction, namely the hydrophobic effect, leads to an increase in the observed adsorption. It remains, now, to develop a scheme that permits an evaluation of the relative contributions of these effects.

4.3.2 A QUALITATIVE DESCRIPTION OF THE HYDROPHOBIC INTERACTION

To a first approximation contributions arising from the electrostatic interactions are expected to be related to the strength of the acid, i.e., the pK_a,

while the role of the hydrophobic effect requires an evaluation of the degree of hydrophobicity of the adsorbate. A scale of relative hydrophobicity can be obtained from the retention times for the adsorbate as derived from analysis by reverse phase liquid chromatography. In this type of chromatography the mobile phase (acetic acid/methanol, in this case) is more polar than the stationary phase and hence the elution order is related to the increasing hydrophobic nature of the solute. The more soluble the solute is in water the faster it will elute. Thus, the longer the retention times on the column, the less polar the molecule and hence the more hydrophobic it must be.

Using this criterion it is possible to list the small molecules in order of increasing hydrophobic nature, as seen in Table 4.3. These data permit a qualitative explanation for some of the apparent anomalies seen in the adsorption data when only pK_a's were considered. For example, it was noted that although both pyrrole-2-COOH and phenyl acetic acid are stronger acids than indole-5-COOH and indole-3-acetic acid, they are less effectively adsorbed by cholestyramine. The data in Table 4.3 indicate that the two indoles are more hydrophobic. Apparently, this secondary interaction contributes to more effective adsorption.

It is also interesting to note that although the difference in acidity of indole-3-COOH (pK_a = 3.25) and indole-3-acetic acid (pk_a = 4.75) is very large, the extents of adsorption by cholestyramine are essentially identical at plateau conditions (0.83 and 0.81, respectively). As noted in Table 4.3, of the molecules studied the indole-3-acetic acid is the most hydrophobic. Thus, even though it is a weaker acid, it is still adsorbed onto the resin to the same extent as the indole-3-COOH. On a qualitative basis, therefore, the hydrophobic effect can explain some of the anomalies in the binding behaviour.

TABLE 4.3

The Order of Increasing Hydrophobic Nature of the Indoles and Pyrroles Studied, Based on Their Retention Times in Reverse Phase Chromatography.

Molecule	Retention time (min)
Pyrrole-2-carboxylic acid	«4.4[a]
Phenyl acetic acid	4.4
2-acetyl pyrrole	4.5
Indole-3-carboxaldehyde	4.7
1-methyl-2-pyrrole carboxylic acid	4.8
Indole-5- and -3-carboxylic acid	5.2
Indole-2-carboxylic acid	5.7
Indole-3-acetic acid	7.2

[a] An accurate relative value cannot be determined for this pyrrole since a different mobile phase was used.

4.3.3 THERMODYNAMIC MODEL OF THE BINDING

To more fully understand the complete binding mechanism a semi-quantitative analysis based on thermodynamic arguments was undertaken. The free energy of the overall adsorption process may be expressed as:

$$\Delta G^{o}_{ads} = \Delta H^{o} - T\Delta S^{o} = -RT\ln K_{ads} \qquad [4.1]$$

Rearrangement of equation 4.1 yields:

$$\ln K_{ads} = \Delta S^{o}/R - (\Delta H^{o}/R)(1/T) \qquad [4.2]$$

If both $\Delta S°$ and $\Delta H°$ are constant within the temperature range studied, a straight line will be obtained by plotting ln K_{ads} as a function of 1/T.

From a thermodynamic point of view, the enthalpy and entropy changes in the adsorption process may be broken down into three steps, each step contributing to the overall change. These steps include:

(1) desolvation of the small molecule;

(2) desolvation of the active site on the resin;

(3) binding of the desolvated molecule by the desolvated resin.

The **overall** enthalpy change of the adsorption process is thus a sum of the enthalpy changes for all three steps. It is clear that the enthalpy change in the binding step, step 3, must be a negative quantity since this step includes the formation of a strong ionic bond. Steps 1 and 2 involve the breaking of interactions between the solvent and the small molecule and the solvent and cholestyramine, respectively, and thus result in positive enthalpy changes. When considering the enthalpy term in the overall adsorption it must be remembered that it is the sum of the enthalpies in these three steps which is important.

Similarly, the **overall** entropy change is the important part of equation 4.1 and it can be viewed as a sum of contributions from the three steps described above. The entropy change of step 3 (the binding step) is negative since the bound state is clearly more ordered than the unbound state. The entropy change due to desolvation involves the breaking of the icebergs, discussed earlier, which leads to a restructuring of the water molecules. The water molecules are now in a more disordered state and thus the overall entropy change associated with these steps must be positive.

The free energy for the adsorption process includes contributions from the overall entropy change and the overall enthalpy change. For a strong interaction, $\Delta G°$ must be negative, which requires that $\Delta S°$ be positive and/or $\Delta H°$ be

effects due to changes in enthalpy and entropy. This can lead to an understanding of the separate contributions to the binding from the electrostatic and hydrophobic interactions. (A positive entropy change, for example, is indicative of a hydrophobic interaction.)

Jacobsen (19) studied the temperature dependence of the binding of bilirubin by human serum albumin, calculated ΔG°, and, using a van't Hoff plot, calculated the enthalpy and entropy of binding. He found that the binding was driven by a strong enthalpic force (-56 kJ/mol) which was counteracted by a decrease in entropy (-35.5 J/mol deg). Since hydrophobic bonding is driven by a positive entropy change, he concluded that hydrophobic forces are not the main binding forces, but that they do contribute to the overall binding.

To this end, the temperature dependence of the adsorption isotherms was studied and binding constants were determined from binding isotherm data (Fig. 4.4 A/B) for indole-2-COOH, indole-5-COOH, phenyl acetic acid and pyrrole-2-COOH at 0 and 40°C, (Table 4.4) in addition to the values at 20°C reported in Table 4.2. The fit to the data is illustrated by the curves in Fig 4.4 A/B. The binding constants were used to determine the free energy change for the overall binding process, ΔG°_{ads}, from equation 4.1.

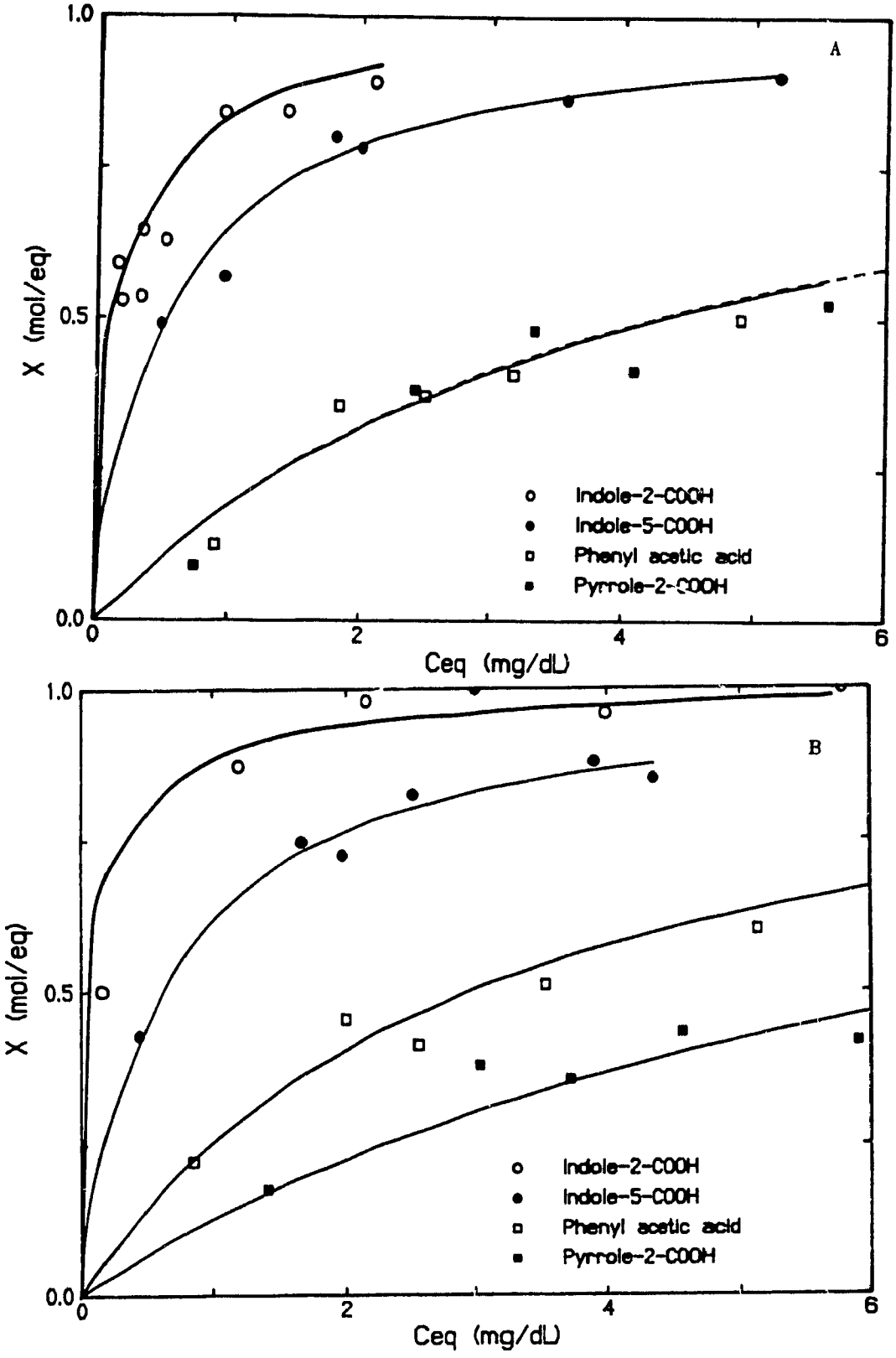

Figure 4.4 A/B Binding isotherms for some substituted indoles, pyrroles and phenyl acetic acid onto cholestyramine at (A) 0°C and (B) 40°C, pH = 7.5. The curves represent the best fit of points to the Scatchard equation based on a one site model.

TABLE 4.4

Calculated Binding Constants for the Interaction of Selected Molecules With Cholestyramine at Three Temperatures (pH=7.5)

Molecule	Temp (°C)	$K_{ads} \times 10^{-4}$	$\Delta G°_{ads}$ (kJ/mol)
Indole-2-COOH	0	8.3	-26
	20	4.1	-26
	40	12	-30
Indole-5-COOH	0	3.0	-23
	20	1.3	-23
	40	2.6	-26
Phenyl acetic acid	0	0.32	-18
	20	0.15	-18
	40	0.46	-22
Pyrrole-2-COOH	0	0.26	-18
	20	0.45	-20
	40	0.16	-19

Within experimental error, estimated to be $\pm$5%, the free energy change of adsorption appears to remain constant between 0 and 20°C for all the molecules tested. (At all times the change in the free energy will be discussed in terms of the magnitude of the free energy, to avoid complications due to the sign). However, on going from 20 to 40°C the free energy change of adsorption for the indoles and the phenyl acetic acid increases significantly while that for the adsorption of pyrrole-2-COOH remains unchanged. Thus, in almost all cases, as the temperature increases the free energy change of adsorption increases, indicating that the higher the temperature the stronger the adsorption.

The change in $\Delta G°_{ads}$ as a function of temperature is best understood if the separate contributions from the enthalpy and the entropy can be evaluated.

However, since the binding is not a simple process the plot of ln K as a function of 1/T, using the data in Table 4.4, does not yield a straight line (equation 4.2). Moreover, the available data are insufficient to permit the evaluation of the separate contributions from the enthalpy and the entropy since the plot is parabolic.

Strong evidence has been given to indicate that the binding mechanism includes contributions from hydrophobic interactions. A quantitative evaluation of this effect should contribute to the understanding of the thermodynamics, which, in turn, can help to understand the variation of $\Delta G°_{ads}$ with temperature. To this end, the following approach is proposed to isolate the contribution from hydrophobic interactions: Ignoring ionic interactions, adsorption can be thought of as corresponding to the transfer of the adsorbate from the aqueous environment to a hydrophobic environment, similar to that which the adsorbate experiences in the pure liquid phase.

The thermodynamic parameters for the transfer of a hydrophobe from water to the pure organic phase are given by a quantity known as the free energy of transfer, ΔG_T, which describes the free energy change associated with the transfer of the hydrophobe molecule from the environment of the pure liquid phase to an environment of pure water. This can be calculated from data for the temperature dependence of the solubility of the hydrophobe in water (20). Solubility measurements of this kind yield the free energy of transfer of the hydrophobe from its pure liquid state to a pure aqueous state. Using the argument given above, this corresponds to the opposite of the adsorption process. However, once $\Delta G°_T$ has been calculated in this fashion, the transfer of the hydrophobe in the other direction, i.e., from an aqueous environment to the organic environment (adsorption process) may be obtained by changing the sign of $\Delta G°_T$.

The thermodynamic terms were calculated as follows:

The free energy for solution is given by:

$$\Delta G^{\circ}_{solution} = -RT\ln X \qquad [4.3]$$

where X = mole fraction of solute at saturation.

It can also be expressed as:

$$\Delta G^{\circ}_{solution} = \Delta H^{\circ}_{fusion}(1 - T/T_f) + \Delta G^{\circ}_{transfer} \qquad [4.4]$$

where T = temperature of experiment;

T_f = melting temperature;

ΔH°_f = heat of fusion.

The effect of temperature on solubility is described by

$$\Delta \ln c_{saturated}/\Delta T = \Delta H^{\circ}/RT^2 \qquad [4.5]$$

where $c_{saturated}$ = solubility;

$\Delta H^{\circ} = \Delta H^{\circ}_{fusion} + \Delta H^{\circ}_{transfer}$, in this case.

In this calculation it was assumed that, for these carboxylic acids, the enthalpy of dissociation is negligibly small, an assumption that is valid for many carboxylic acids since $d(\ln K_a)/dT$ is almost zero for small dT near room temperature (21).

The solubilities of indole-2-COOH, indol- 3-COOH, pyrrole-2-COOH and phenyl acetic acid as a function of temperature are plotted in Fig. 4.5. (The only literature values found were for phenyl acetic acid at 20°C. They were 0.1219 moles/l (22) and 0.1175 moles/l (23) and can be compared to 0.105 moles/l determined here. The error, 12-15%, is unexpectedly high and may reflect differences in pH.) Clearly, the indole-carboxylic acids have a much lower solubility than the pyrrole-2-COOH, which is less soluble than phenyl acetic acid.

The heats of fusion, calculated from DSC measurements, are given in Table 4.5. Because pyrrole-2-COOH decomposes on melting, a heat of fusion could not be determined directly. A value was estimated based on the entropies of fusion. For the other three carboxylic acids these vary between 41 and 49

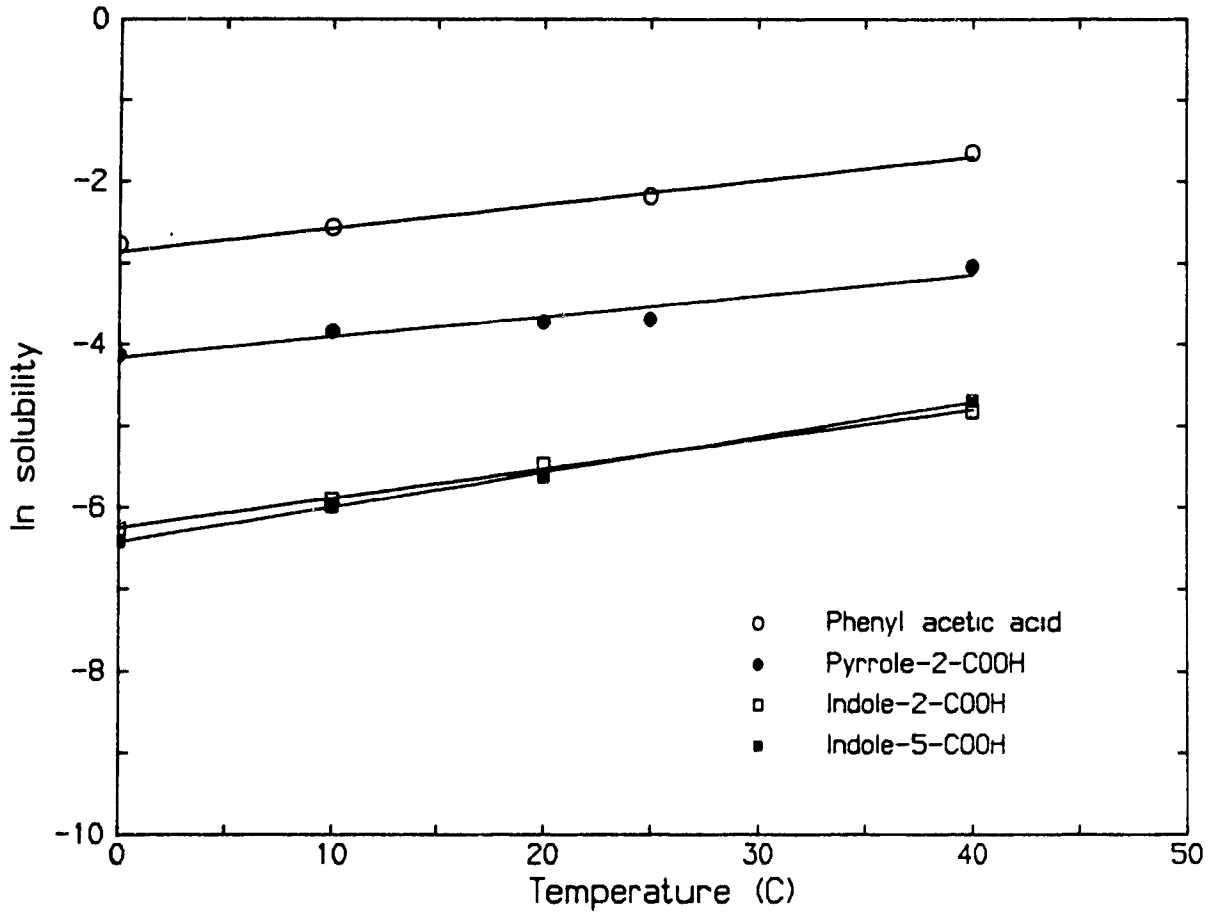

Figure 4.5 The variation in the natural log of the solubility of specific small molecules studied as a function of temperature.

J/mol deg, with an average of 44.7. Using this value, together with the experimentally determined melting point, results in a value of 21.4 kJ/mol for the heat of fusion of pyrrole-2-COOH.

TABLE 4.5

The Heats of Fusion Determined by DSC Measurements.

Molecule	$\Delta H^{\circ}_{fusion}$ (kJ/mol)	T_{fusion} (K)	$\Delta S^{\circ}_{fusion}$ (J/mol deg)
Indole-2-COOH	21.0	480.4	43.7
Indole-5-COOH	23.6	480.8	49.1
Pyrrole-2-COOH	21.4[a]	479.5	44.7[a]
Phenyl acetic acid	14.48	349.7	41.4 (16)

[a] Estimated as described in text because of decomposition on melting.

Thus, using the slope of the natural log of the solubility as a function of temperature and the heats of fusion, (Table 4.5) the free energies of transfer and the enthalpies of transfer can be calculated. These can be combined to obtain the entropy of transfer from the Gibbs free energy equation. The data may be found in Table 4.6.

As stated previously, the explanation for the change in the free energy of adsorption (Table 4.4) as a function of temperature must lie in the overall entropy and enthalpy changes in the process, but separate contributions could not easily be calculated. However, separate contributions of enthalpy and entropy can be determined for the **transfer** process, and the results obtained here can be used to understand the change in free energy of **adsorption** as a function of temperature.

TABLE 4.6

The Free Energy, Enthalpy and Entropy of Transfer of Some Small Molecules From the Liquid Phase into Pure Water at Various Temperatures.

Molecule	Temp (°C)	$\Delta G°_{transfer}$ (kJ/mol)	$\Delta H°_{transfer}$ (kJ/mol)	$\Delta S°_{transfer}$ (J/moldeg)
Indole-2-COOH	0	14.2	1.62	-46.1
	20	14.9	5.05	-33.6
	40	15.7	8.73	-27.9
Indole-5-COOH	0	13.5	3.11	-38.1
	20	14.3	7.16	-24.3
	40	14.5	11.5	-9.58
Pyrrole-2-COOH	0	9.30	-5.70	-54.9
	20	10.5	-3.30	-47.1
	40	11.0	-0.80	-37.7
Phenyl acetic acid	0	12.2	2.68	-34.9
	20	12.6	5.29	-24.8
	40	13.2	8.08	-16.4

The thermodynamic quantities of transfer of a hydrophobic group from an inert solvent into water ordinarily have the following signs (4): $\Delta G°_T>0$, $\Delta H°_T<0$, $T\Delta S_T<<0$. However, the enthalpy change can, on occasion, be positive, the sign depending on the net change in the number of hydrogen bonds and the strength of these bonds. The data in Table 4.6 indeed show small positive ΔG_T and large negative $\Delta S°_T$ terms. For three of the four compounds positive enthalpy terms are obtained for the transfer from an organic environment to water indicating an unfavourable energy change. This reflects an increase in the number, or strength of the hydrogen bonds broken and then reformed after the hydrophobe is

dissolved in water. The opposite is true for the pyrrole-2-COOH for which the enthalpy change is favourable which is consistent with the smaller free energy of transfer of this molecule into water (hence it is not very hydrophobic) and its very short retention time in reverse phase chromatography. All of these data point to a special case for this molecule. The hydrogen on the pyrrole nitrogen apparently is very susceptible to hydrogen bonding with the water. This is in accord with the retention times, in Table 4.3, which give a measure of the hydrophobicity of the molecule. The pyrrole-2-COOH (R_T = «4.4 min) is much less hydrophobic than the 1-methyl-2-pyrrole-COOH (R_T = 4.8 min). The replacement of the hydrogen on the nitrogen by a methyl group, eliminates the possibility of hydrogen bonding at this point of the molecule. This changes the pyrrole-2-COOH from being the most hydrophilic to being even more hydrophobic than the indole-3-carboxaldehyde (Table 4.3).

As can be seen from Table 4.6, $\Delta G°_T$ increases in magnitude as a function of temperature, which is consistent with the view that iceberg formation decreases with temperature, as confirmed by Shinoda's theoretical approach. According to Tanford, the increase in the free energy of transfer represents an increase in the hydrophobic effect.

It is generally known that, whereas $\Delta G°_T$ increases with increasing hydrophobicity, the corresponding enthalpy and entropy functions show no corresponding regularity (18). According to Tanford, this indicates that the water molecules at a hydrocarbon-water interface do not have a unique way of arranging themselves, but that different arrangements are possible leading to different enthalpy and entropy terms. However, the terms must change together since $\Delta G°_T$ remains constant. Thus, the high values of $\Delta S°$ for pyrrole-2-COOH do not reflect a higher hydrophobic nature, but, rather, it is the low $\Delta G°_T$ which reflects a low hydrophobicity. Note also that the $\Delta G°_{ads}$ and the $\Delta G°_T$ are opposite

in sign, as expected, and in both cases the magnitude of the free energy increases with temperature.

It is important also to note that the entropy of transfer is negative in all cases, as expected for the formation of icebergs. As the temperature is increased the entropy effect decreases, indicating that there are less cages being formed, or the cages being formed are less ordered. Thus, at higher temperatures the molecule is in a less favourable environment and can bind more easily, as verified by the increased free energy of binding in Table 4.4.

TABLE 4.7

A Summary of Data in Tables 4.3, 4.4 and 4.7 Showing the Trends in Hydrophobicity and Absolute Values of Their Free Energies at 20°C. The Molecules are Listed in Order of Decreasing Hydrophobicity.

Molecule	$\Delta G^\circ_{adsorption}$ (kJ/mol)	$\Delta G^\circ_{transfer}$ (kJ/mol)
Indole-2-COOH	26	14.9
Indole-5-COOH	23	14.3
Phenyl acetic acid	18	12.6
Pyrrole-2-COOH	20	10.5

The summary of the data, given in Table 4.7, shows that increases in the the hydrophobicity, as determined by retention times, cause increases in both ΔG°_{ads} and ΔG°_T. The table above also shows that, with the exception of pyrrole-2-COOH, as the hydrophobicity decreases the free energy of adsorption decreases. In accordance with Tanford (20), it is seen that molecular size is an important factor in the hydrophobic effect. The larger molecules are more hydrophobic and show a larger change in the free energy, both in binding and

transfer. This explains the trend in capacities of some of the molecules.

From the isotherm and binding data it is clear that the primary interaction between solute and cholestyramine is electrostatic, and thus the pK_a is the most important factor. However, the pK_a alone does not explain the variation in adsorption capacities when comparing the smaller pyrroles to the larger indoles. Specifically, regardless of the pK_a, the indoles always adsorb better than the pyrroles and phenyl acetic acid. This can now be explained by the hydrophobic effect. The larger molecules have larger free energy changes associated with transfer and thus stronger hydrophobic interactions. This leads to an overall stronger binding for the larger molecules.

Using the values of the free energies of adsorption and transfer, it is now possible to give a semi-quantitative description of the overall binding process, ΔG°_{ads}. Recall that the binding mechanism proposed above consists of three steps: 1) desolvation of the small molecule; 2) desolvation of the cholestyramine; and 3) binding of the desolvated cholestyramine and small molecule. The semi-quantitative approach involves an estimation of the free energies involved in each step:

Step 1 (desolvation of the small molecule) can be considered equivalent to the free energy of transfer of the small molecule from the aqueous environment to a non-aqueous environment. The free energy of transfer, ΔG°_T, calculated above from data on the solubility of the small molecules in water reflects the free energy of transfer of the hydrophobe from its pure liquid to the pure aqueous environment. Therefore, the free energy for step 1 is the negative of the free energy of transfer.

Step 2 (desolvation of the active site on the cholestyramine) cannot easily be calculated. However, to a first approximation the free energy associated with this step is expected to be constant for these small molecules and hence can be

this step is expected to be constant for these small molecules and hence can be disregarded in this comparative treatment without adversely affecting the results. It will be shown, by consideration of the adsorption behaviour of uncharged molecules, that this approximation is warranted.

The binding step, step 3, is the force of attraction between the resin and the small molecule. In the case of ionized small molecules this is predominantly the free energy associated with the electrostatic interaction, although contributions from hydrogen bonding and dipole interactions may be included.

Based on this model, the overall free energy for the adsorption, measured from the binding constants, must be due to the combination of steps 1 and 3, i.e., the free energy of transfer (hydrophobic interactions), the electrostatic interactions and other contributions discussed in step 3. The term for the hydrophobic interaction may, to a good approximation, be replaced by the negative of the free energy of transfer of the molecule from the pure hydrocarbon to water, yielding the following equation:

$$\Delta G^\circ_{ads} = -\Delta G^\circ_T + \Delta G^\circ_{elec} + \Delta G^\circ_{other} \qquad [4.6]$$

where ΔG°_{other} = the contributions from interactions other than hydrophobic or electrostatic, as discussed in step 3.

Rearrangement of equation [4.6] gives:

$$\Delta G^\circ_{other} = \Delta G^\circ_{ads} + \Delta G^\circ_T - \Delta G^\circ_{elect} \qquad [4.7]$$

The contribution to the overall free energy from these other contributions may be evaluated by eliminating the free energy due to the electrostatic interactions term, i.e., in a non-ionized molecule. The electrostatic interaction is

obviously very nearly zero in the case of indole-3-COOH at pH = 2.25, indole-3-carboxaldehyde and 2-acetyl pyrrole. Using the data in Table 4.2, ΔG°_{ads} can be calculated as before. The values for ΔG°_T were not determined directly but can be estimated from the retention times in Table 4.3 in conjunction with the free energy of transfer for the molecules listed in Table 4.6. (In fact, there is a linear relationship between the retention time and ΔG°_{ads}.) In particular, since the retention time for indole-3-COOH is identical to that of indole-5-COOH, the free energies of transfer should be very similar. Using these data and approximations, it is seen that ΔG°_{other} is ca. -1 kJ/mole.

TABLE 4.8

The Free Energy of Adsorption and the Free Energy of Transfer for the Non-Ionized Molecules.

Molecule	ΔG°_{ads} (kJ/mol)	ΔG°_T (kJ/mol)
Indole-3-COOH (at pH = 2.25)	-15	14
Indole-3-carboxaldehyde	-12	13
2-acetyl pyrrole	-11	7

Having estimated a value for ΔG°_{other}, it is now possible to evaluate the contributions to the free energy of adsorption from the electrostatic interactions, since:

$$\Delta G^{\circ}_{elect} = -\Delta G^{\circ}_{other} + \Delta G^{\circ}_{ads} + \Delta G^{\circ}_{T} \quad [4.8]$$
$$= +1.0 + \Delta G^{\circ}_{ads} + \Delta G^{\circ}_{T}$$

Using the same estimates of ΔG°_{T} as before, the values obtained for the electrostatic interaction are tabulated in Table 4.9.

TABLE 4.9

Estimated Values of the Free Energy of Binding due only to the Contribution from the Electrostatic Interaction at 20°C

Molecule	ΔG°_{elect} (kJ/mol)	pKa
Indole-2-COOH	-10	1.75
Indole-3-COOH	-9.0*	3.25
Indole-3-acetic acid	-7.0*	4.75
Indole-5-COOH	-7.7	4.83
Phenyl acetic acid	-4.4	4.28
Pyrrole-2-COOH	-8.5	4.45
1-methyl-2-pyrrole-COOH	-13*	4.4
Non-ionized molecules:		
Indole-3-COOH (pH = 2 25)	0*	
2-acetyl pyrrole	-1.0*	
Indole-3-carboxaldehyde	2.0*	

* Estimated values

The data in Table 4.9 show that for a non-ionized molecule the contribution from the electrostatic interaction to the free energy of adsorption is, within experimental error, zero. In addition, the free energy of binding due to the contribution from the electrostatic interaction closely parallels the pK_a. Within

the group of indoles the strongest acid, indole-2-COOH also has the strongest electrostatic interaction, followed closely by indole-3-COOH, indole-5-COOH and indole-3-acetic acid.

In summary, the total free energy of adsorption can be represented by a sum of the free energy due to electrostatic interactions, the free energy due to hydrophobic interactions and a small contribution due to other interactions, such as dipole interactions and hydrogen bonding. In general, the larger molecules have larger contributions from the hydrophobic effects and smaller contributions from the electrostatic interactions. Thus, the binding mechanism is a function of the size of the molecule, and the acidity, and this leads to different hydrophobic and electrostatic effects.

4.4 SUMMARY

The binding of small molecules such as the carboxylic acids of indoles and pyrroles to cholestyramine from aqueous solution involves an electrostatic interaction between the oppositely charged moieties of the adsorbent and adsorbate as well as a secondary effect known as the hydrophobic effect. High binding constants are found when the adsorbate is both a strong acid and a large hydrophobic molecule. If the pH is decreased below the pK_a of the acid the binding constant is decreased dramatically. If the hydrophobic nature is decreased (indole-3-acetic acid as compared to phenyl acetic acid) the binding constant decreases as well.

The concept of the free energy of transfer of a hydrophobe from water to a more organic environment, i.e., the adsorbent, can be used quantitatively in a description of the binding from aqueous solution. Using the free energy of transfer it is possible to isolate the contributions to the binding from electrostatic interactions as well as other contributions, e.g., dipole-dipole and hydrogen

interactions as well as other contributions, e.g., dipole-dipole and hydrogen bonding.

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CHAPTER 5

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE AND SUGGESTIONS FOR FUTURE WORK

5.1 CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The research conducted for this thesis concerns the adsorption of bilirubin and its photoproducts onto cholestyramine and peptide substituted polyacrylamide resins. The investigation also includes a study of the binding behaviour of bilirubin model compounds by cholestyramine. The contributions to knowledge, presented in detail in each chapter, are summarized below.

The effect of environment on the photoreactions of bilirubin was studied, both in aqueous and organic solutions, with respect to photoisomerization reactions. There has been much controversy over certain aspects of the behaviour of bilirubin upon photolysis. In particular, there is still no firm agreement as to the role of protein in photoisomerization of bilirubin; there is still some uncertainty as to the possibility of forming bilirubin isomers in aqueous solution; and there is no consensus on whether the lumirubins are formed directly from bilirubin in the Z,Z form or from the EZ/ZE isomers. Conclusions to these questions as well as others have been reached in this thesis:

1) In the presence of human serum albumin (HSA) in aqueous solution the photoisomerization reaction involves bilirubin that is bound to the protein;

2) In the presence of protein bilirubin photoproducts can be formed only in small amounts in aqueous solution, and they rapidly revert to the native Z,Z bilirubin unless "trapped" by HSA;

3) The photoproduct known as lumirubin must be formed from the EZ/ZE isomers and not directly from bilirubin itself;

4) The binding of bilirubin to proteins strongly affects the amount and types of photoproducts formed. When bilirubin is bound to HSA the amount of EZ/ZE isomers formed is very high, as compared to when it is bound to a very similar type of protein, bovine serum albumin (BSA). In the presence of poly-l-lysine, there is little photoisomerization of bilirubin because the photooxidation reaction is preferred;

5) The presence of ethanol in $CHCl_3$ decreases the amount of photoproducts formed in this solvent, whether it is added before or after the start of irradiation;

6) The presence of oxygen increases the rate of formation of photooxidation products as well as the amount of photoproducts formed in $CHCl_3$ and dimethylsulfoxide (DMSO);

The effect of irradiation of the bilirubin solution on the binding behaviour was also studied and it was demonstrated that:

1) The presence of photoproducts increases the initial rate of adsorption onto cholestyramine, and possibly TMG-Ala_3-Support, from aqueous solution. This photoenhanced adsorption is not observed when polyacrylamide resins with pendants consisting of Ala-Arg_5-Ala_3-, Lys_5-Ala_3- and Arg-Ala_4- are used;

2) The adsorbents used in this study did not binding bilirubin from DMSO. However, the lumirubins, formed upon irradiation of bilirubin in DMSO, do in fact bind onto cholestyramine and TMG-Ala_3-Support from DMSO solutions.

Carboxylic acid derivatives of indoles and pyrroles were chosen as bilirubin model compounds and were used to study their binding behaviour from aqueous solution onto cholestyramine. The results obtained here show that:

1) The binding is primarily due to electrostatic interactions and, within a specific group of compounds, for example the indole carboxylic acids, the trend in binding can be predicted from an examination of the respective pK_a's;

2) The secondary interaction involved in the binding, known as the hydrophobic effect, can be quantified using the free energy of transfer term, ΔG_T;

3) When electrostatic interactions are eliminated by either eliminating the carboxyl group or by ensuring its protonation by controlling the pH, the binding behaviour is shown to depend upon only the hydrophobic interaction. Thus the binding of a non-ionized molecule onto cholestyramine can be quantified using the free energy of transfer calculated from a thermodynamic treatment of solubility data as a function of temperature;

4) It is possible to quantify the electrostatic contribution to the overall binding by subtracting out the free energy of transfer term (i.e., the hydrophobic term) from the free energy of binding calculated from binding constants;

5.2 SUGGESTIONS FOR FUTURE WORK

The photoisomerization and photooxidation of bilirubin has been shown in this thesis to be dependent upon the environment of bilirubin during photolysis. Among the more interesting results from these studies concerns the behaviour of bilirubin when it is in the bound state. Specifically, bilirubin bound to human serum albumin (HSA) yields large amounts of EZ/ZE isomers as well as lumirubins, whereas the concentration of all photoproducts decreases dramatically when bilirubin is bound to a very similar protein, bovine serum albumin (BSA). Furthermore, when bilirubin is bound to polylysine almost no photoproducts are formed, and, in fact, photooxidation products predominate. These data raise interesting questions as to the exact nature of the binding of bilirubin to these, as well as other proteins. Further studies should be done to better understand the

interactions of bilirubin and these proteins to understand the differences in the photolysis behaviour.

In Chapter 2 data were presented indicating that HSA could "trap" photoproducts by binding and protecting the EZ/ZE isomers. A study of the binding of the EZ/ZE isomers onto BSA and polylysine should provide insight about the binding site and binding mechanism of the photoproducts. Indeed, it would be interesting to isolate the lumirubins and study the binding of these photoproducts by serum albumin and polylysine, both alone and in competition with bilirubin and the EZ/ZE isomers.

The trapping of photoprouucts by HSA as described in Chapter 2 also implies that the photoproducts bind immediately to the protein. A study of the kinetics of binding of the photoproducts as compared to the kinetics of binding of bilirubin should also be considered. This could lead to more information about the binding site and binding mechanism.

In Chapter 3 a discussion is presented and it is concluded that it could not be determined if the polyacrylamide resins studied showed any specificity for the photoproducts relative to bilirubin. This question could be answered by changing some of the conditions of the experiment. For example, it was shown that :

$$BR + resin \xrightarrow{k_1} BR\text{-}resin$$

$$BR + h\nu \xrightarrow{k_2} EZ/ZE \text{ isomers}$$

$$EZ/ZE \text{ isomers} + resin \xrightarrow{k_3} EZ/ZE \text{ isomers-resin}$$

The rate of adsorption of bilirubin and/or photoproducts is shown to be too fast relative to the formation of photoproducts (EZ/ZE isomers, which revert to bilirubin) and thus it is not possible to determine absolutely if the resin would adsorbs the EZ/ZE isomers preferentially to bilirubin. This may be overcome by

increasing the light intensity of the irradiating light source, since it is shown in Chapter 2 that such an increase would lead to an increased rate of formation of photoproducts, hence the rate of the second reaction in the above scheme would increase. This can be done by either changing the light source or by using more than one source. In this way the rate of production of photoproducts may be high enough to be able to study the effect of the EZ/ZE isomers on these resins.

Alternatively, lumirubins (recall that the lumirubins will not revert back to bilirubin) could be isolated and prepared with a bilirubin solution to study the relative rates of adsorption of these photoisomers, which would be exactly k_1/k_3. These experiments would lead to important information regarding the strength of binding of the lumirubins versus bilirubin. It would lead to more evidence about the importance of the more accessible COO^- group and the importance of hydrophobic and polarity effects.

Once the specifics of the binding of both the EZ/ZE isomers and the lumirubins has been determined, it would be interesting to try competition experiments with HSA to see how strongly these photoisomers are bound to the resin.

The work presented in Chapter 4 provides important information as to the importance of hydrophobic interactions in the binding of small molecules to cholestyramine. This was done by eliminating electrostatic interactions by suppressing the proton leaving capability of the acid. Further systematic studies of the effect of pH on the binding of indoles and pyrroles onto cholestyramine would give more information as to the importance of this interaction. Based on the preliminary work done here it should be possible to do a quantitative study of electrostatic and hydrophobic effects. Along with a study of pH, a complete quantitative study should include the use of larger molecules, and dicarboxylic acids.

6.1 EXPERIMENTAL DATA

DATA FOR FIGURE 2.2A

The absorbance difference of the gain peak at 500 nm in $CHCl_3$ and $CHCl_3$/EtOH, irradiated with and without O_2.

time (min)	Abs. diff $CHCl_3$		Abs. diff.$CHCl_3$/EtOH	
	O_2	no O_2	O_2	no O_2 (x 10^3)
0.00	0.00	0.00	0.00	0.00
1.00	1.38	3.45	1.19	1.19
2.00	6.21	6.90	-	3.57
3.00	5.52	8.28	3.57	4.76
4.00	8.28	9.66	3.57	4.76
5.00	12.4	11.0	3.13	5.95
7.00	13.1	13.8	5.95	7.14
10.0	17.9	16.6	5.95	9.52
15.0	23.4	20.7	10.7	9.52
20.0	27.6	23.4	13.1	11.9
25.0	31.7	24.8	-	-
30.0	35.9	26.3	15.6	10.7
45.0	37.9	28.0	19.0	10.2
60.0	39.1	28.0	19.0	10.2
90.0	34.8	25.5	18.8	10.2
100.	32.6	24.5	18.1	-

DATA FOR FIGURE 2.2B

The absolute value of the absorbance difference of the loss peak at 460 nm in $CHCl_3$ and $CHCl_3$/EtOH, irradiated with and without O_2.

time (min)	Abs. diff $CHCl_3$		Abs. diff. $CHCl_3$/EtOH	
	O_2	no O_2	O_2	no O_2 (x 10^2)
0.00	0.00	0.00	0.00	0.00
1.00	0.552	0.414	0.952	0.833
2.00	0.966	1.24	1.51	1.90
3.00	1.79	1.38	2.26	2.62
4.00	2.34	2.76	2.86	3.09
5.00	1.93	3.31	3.33	3.33
7.00	3.72	4.41	4.05	4.28
10.0	5.03	5.79	5.36	5.36
15.0	6.89	7.59	6.66	6.66
20.0	8.48	9.10	7.97	7.62
25.0	9.93	10.2	-	-
30.0	10.8	11.4	9.70	9.76
45.0	13.6	14.0	13.1	12.7
60.0	15.8	16.3	16.8	14.3
90.0	20.5	20.2	23.6	18.6
100.	21.7	21.3	-	-

DATA FOR FIGURE 2.3

The absolute value of the absorbance difference of the gain and loss peaks for the irradiation of bilirubin in pure $CHCl_3$.

time (min)	Absorbance difference (x 10^3) loss peak (460 nm)	gain peak (500 nm)
0.0	0.00	0.00
1.0	8.40	1.10
3.0	18.9	4.20
7.0	50.5	13.7
15.0	65.8	17.9
20.0	77.9	21.1
25.0	87.4	23.2
30.0	96.8	24.2
31.0	97.9	13.7
45.0	97.9	12.6
60.0	110	10.5

DATA FOR FIGURE 2.5A/B

The absolute value of the absorbance difference for the photolysis of bilirubin in DMSO as a function of the time of irradiation with and without O_2.

time (min)	With O_2 Absorbance difference ($\times 10^2$) 430 nm	460 nm	490nm
0.00	0.00	0.00	0.00
1.00	0.00	1.60	0.091
2.00	0.00	2.90	0.11
3.00	0.00	3.80	0.14
4.00	0.00	4.30	0.23
7.00	1.60	4.30	0.55
10.0	2.20	4.50	0.72
15.0	3.40	5.30	0.91
20.0	4.20	5.60	1.1
25.0	4.60	6.10	1.2
30.0	5.60	6.80	1.3
40.0	6.90	8.00	1.7
60.0	9.40	9.50	1.9
70.0	10.2	102	1.7
90.0	12.1	11.9	1.6
120.0	13.5	13.4	1.1

DATA FOR FIGURE 2.5 A/B CONTINUED

time (min)	No O_2 Absorbance difference (x 10^2) 430 nm	460 nm	490nm
0.00	0.00	0.00	0.00
1.00	0.00	1.44	0.11
3.00	0.00	3.44	0.28
4.00	0.00	3.78	0.33
7.00	1.56	4.78	0.39
10.0	2.00	5.67	0.44
15.0	2.20	6.22	0.44
20.0	2.22	6.56	0.50
25.0	2.33	6.78	0.56
30.0	1.56	7.22	0.56
45.0	3.30	8.33	0.67
60.0	4.00	9.44	0.67

DATA FOR FIGURE 2.9

The absorbance difference of the gain peak (490 nm) resulting from the irradiation of a bilirubin solution in the presence of HSA at different light intensities.

time (min)	Absorbance difference (x 10^2) High Intensity	Low Intensity
0.00	0.00	0.00
2.00	57.0	23.0
3.00	92.0	36.0
8.00	101	66.0
12.0	127	83.0
15.0	134	85.0
20.0	140	-
25.0	147	102
30.0	148	111
40.0	145	136
50.0	-	139
70.0	-	142
90.0	-	143

DATA FOR FIGURE 2.11

The absolute value of the absorbance difference of the gain peak (490 nm) and the two loss peaks (448 nm and 408 nm) for a solution of bilirubin and HSA pH = 7.4, after 7.0 minutes of irradiation.

[HSA]/[BR]	Absorbance difference		
	490 nm	448 nm	408 nm
0.20	16	14	14
0.33	38	18	21
0.50	56	16	26
0.77	96	19	30
0.91	--	18	38
1.0	140	20	44
1.1	76	17	--
1.3	140	19	55
1.5	136	--	51
1.8	141	16	52
3.0	128	15	49
4.0	124	--	--
5.0	122	15	49
7.0	122	14	48
10	104	16	45

DATA FOR FIGURE 2.12

The absorbance difference of the gain peak (490 nm) for a solution of bilirubin and HSA pH = 7.4, after 7.0 minutes of irradiation, at 1/10 the concentration of both HSA and BR when compared to Fig. 2.11.

[HSA]/[BR]	Absorbance difference 490 nm
0.2	0.0
0.5	6.0
1.0	16.0
1.3	16.0
1.8	15.0
3.0	15.0
5.0	15.0
7.0	16.0

DATA FOR FIGURE 2.13

The absolute values of the absorbance difference at 490 nm, 460 nm and 410 nm for a photolyzed solution of bilirubin after mixing with HSA.

time (min)	ΔA (490nm) (x 1000)	ΔA (460nm) (x 1000)	ΔA (410nm) (x 1000)
0.0	0.0	0.0	0.0
5.0	14.0	---	---
9.0	16.0	-2.0	---
10.0	31.5	---	---
11.0	---	---	-1.8
12.0	29.0	-5.5	---
15.0	44.9		-5.0
17.0	47.0	-1.5	---
20.0	---	-2.8	-11.8
25.0	---	---	-18.0
30.0	---	---	-29.8

DATA FOR FIGURE 2.15

Percentage loss of bilirubin as a function of time for a solution of bilirubin and polylysine, DP = 1150, pH = 11.0, L/B = 10.

Time (min)	Percentage loss of bilirubin (448 nm)
1.0	0.678
2.0	1.22
3.0	1.58
4.0	1.76
5.0	2.83
7.0	4.62
9.0	6.87
11.0	8.56
13.0	10.2
15.0	12.4
17.0	14.3
19.0	15.8
21.0	18.4
23.0	20.3
25.0	22.3
27.0	24.6
29.0	26.2
31.0	27.8
33.0	30.4
35.0	31.8
37.0	34.4
39.0	35.8
41.0	37.2
43.0	38.8
45.0	40.4
47.0	41.5
50.0	43.7
53.0	45.6
55.0	46.8
60.0	49.1
63.0	50.5
65.0	52.1
70.0	53.0
75.0	50.9

DATA FOR FIGURE 3.3 A-E

A. The amount of bilirubin (in moles/eq) adsorbed onto cholestyramine from aqueous solution as a function of time for both irradiated and unirradiated solutions.

Time (min)	X (mol/eq) unirradiated	X (mol/eq) irradiated
5.0	0.00753	0.0119
10	0.0101	0.0139
15	0.0136	0.0163
25	0.0179	0.0171
30	0.0208	0.0207
40	0.0228	0.0227
60	0.0260	0.0236

B. The amount of bilirubin (in moles/eq) adsorbed onto TMG-Ala_3-Support from aqueous solution as a function of time for both irradiated and unirradiated solutions.

Time (min)	X (mol/eq) unirradiated	X (mol/eq) irradiated
5.0	0.0488	0.0665
10	0.0105	0.0791
15	0.0872	0.120
30	0.181	0.196
60	0.258	0.260

DATA FOR FIGURE 3.3 C

C. The amount of bilirubin (in moles/eq) adsorbed onto Ala-Arg_5-Ala_3-Support from aqueous solution as a function of time for both irradiated and unirradiated solutions.

Time (min)	X (mol/eq) unirradiated	X (mol/eq) irradiated
5.0	0.525	0.371
10	0.750	0.647
15	0.967	0.880
30	1.09	1.10
60	1.33	1.37

D. The amount of bilirubin (in moles/eq) adsorbed onto Lys_5-Ala_3-Support from aqueous solution as a function of time for both irradiated and unirradiated solutions.

Time (min)	X (mol/eq) unirradiated	X (mol/eq) irradiated
5.0	0.359	0.351
10	0.557	0.554
15	0.695	0.694
30	1.02	0.955
60	1.23	1.12

DATA FOR FIGURE 3.3 E

E. The amount of bilirubin (in moles/eq) adsorbed onto ARG-Ala_4-Support from aqueous solution as a function of time for both irradiated and unirradiated solutions.

Time (min)	X (mol/eq) unirradiated	X (mol/eq) irradiated
5.0	0.289	0.251
10	0.333	0.332
15	0.380	0.399
30	0.373	0.394
60	0.409	0.394

DATA FOR FIGURE 3.4

Control experiment

Time (min)	Absorbance
0.0	0.830
5.0	0.820
10	0.791
15	0.820
20	0.839
30	0.821
50	0.833
55	0.819

DATA FOR FIGURE 3.6

The absorbance difference at 456 nm as a function of time of irradiation for a solution of bilirubin in DMSO in the absence of any resin.

Time (min)	Absorbance change
5.0	0.0084
10	0.0175
20	0.0218
40	0.0350
90	0.0550

DATA FOR FIGURE 3.7

The amount of bilirubin (in moles/eq) adsorbed onto TMG-Ala_3-Support from DMSO as a function of time for both irradiated and unirradiated solutions.

Time (min)	X (mol/eq) unirradiated	X (mol/eq) irradiated
5.0	0.012, 0.00	0.010, 0.00
10	0.0098, 0.0076	0.041, 0.033
20	0.020, 0.019	0.046, 0.109
45	0.0098, 0.046	0.133, 0.169
90	0.0098, 0.046	0.144

DATA FOR FIGURE 3.8

Adsorption isotherms for the lumirubins onto TMG-Ala_3-Support

T = 20°C		T = 40°C	
Ceq (mg/dl)	X (mol/eq)	Ceq (mg/dL)	X (mol/eq)
1.43	0.108	1.92	0.0734
3.10	0.165	3.45	0.137
5.24	0.173	4.17	0.0734
8.87	0.163	7.39	0.109

DATA FOR FIGURE 3.9

The amount of bilirubin (in moles/eq x 10^4) adsorbed onto cholestyramine from DMSO as a function of time for unirradiated and pre-irradiated solutions.

Time (min)	X (mol/eq) pre-irr . 20 min	X (mol/eq) no pre-irr.	X (mol/eq) pre-irr. no irr. 90 min	X (mol/eq)
5.0	0.00	0.00	1.87	0.00
10	0.00	0.00	0.806	---
15	---	0.00	3.85	0.00
20	2.90	0.00	2.42	---
25	---	---	2.36	0.00
45	5.88	5.52	2.82	0.00
60	---	---	3.13	---
70	---	4.20	---	---
90	6.56	4.40	5.24	---
120	---	5.52	---	---

DATA FOR FIGURE 4.1

Isotherm for indole-3-carboxaldehyde at 20 °C pH = 7.5.

Ceq (mg/dL)	X (mol/eq)
3.47	0.0413
4.32	0.0323
4.93	0.0876
7.09	0.0522
8.83	0.0426

Isotherm for indole-3-COOH at 20 °C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.192	0.138
0.215	0.188
0.365	0.252
0.306	0.258
0.434	0.321
0.493	0.243
1.20	0.568
1.75	0.581
3.67	0.748
4.58	0.856

Isotherm for indole-3-COOH at 20 °C and pH = 2.25

Ceq (mg/dL)	X (mol/eq)
1.95	0.00
3.26	0.00
3.84	0.0714
4.39	0.0874
6.10	0.168
7.40	0.183

DATA FOR FIGURE 4.2

Isotherm for indole-3-COOH at 20°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.192	0.138
0.215	0.188
0.365	0.252
0.306	0.258
0.434	0.321
0.493	0.243
1.20	0.568
1.75	0.581
3.67	0.748
4.58	0.856

Isotherm for indole-2-COOH at 20 °C and pH = 7.5.

Ceq (mg/dL)	X (mol/eq)
0.181	0.470
0.583	0.513
0.506	0.592
0.978	0.753
3.27	0.995
3.98	0.846

Isotherm for indole-5-COOH at 20 °C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.387	0.290
0.524	0.349
1.27	0.486
1.53	0.493
1.41	0.443
3.16	0.721
3.82	0.641

DATA FOR FIGURE 4.2 CONTINUED

Isotherm for indole-3-acetic acid at 20 °C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.408	0.569
1.26	0.531
1.54	0.576
2.83	0.747
4.10	0.794

DATA FOR FIGURE 4.3

Isotherm for pyrrole-2-COOH at 20 °C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.263	0.247
0.690	0.250
1.17	0.393
1.34	0.392
2.12	0.527
2.55	0.500

Isotherm for 1-methyl-2-pyrrole-COOH at 20 °C and pH=7.5.

Ceq (mg/dL)	X (mol/eq)
0.812	0.198
1.97	0.358
2.27	0.298
3.01	0.318
3.96	0.440
5.41	0.500

DATA FOR FIGURE 4.3 CONTINUED

Isotherm for phenyl acetic acid at 20 °C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.511	0.200
0.548	0.165
1.61	0.226
2.83	0.329
3.31	0.283
4.23	0.321
4.34	0.388
5.30	0.400
6.49	0.388

Isotherm for 2-acetyl pyrrole at 20 °C and pH = 7.5.

Ceq (mg/dL)	X (mol/eq)
2.07	0.0137
4.05	0.0473
5.17	0.0251
6.18	0.0421
8.30	0.0336

DATA FOR FIGURE 4.4 A/B

Isotherm for Indole-2-COOH at 0°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.141	0.590
0.332	0.645
0.171	0.528
0.32	0.534
0.947	0.839
0.525	0.629
1.44	0.842
2.11	0.891

Isotherm for Indole-5-COOH at 0°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.49	0.490
0.96	0.568
1.78	0.800
2.00	0.782
3.58	0.863
5.19	0.901

Isotherm for Phenyl acetic acid at 0°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.91	0.125
1.83	0.356
2.48	0.372
3.16	0.407
4.89	0.500
6.14	0.610

Isotherm for Pyrrole-2-COOH at 0°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.76	0.09
2.41	0.382
3.32	0.480
4.08	0.414
5.55	0.525

Isotherm for Indole-2-COOH at 40°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.16	0.501
1.19	0.873
2.17	0.980
3.00	1.00
3.99	0.958
5.78	1.00

Isotherm for Indole-5-COOH at 40°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.43	0.428
1.68	0.747
1.97	0.725
2.51	0.825
3.91	0.879
4.35	0.851

Isotherm for Phenyl acetic acid at 40°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
0.86	0.222
2.00	0.455
2.55	0.411
3.53	0.510
5.14	0.601
6.03	0.772

Isotherm for Pyrrole-2-COOH at 40°C and pH = 7.5

Ceq (mg/dL)	X (mol/eq)
1.42	0.176
3.04	0.379
3.74	0.355
4.58	0.432
5.91	0.418

DATA FOR FIGURE 4.5

The variation in the natural log of the solubility of specific small molecules studied as a function of temperature.

T (°C)	ln solubility			
	Phenyl acetic	Pyrrole -2-COOH	Indole-2-COOH	Indole-5-COOH
0.0	-2.77	-4.13	-6.26	-6.42
10.0	-2.56	-3.84	-5.90	-5.99
20.0	-----	-3.72	-5.47	-5.62
25.0	-2.18	-3.69	-----	-----
40.0	-1.65	-3.04	-4.82	-4.69

6.2 PUBLICATION