A Thesis

entitled

BILIRUBIN AND ITS ESTERS.

Submitted by

Brian Johnson

In partial fulfilment

for the degree

of

DOCTOR OF PHILOSOPHY

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To my Parents....

ACKNOWLEDGEMENTS.

The work described in this thesis is the original work of the author, except where specific acknowledgement is made, and has not been submitted for a degree at any other University.

I am indebted to Dr. D.W. Hutchinson, who directed this work, and Dr. A.J.Knell for their advice, interest and constant encouragement.

I am also grateful to Professor V.M. Clark for the research facilities in the School of Molecular Sciences, University of Warwick, Coventry, between February 1970 and September 1972.

Thanks are also due to Vanessa Aris, who carefully typed the script.

The award of the post of Junior Research Associate at the University of Warwick by the Medical Research Council is gratefully acknowledged.

"Now to assert that these things are exactly as I have described would not be reasonable. But that these things, or something like them, are true ....seems to me fitting.."

Phaedo

Plato.

C O N T E N T S.

ABBREVIATIONS.

The following abbreviations are used in the text:-

ALA	-	δ-Aminolaevulinic acid.
NADPH	-	Reduced nicotinamide adenine dinucleotide phosphate.
HSA	-	Human serum albumin.
BSA	-	Bovine serum albumin.
ORD	-	Optical rotatory dispersion.
CD	-	Circular dicroism.
UDP(G)	-	Uridine diphosphate (-α- glucuronic acid).
EDTA	-	Ethylenediamine tetraacetic acid.
DMSO	-	Dimethyl sulphoxide.
DMF	-	N,N -Dimethylformamide.
TLC	-	Thin layer chromatography.
PLC	-	Preparative layer chromatography.
GLC	-	Gas liquid chromatography.
ER	-	Endoplasmic reticulum.
i.r.	-	Infra red.
u.v.	-	Ultra violet.
n.m.r.	-	Nuclear magnetic resonance.
m.p.	-	Melting point.
b.p.	-	Boiling point.

The following abbreviations are used in the diagrams:-

M	-	$-CH_3$
V	-	$-CH=CH_2$
E	-	$-CH_2CH_3$
P	-	$-CH_2CH_2COOH$
DPM	-	Dipyrrylmethene subunit.

NOTES FOR THE EXPERIMENTAL SECTIONS.

Infra red spectra (i.r.) were recorded with either a Perkin - Elmer 257 or 621 spectrophotometer. Frequencies of the significant absorptions are recorded in $cm.^{-1}$, calibrated against a polystyrene film. The absorptions are designated as w(weak), m(medium), s(strong), and br(broad).

Ultra violet and absorption spectra (u.v.) were recorded with either a Unicam SP800 or a Cary 14 spectrophotometer. Wavelengths are recorded in nm, calibrated against a holmium filter. Extinction Coefficients (ε) were determined using a Unicam SP500 spectrophotometer.

Nuclear magnetic resonance spectra (n.m.r.) were recorded with either a Perkin - Elmer R12 spectrometer at 60MHz or a Varian HA - 100 spectrometer at 100MHz at PCMU, Harwell. The resonances are designated by their chemical shifts (τ) from the internal standard(TMS) at τ=10. Following the τ value in each case, where appropriate, is given, in parentheses, the multiplicity, s(singlet), d(doublet), t(triplet), q(quartet), sp(septet), m(multiplet), br(broad), the integration (H) and the spin - spin coupling constant (J) in Hz.

Elemental analyses were determined by either Alfred Bernhardt (W. Germany) (B) or Dr. F.B. Strauss (Oxford) (S).

Mass spectra were recorded with either an AEI MS902 spectrometer at the University of Hull (Hull) or an AEI MS9 spectrometer at PCMU, Harwell (PCMU). The major peaks are recorded in m/e units, with the relative abundances (as a % of the base peak) in parentheses. The molecular ion is designated by M^+ and the base peak by B^+.

Melting points (m.p.) were determined using a Reichert heated microscope stage and are uncorrected.

Thin layer chromatography (TLC) and preparative layer chromatography (PLC) were carried out on silica gel PF 254 (u.v. sensitive) at thicknesses of 0.25mm and 0.5mm respectively, and on 5 x 20cm and 100 x 20cm plates respectively. The solvents used are specified in the parentheses.

Specific activities of radioactive samples were determined using a Packard 4000 scintillation counter.

SUMMARY

In this thesis some chemical aspects of the bile pigment bilirubin have been examined.

Bilirubin has been shown to exist as the bislactam tautomer, with an intramolecularly hydrogen bonded structure which is independent of the medium. Possible structures are discussed in Chapter 2.

The clinical determination of bilirubin involves treatment of the pigment with diazotised sulphanilic acid (the van den Bergh reaction) when bilirubin is cleaved at the central methylene bridge to form two azopigments. The fate of the central methylene bridge carbon atom was unknown until the present work, but it had been postulated as being liberated as formaldehyde. In Chapter 3, it is shown that formaldehyde is formed during this reaction , and that it can be detected by the product of its reaction with dimedone.

Some metal complexes of bilirubin formed in dipolar aprotic solvents are discussed in Chapter 4. A possible structure for the complex with Zn(II), isolated from DMF solution, is suggested.

In Chapter 5, a potentially extremely useful method for the synthesis of bilirubin conjugates of defined structures is examined, using substituted aryl triazenes, under mild conditions. This method allows the esterification of a specific hydroxyl group in a polyhydroxylated mono- or oligosaccaride without protection of the other hydroxyl groups present. Thus, in this work, using 1 - alkyl -3-p- tolyltriazenes, the hitherto unknown diethyl, diisopropyl and dibenzyl esters of bilirubin have been prepared and characterised.

CHAPTER I.

GENERAL INTRODUCTION.

1. GENERAL INTRODUCTION.

1.1. Historical.

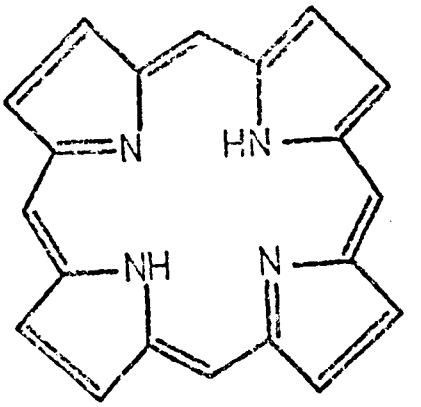
As early as 1347, Virchow had suggested that bile pigments are derivatives of haemoglobin and had described the isolation of " haematoidin" crystals.[1] Hans Fischer, showed that haematoidin was identical to bilirubin.[2] Having determined the structure of haemin [3] and bilirubin [4] Fischer showed that bilirubin was formed from haemin [4] and also synthesised both haemin [5] and bilirubin.[6]

1.2. General Structure of the Bile Pigments.

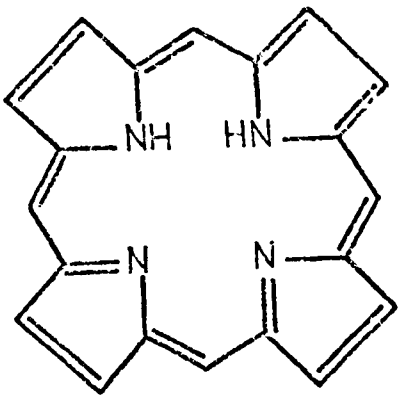
The bile pigments are open chain tetrapyrrole compounds, the pyrrole rings being joined together by single carbon links, which may be either methylene ($-CH_2-$) or methine ($-CH=$) bridges. They are derived from the porphyrins, which are cyclic tetrapyrroles, having the individual pyrrole rings linked by four methine bridges, designated α, β, γ, and δ. The porphyrins are formed by substituting the hydrogen atoms in positions 1 - 8 of the porphin nucleus (1.I.) with various radicals, such as methyl, ethyl, vinyl, carboxymethyl, and 2 -carboxyethyl. The two mesomeric forms of porphin are shown in Figure 1.1, but the true structure of porphin is intermediate between these two extremes.[7]

If the porphyrin ring is opened by oxidation and subsequent loss of one of the methine bridge carbon atoms, open chain tetrapyrroles, bilatrienes, are obtained. Progressive hydrogenation of the remaining methine bridges produces biladienes, bilenes, and bilanes. If the carbon bridges are designated a, b, c, [8a], then the series of compounds shown in Figure 1.2 is obtained.

In the case when the porphin nucleus is substituted with four methyl groups, two vinyl groups, and two 2 -carboxyethyl



(1.I)



(1.I)

FIGURE 1.1. TAUTOMERIC FORMS OF PORPHIN

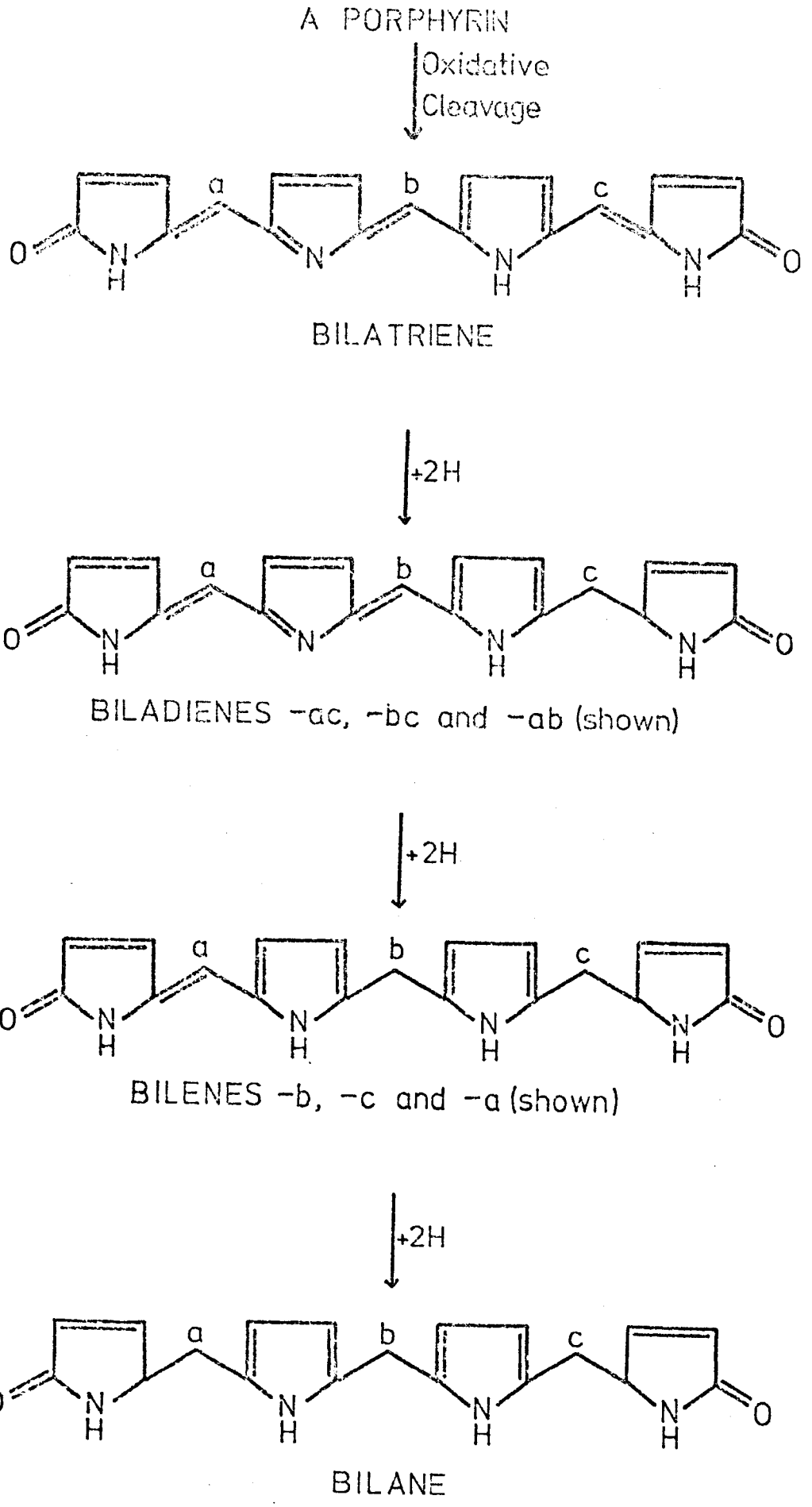


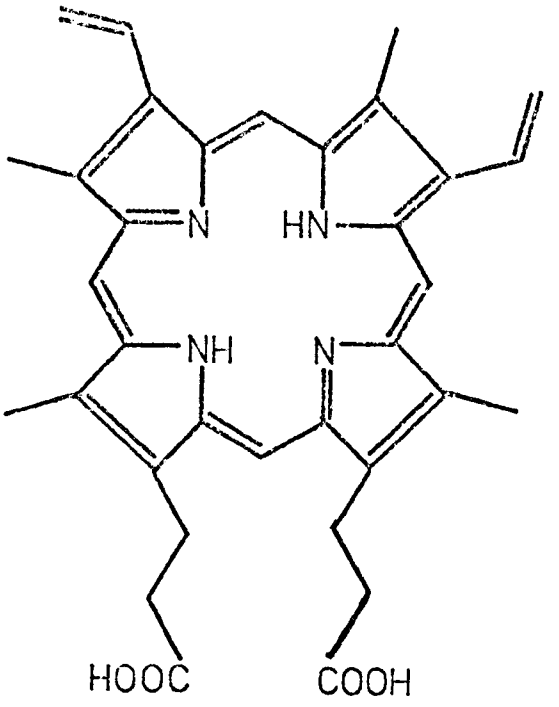
FIGURE 1.2. FOUR CLASSES OF BILE PIGMENTS

groups, the protoporphyrins are formed. In theory, 15 isomers are possible, but in nature, only the form IX occurs.[3] If protoporphyrin IX, (1.II.), is cleaved specifically at the α- methine bridge, the IXα series of bile pigments is formed. This series comprises the verdins (bilatrienes), rubins (biladienes - ac), rhodins and violins (biladienes - ab), urobilins (bilenes), and urobilinogens (bilanes). This thesis is concerned with one member of this series, namely bilirubin IXα, which is a biladiene - ac (1.III.). The other members of the series are fully discussed elsewhere.[8a, 9, 10a]

1.3. Biosynthesis of Bilirubin.

In man, 80 - 90% of the total bilirubin formed daily, (200 - 300mg), originates from the breakdown of the haem moiety (1.IVa.) of haemoglobin liberated during the breakdown of red blood cells. The remaining 10 - 20% originates from other haemoproteins, such as myoglobin and the cytochromes.

Studies of bile pigment formation using ^{15}N- labelled glycine, showed that 10 - 20% of the label was excreted within a few days of administration.[11, 12] Most of the remainder was excreted after 120 - 140 days, and corresponded to the normal red-cell life - span. Between these two peaks there was a low plateau of excretion of the label. In contrast to the 120 day life span of the haem from haemoglobin, the haem from other haemoproteins, for example myoglobin and the cytochromes, shows no definite life span. It is likely that the degradation of the haem from these proteins accounts for the "low plateau" of bile pigment excretion, especially since it has now been shown that myoglobin haem can be converted into bilirubin, but at a turnover rate too slow to contribute a major part of the "early labelled" peak.[13]



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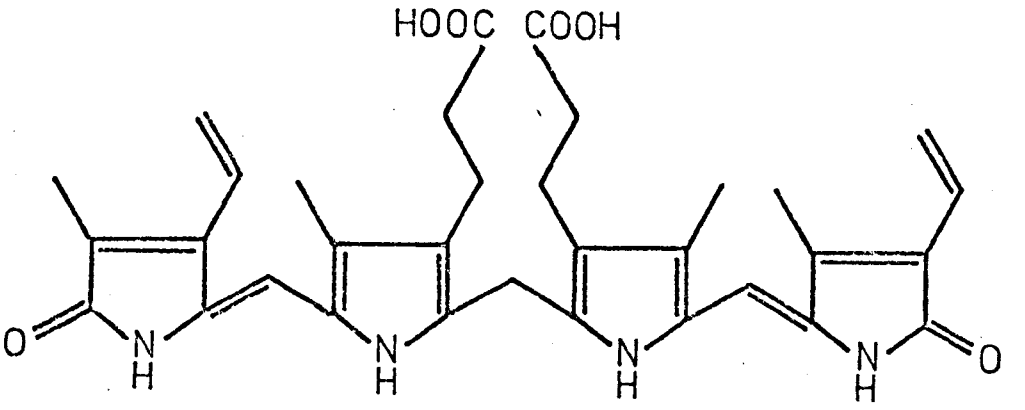

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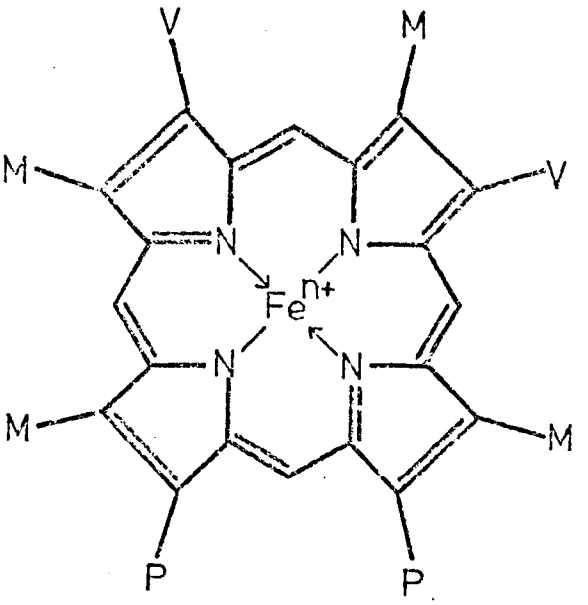
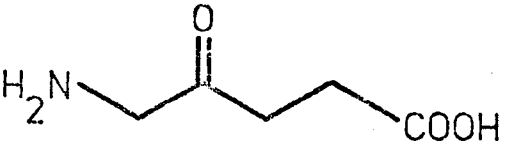


(1.IVa)	n=2	NO COUNTER ION,	HAEM
(1.IVb)	n=3	X=OH,	HAEMATIN
(1.IVc)	n=3	X=Cl,	HAEMIN



(1.V)

Up to about 1963, it was generally accepted that the "early labelled" bilirubin arose via the destruction of erythrocytes shortly after their formation, although no direct evidence had ever been presented. The observation that the incorporation of the porphyrin precursor, δ- aminolaevulinic acid (ALA) (I.V.) into the haem of haemoglobin is very much lower than that resulting from an equivalent amount of glycine [14] has lead to clarification of the origin of the "early labelled" peak. Analysis of the radioactivity of plasma bilirubin after the administration of ^{14}C - labelled ALA has shown that an "early labelled" peak appears within two hours. [15] In contrast, when ^{15}N - labelled glycine is administered, the "early labelled" peak does not appear until after four hours. [15] Thus the "early labelled" peak consists of two fractions, one independent of, and the other associated with haemoglobin haem synthesis. This was confirmed in kinetic studies, which also demonstrated that the appearance and disappearance of radioactivity in hepatic haem- containing enzymes precedes the appearance of ^{14}C -labelled bilirubin in the bile. [16, 17] Thus it appears that both erythroid and non - erythroid components normally contribute to the "early labelled" peak in bile pigment formation.

1.4. Catabolism of Haemoglobin.

The haem moiety of haemoglobin is converted into bilirubin via biliverdin, but the metabolic steps involved in this conversion are not well understood. The presence of iron-bound protoporphyrin is essential for the conversion, both *in vitro*, [18] and *in vivo*. Although free protoporphyrin is converted to bilirubin *in vivo*, [15, 19] the turnover rate is much slower than that for the conversion of

haematin (1. IVb.) to bilirubin. [20] The small quantities of protoporphyrin excreted as bilirubin in these studies can be explained by assuming that the protoporphyrin is first converted to haem. It is uncertain whether the protein moiety of haemoglobin is required for the breakdown of haem. In vivo studies show that exogenous haematin (1.IVb.) is catabolised at a rate similar to that observed for haemoglobin, suggesting that the binding of haem to globin is not necessary for the porphyrin ring opening.[21] However, other experiments, in vitro, indicate that globin is necessary for the catabolism of haem. [22] The mechanism of the conversion of haemoglobin to bile pigments has been the subject of several recent investigations and evidence has been presented which suggests that the reaction can either be non - enzymatic or enzymatic in nature.

A non - enzymatic mechanism for the degradation of haem has been proposed [8b] and strong evidence supporting this has been reported. [22 - 27] In vitro studies of the coupled oxidation of various haem - containing substrates with ascorbate, under physiological conditions of temperature, pH, and partial pressure of oxygen, have shown that green oxidation products, which yield biliverdin on hydrolysis, can be obtained. (Figure 1.3.)It is suggested that such a coupled oxidation may be of significance in vivo. The basic mechanism has been modified by supposing that apo - haemoproteins (eg. apo - haemoglobin, apo - myoglobin) can act as enzymes, with the haem binding sites being equivalent to the enzyme active sites. [22] This modification was introduced for several reasons. Firstly, random cleavage of all four methine bridges occurs during the in vitro ascorbate - coupled oxidation of haemin [28, 29] (1.IVc.) whereas in vivo, the haem is cleaved specifically at the α- bridge. [30] Secondly, the ascorbate

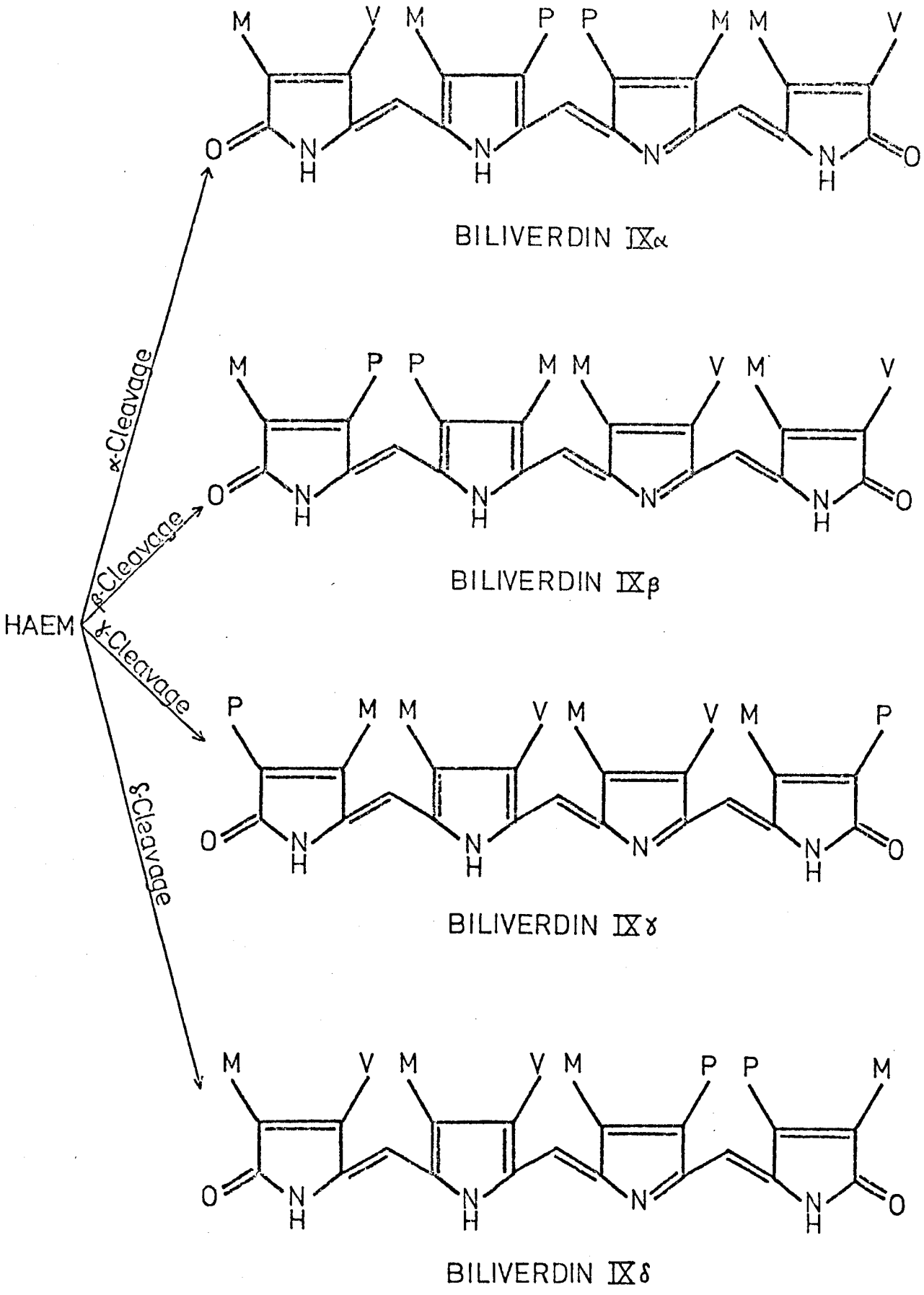


FIGURE 1.3. THE CONVERSION OF HAEM INTO THE FOUR ISOMERS OF BILIVERDIN IX

coupled oxidation of haemoglobin or myoglobin in the presence of free haem yields specifically-cleaved degradation products in excess of those possible from the original haem protein; in the absence of the haemoprotein, the haem is not significantly degraded.[22]

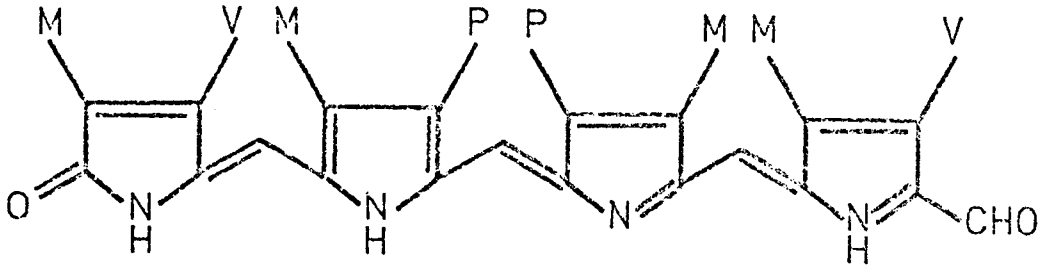
X -Ray diffraction analysis of haemoglobin [31] and myoglobin [32] has shown that in both cases, the haem groups lie in non - polar crevices near the surface of the globin chains with the α - methine bridges lying deep inside the crevices, leaving the other bridges (particularly the γ - bridge) exposed. The specific cleavage of the α - methine bridge must, therefore, be a positive effect of the haem binding site, rather than the masking of the other three bridges.[22, 33] Thus, when the cleavage is carried out under denaturing conditions, the specificity is lost and a random mixture of all four isomers of biliverdin is obtained [34] (Figure 1.3.). The observation[21] mentioned earlier, that the protein moiety is apparently not necessary for haem cleavage is only compatible with this theory if it is assumed that the exogenous haem has immediate access to apo - haemoprotein.

For the enzymatic catabolism of haem, two different systems have been described: haem - α - methenyl oxygenase from liver [35-37] and microsomal haem oxygenase.[38-42] The so called haem - α - methenyl oxygenase was thought to convert haem into formylbiliverdin (1.VI.) in a reaction involving ascorbate, which appeared to be essential for the activity of the enzyme. The formylbiliverdin was then converted into biliverdin by another enzyme, haem - α - methenyl formylase. It was also claimed that the formylbiliverdin produced was entirely IXα in configuration. Reinvestigation of this system has shown the cleavage to be non - α - bridge specific [26, 27] and non - enzymatic, the apparent activity being due to the ascorbate

coupled oxidation already described.[24, 25]

Microsomal haem oxygenase is a mixed function enzyme system, with cytochrome P - 450 as the terminal oxidase, [42] which catalyses the oxidation of haem specifically at the α- methine bridge to form biliverdin IXα. This step is coupled with a soluble NADPH-dependent biliverdin reductase, which selectively converts the biliverdin IXα into bilirubin IXα.[43 - 45] Haem oxygenase has an absolute and stoichiometric requirement for the NADPH and molecular oxygen, generates carbon monoxide in amounts equimolar to the bilirubin formed, and is inhibited by carbon monoxide.[39] Using molecular $^{18}O_2$, it has now been shown that the bilirubin formed from haem, using this enzyme system, contains two atoms of ^{18}O and an additional ^{18}O atom appears in the carbon monoxide that originates from the α-methine bridge carbon atom of the haem. No ^{18}O was found in the bilirubin when the reaction was carried out in a medium containing $H_2{}^{18}O$ instead of molecular $^{18}O_2$.[42]

These observations, together with those of the metabolism of the α- and β- oxymesoferrihaems (1.VIIa. and 1.VIII.) have enabled a possible pathway for the conversion of haem into bilirubin to be elucidated. α- Oxymesoferrihaem is extensively converted into the expected bile pigment, while the β isomer is poorly converted into bile pigment.[45] By analogy with these results it is assumed that α-oxyprotoferrihaem (1.VIIb.) is an intermediate in the catabolism of haem. The possible pathway is shown in Figure 1.4, and is a modified version of that already published.[46] In the light of the experiments using $^{18}O_2$, it is now unnecessary to retain the last two steps in the published scheme. The compounds involved in these steps are apparently not intermediates in the conversion in vivo, otherwise the final bile pigment would have only one ^{18}O atom per molecule.



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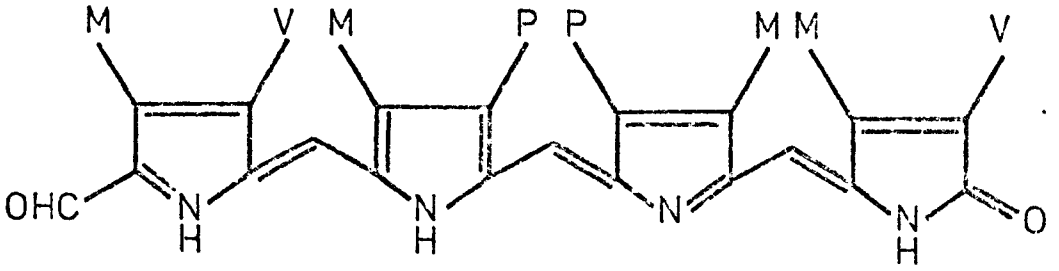

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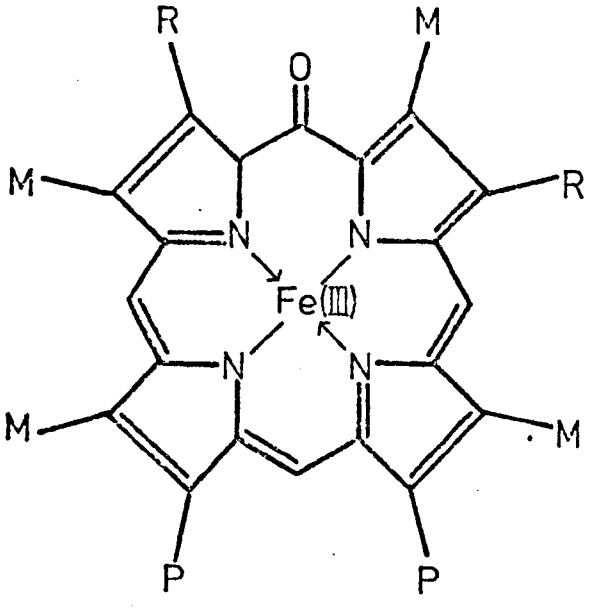
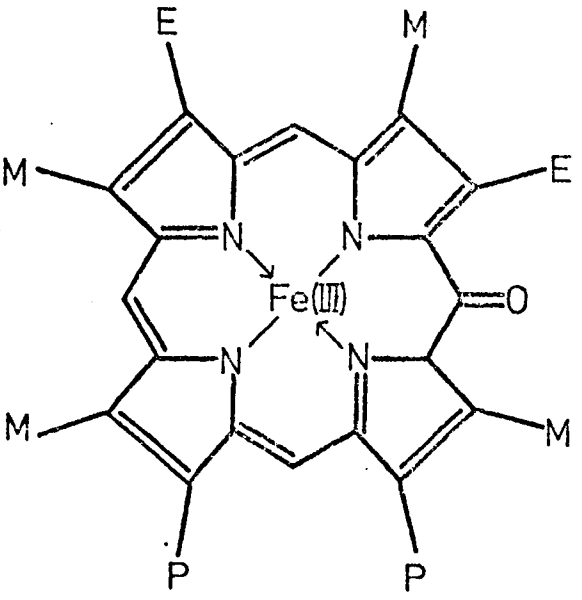


X^-

(1.VIIa) R=E

(1.VIIb) R=V

X= OH or Cl



X^-

(1.VIII)

X= OH or Cl

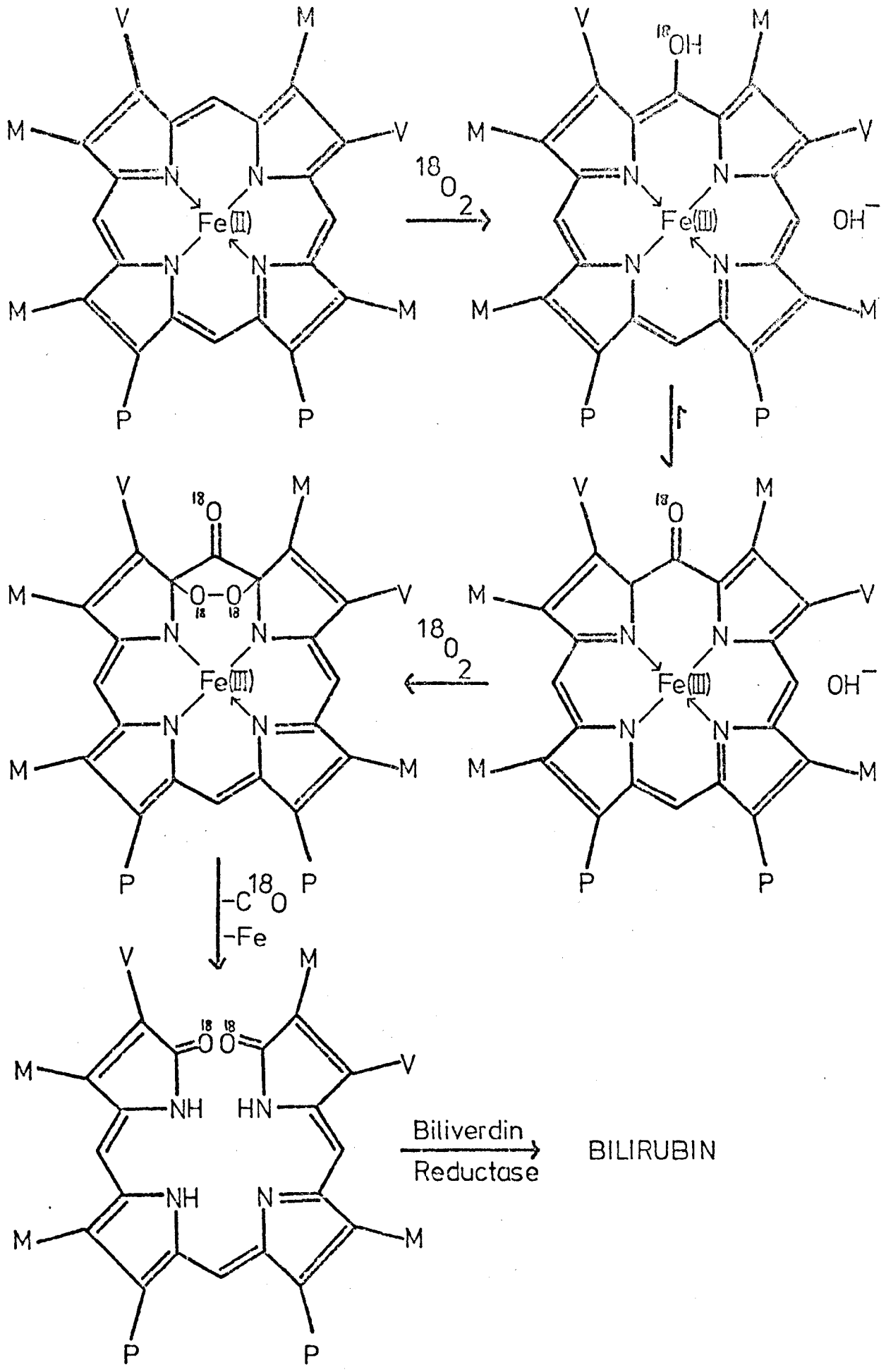


FIGURE 1.4. A PLAUSIBLE PATHWAY FOR THE CONVERSION OF HAEM INTO BILIRUBIN.

In summary: Haem -α- methenyloxygenase is unacceptable, and while microsomal haem oxygenase has allowed a metabolic pathway to be deduced, the concept that bile pigments may be formed non-enzymatically via ascorbate and apohaemoproteins should not be ignored.

Recently, the degradation of haem compounds to bile pigments has been reviewed. [47]

Brief mention should be made here of the sites of formation of bilirubin. Most bile pigment produced in mammals is formed in the reticuloendothelial system and parenchymal cells of the liver. The remainder is produced extrahepatically, and bone marrow, circulating blood and the spleen appear to be important here. It is of interest to note that the studies on microsomal haem oxygenase have shown the highest activity of the enzyme to be in the spleen, liver, brain, kidney, and lung, (in order of decreasing activity), [39] in keeping with the classical view of the importance of the reticuloendothelial system in the catabolism of haemoproteins. [10b]

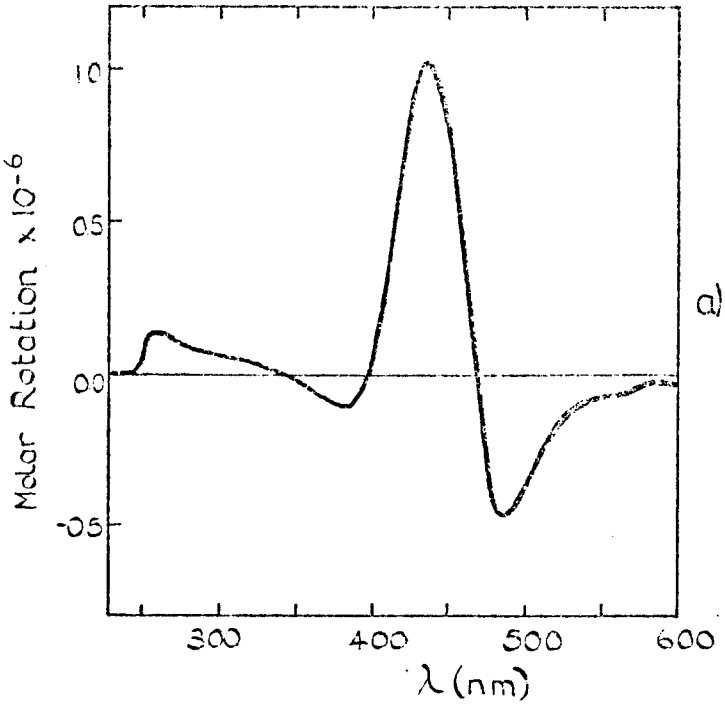
1.5. Metabolism of Bilirubin.

1.5.1. Binding to Albumin.

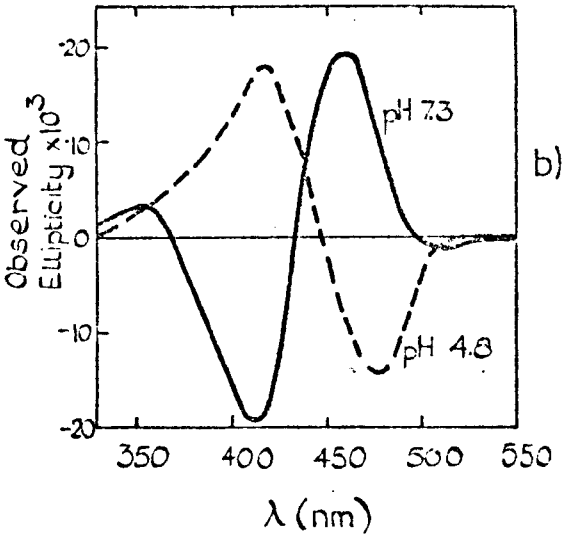
After release from the sites of haemoglobin breakdown, since bilirubin is essentially water insoluble, it is transported in plasma bound exclusively to albumin. Both bovine serum albumin (BSA) and human serum albumin (HSA) have been used to study the properties of bilirubin bound to albumin. Studies using several different techniques in vitro, [10c, 48 - 52] have shown that one, two or three molecules of bilirubin are bound per molecule of albumin, depending on conditions and method of analysis. However, this ratio is probably lower in vivo, since several endogenous

and exogenous substances, particularly organic anions, may compete for the bilirubin binding sites on the protein. [48, 53] The binding is non - covalent, involving histidine residues, [54] and if more than one bilirubin molecule is bound per HSA molecule, there appear to be two types of binding sites, with one bilirubin molecule bound more tightly than the rest. [49] On binding to BSA or HSA, either bathochromic or hypsochromic shifts in the visible absorption spectrum of bilirubin are observed, depending on the conditions (eg. pH, ionic strength, ions present). [55] These shifts could be caused by the influence of the amino acid residues near the binding site or could indicate changes in conformation of the bilirubin molecule. Optical rotatory dispersion (ORD) and circular dichroic (CD) spectral studies have been useful regarding the latter possibility. [50 - 53] While free bilirubin is usually considered optically inactive because of the commonly accepted "linear" formula, the ORD spectral curve of BSA - bilirubin complex at pH 5 exhibits the largest Cotton effect reported in the visible region (Figure1.5a.).[51] This effect is very likely associated with a huge degree of inherent dissymmetry and dipole - dipole coupling between the two dipyrrylmethene chromophores. Therefore, it is proposed that bilirubin adopts a helical conformation, with the sense of a right handed helix, when bound to BSA. [52]

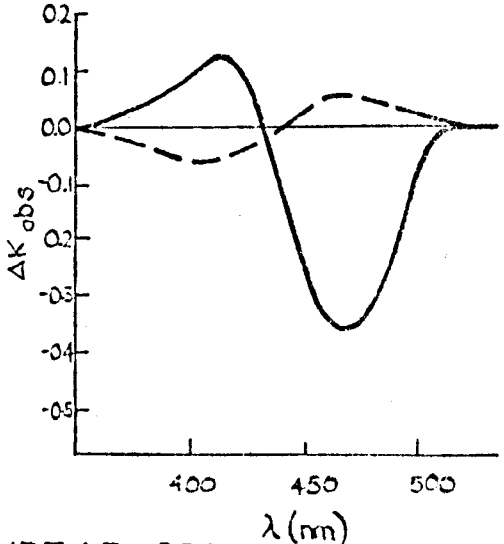
While the binding characteristics and the absorption spectra of BSA - and HSA - bilrubin complexes are very similar, the Cotton effects generated by the HSA - bilirubin complex are very different from those obtained with BSA. [50, 53] The general differences are shown in Figure 1.5.By analogy with the suggested structure for the BSA - bilirubin complex, the observed inversion of the sign of the main CD bands with changing pH may be due to a change in the dissymmetric mode of binding of bilirubin to HSA, resulting



a) ORD Spectrum of the BSA-Bilirubin Complex at pH 5. From ref. 51.



b) CD Spectra of the 2:1 HSA-Bilirubin Complex. From ref. 50.



c) CD Spectra of the 2:1 BSA-Bilirubin —— and HSA-Bilirubin---- Complexes at pH 7.4. From ref. 53.

FIGURE 1.5. ORD & CD SPECTRA OF BILIRUBIN-ALBUMIN COMPLEXES

in a different, opposite relative orientation between the dipole moments of the dipyrrylmethene chromophores. [59]

1.5.2. Conjugation.

In order that it may be excreted, free bilirubin (water insoluble) is converted into a water soluble derivative - a so called "conjugate". This process occurs within the liver cells and the uptake of bilirubin (ie. that which is not formed in the liver) by the liver cells is rapid and is preceded by the dissociation of the bilirubin - albumin complex. Within the cell, the bilirubin is conjugated with glucuronic acid, derived from uridine diphosphate - α - glucuronic acid (UDPG), by the enzyme bilirubin glucuronyl transferase. This enzyme is probably one of several glucuronyl transferases which are bound to the endoplasmic reticulum (ER) of the liver cells. Using bilirubin as substrate (normally glucuronyl transferases are estimated with glucuronide acceptor molecules other than bilirubin, eg. p - nitrophenol) the greatest enzyme activity lies in the rough ER, [56] although activation of the enzyme with EDTA causes rough ER to be converted into smooth ER. [57]

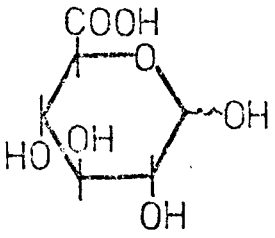
The existence of bilirubin glucuronides ("conjugates") was demonstrated in 1956. [58 - 60] These compounds can be cleaved by β - glucuronidase to yield bilirubin and glucuronic acid, indicating that the glucuronic acid is attached to bilirubin molecule in the β - configuration, and are found to be unstable towards alkali. It was soon shown that the conjugates are esters between the carboxylic acid groups of bilirubin and the C(1) hydroxyl group of glucuronic acid. [61 - 64] Since bilirubin contains two carboxylic acid groups and is asymmetric, a diglucuronide and two monoglucuronides can exist. Initially, while the diglucuronide was readily identified, it was suggested that the monoglucuronide could be a 1 : 1 complex of bilirubin and its diglucuronide. [61]

However, it has now been shown that bilirubin monoglucuronide (esterified at either carboxyl group) exists as a chemical entity.[65 - 67] The sequence of reactions involved in the conjugation of bilirubin is shown in Figure 1.6.

For many years it was thought that bilirubin was conjugated solely with glucuronic acid (as has been assumed in the foregoing discussion). Within the past few years, it has been shown that bilirubin can be conjugated with other sugars. Thus, in vivo, in dog bile, β- D - glucose (IX) and β- D - xylose (X) conjugates of bilirubin have been identified.[68, 69] In human bile, it has been shown that the major bilirubin conjugates are excreted as the acyl glycosides of three aldobiouronic acids (1.XI.- 1.XIII.), a pseudoaldobiouronic acid (1.XIV.) and a hexuronosylhexuronic acid (1.XV.)[70, 71] In vitro, using rat liver preparations, bilirubin has been conjugated with β - D - glucose[72 - 75] and β - D - xylose[73-75] from UDP - α - glucose and UDP - α - xylose respectively.

Clearly, the view that bilirubin is excreted solely as its diglucuronide must be modified in the light of this recent work. It has been suggested that bilirubin is linked to a mucopolysaccharide;[76] if this is so, then it is interesting to speculate that perhaps bilirubin could be conjugated with a polysaccharide initially, which during isolation procedures [eg. 61, 77 - 79] is progressively degraded until only one, or perhaps two sugar residues remain attached to the bilirubin.

In whichever way bilirubin is conjugated, conjugation greatly alters the physiological and chemical properties of bilirubin, and the relationship between conjugation and excretion has yet to be fully explained. The conjugated bilirubin is excreted into the bile and thence into the intestine where eventually bacterial β - glucuronidase hydrolyses the conjugates



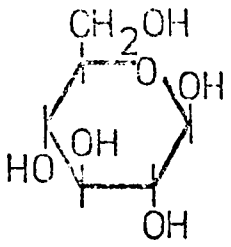
GLUCURONIC ACID

Glucose-1-α-Phosphate + UTP $\xrightarrow[\text{Pyrophosphorylase}]{\text{UDP-Glucose}}$ UDP-α-Glucose + Pyrophosphate

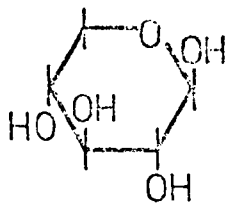
UDP-α-Glucose + $2NAD^+$ $\xrightarrow{\text{Dehydrogenase}}$ UDP-α-Glucuronic Acid + 2NADH + $2H^+$

UDP-α-Glucuronic Acid + Bilirubin $\xrightarrow{\text{Bilirubin glucuronyl transferase}}$ Bilirubin Monoglucuronide + Bilirubin Diglucuronide + UDP

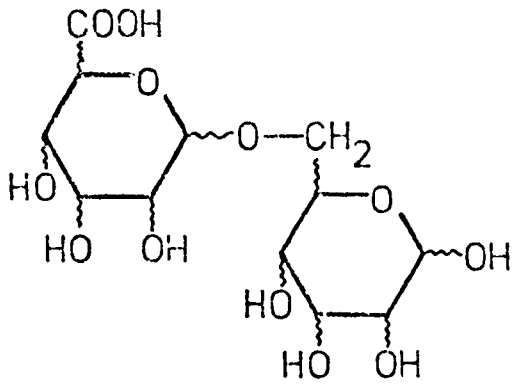
Figure 1.6. The Formation of Bilirubin Conjugates.



(1.IX)



(1.X)



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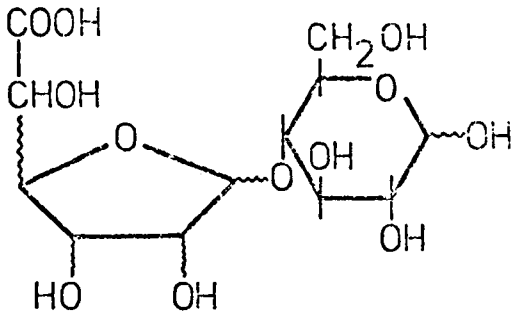
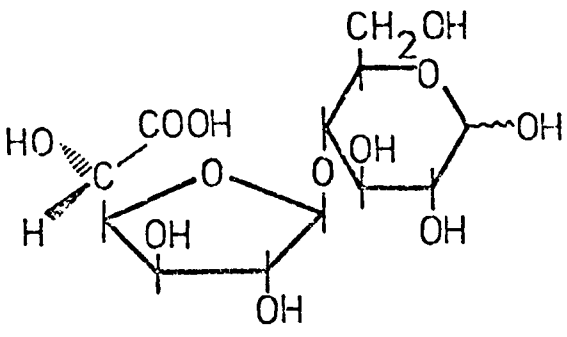


(1.XII)



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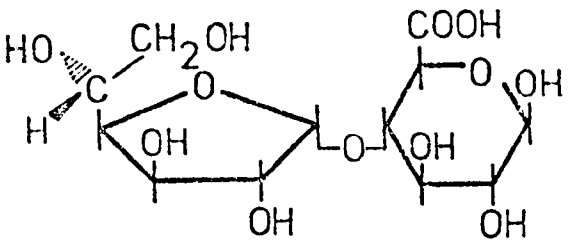

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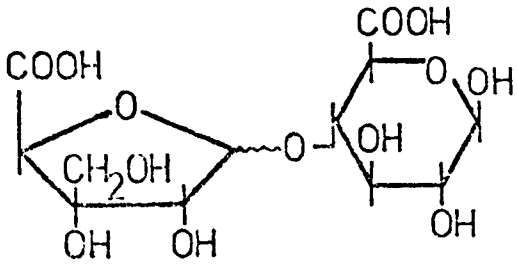


(1.XV)

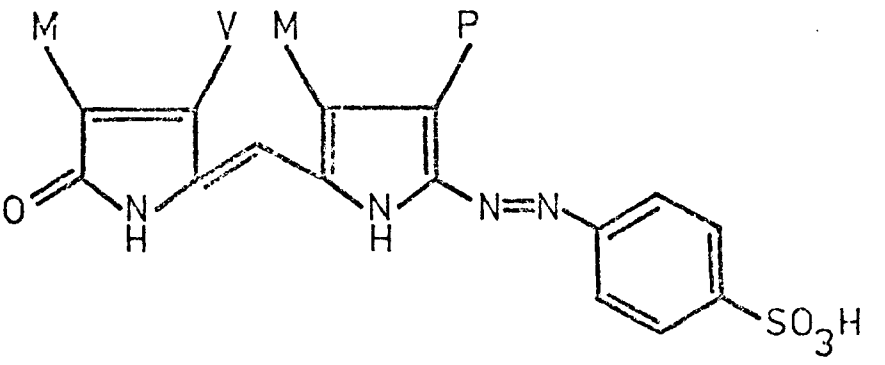
to bilirubin, which is further reduced to urobilinogens (bilanes) and urobilins (bilenes) (Figure 1.2.).[80]

1.6. Determination of Bilirubin and the Diazo Reaction.

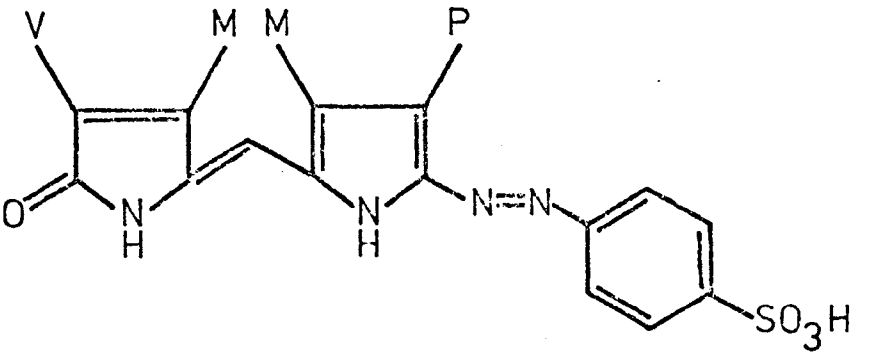
Bilirubin is generally estimated by the van den Bergh reaction in which the bile pigment is allowed to react with diazotised sulphanilic acid. The bilirubin is cleaved at the central methylene carbon bridge, to form two isomeric azopigments (1.XVI, 1.XVII.) which are estimated spectrophotometrically at 520 - 600 nm. Two types of reaction can be identified: the "direct" reaction in which the serum bilirubin reacts immediately with diazo reagent, and the "indirect" reaction, to which alcohol must be added before any appreciable reaction occurs. The distinction between the two reactions is straightforward, the direct reaction being given by conjugated bilirubin while the indirect reaction is given by free, unconjugated bilirubin. The diazo reaction is discussed more fully in a later chapter, while a full description of the modifications of the van den Bergh reaction, and the techniques used in the estimation of bilirubin is given elsewhere.[10d]

1.7. Isomers of Bilirubin, Other Than IXα.

Naturally occurring bilirubin is generally assumed to be formed from the stereospecific cleavage of the α - methine bridge of haem. It has been reported that natural bilirubin consists solely of the IXα isomer (1.III.),[30] but more recent studies have revealed the presence of other isomers. Mass spectrometric investigations of the bilirubin obtained from ox bile and emu egg shells have shown the presence of 2 - 5% of either of, or a mixture of, the isomers IXβ (1.XVIII.) and IXδ (1.XX.).[81] This method relies on the fragmentation of the bile pigments at



(1.XVI)



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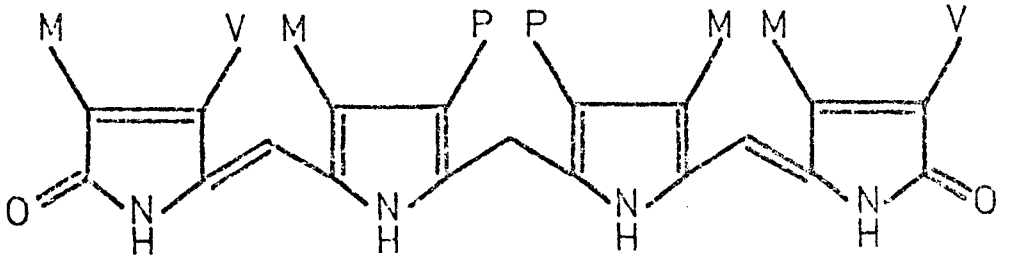
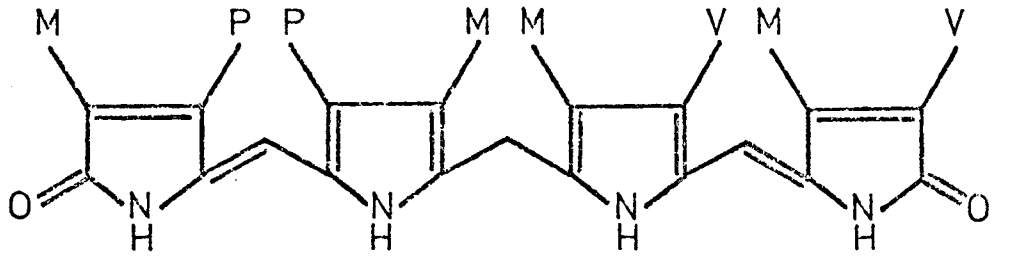
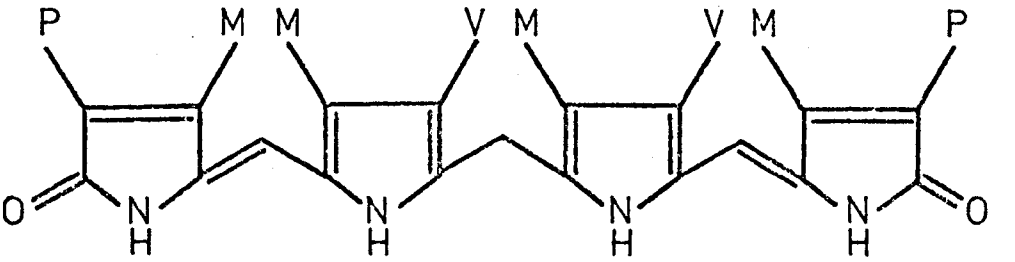


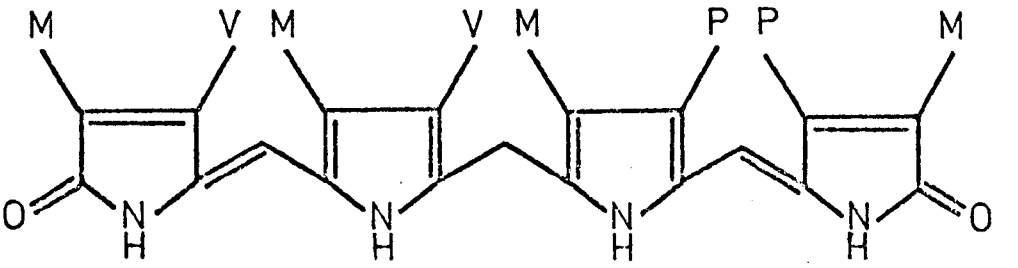
(1.III) BILIRUBIN IXα



(1.XVIII) BILIRUBIN IXβ



(1.XIX) BILIRUBIN IXγ

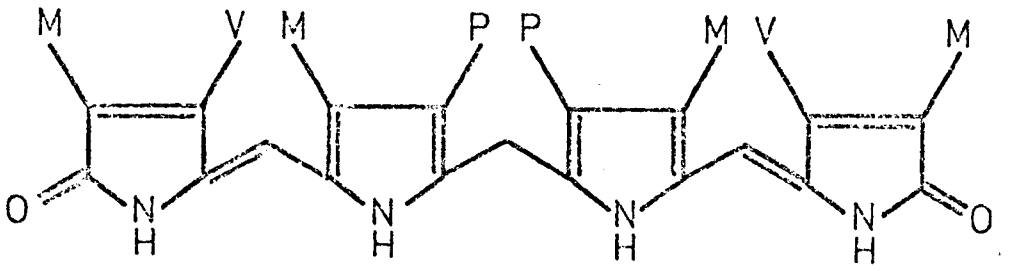


(1.XX) BILIRUBIN IXδ

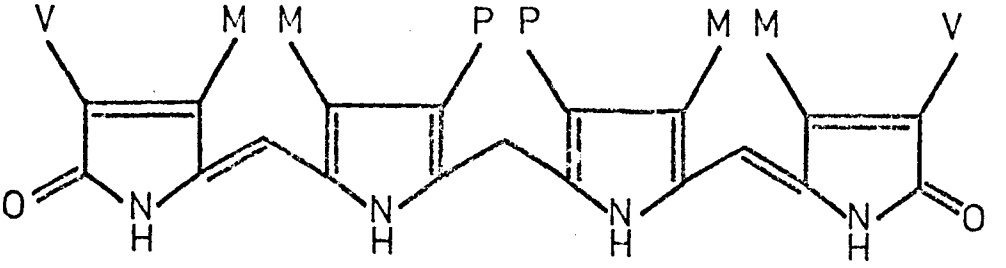
the central methylene bridge to give two dipyrryl methene subunits. However, it will be seen that the IXα and the IXγ (1.XIX.) isomers will yield subunits of the same mass, while the subunits from the IXβ isomer will be identical in mass to those from the IXδ isomer. Thus one cannot distinguish between the IXα and IXγ or the IXβ and IXδ isomers. Oxidation of bilirubin to biliverdin with glacial acetic acid and ferric chloride, followed by esterification and separation of the esters using TLC has shown that pig bile bilirubin contains 99·6 % of the IXα isomer with traces of the IXβ and IXδ isomers, but no IXγ isomer.[82] Oxidation of bilirubin with alkaline potassium permanganate, to convert the middle pyrrole rings into α, α' - dicarboxylic acids, identifiable by GLC of their ethyl esters, has revealed that ox gallstone bilirubin contains 1 - 3 % of non - IXα isomer, if that isomer is solely IXγ or 2 - 6 % if it is IXβ or IXδ.[83] Again, this method does not allow one to distinguish between the IXβ or IXδ isomers. Finally, in vitro oxidations of haem yield all four IX configuration isomers.[22,28,29]

All the non - IXα isomers discussed so far have been of the IX configuration. However, bilirubin from a number of commercial sources is separable, by TLC, into three components, bilirubin IIIα (1.XXI.) in quantities ranging from trace amounts to 16%, bilirubin XIIIα (1.XXII.) (trace amounts to 22%) and bilirubin IXα (100% to 62%).[84] In addition, it was found that under the condition used for TLC (1% glacial acetic acid in chloroform, silica gel), pure bilirubin IXα disproportionates into a mixture of isomers containing IIIα (1%), IXα (95%) and XIIIα (4%).[84] These findings are unusual since haem occurs only in the IX configuration, hence the new isomers cannot be formed naturally.

Dehydrogenation of bilirubin with benzoquinone in acetic



(1.XXI) BILIRUBIN IIIα



(1.XXII) BILIRUBIN XIIIα

acid yields biliverdin IIIα , IXα and XIIIα [85] and when this is compared with the result described above, [84] three possibilities arise :- a) if pure bilirubin IXα was used then the reaction conditions allowed the isomerisation to occur, b) the bilirubin initially contained the three isomers and the reaction conditions had no effect on the isomer ratio or c) the reaction conditions served to enhance the isomer ratio. Treatment of bilirubin IXα in DMSO with glacial acetic acid at 85°C or with concentrated hydrochloric acid at room temperature causes isomerisation and formation of bilirubin IIIα and XIIIα .[86] The disproportionation also occurs under much less vigorous conditions, namely in dilute aqueous solution in the pH range 7.4 - 11. [87] In the pH range 11-14, no disproportionation could be detected. [86, 87]

Nuclear magnetic resonance spectroscopy has also been used in the detection of non - IX configuration isomers. The n.m.r. spectra of human bile bilirubin and commercial bilirubin were compared and found to be identical, with the integrals of all four methyl signals equal. [88] The occurrence of bilirubin IIIα and XIIIα along with bilirubin IXα would have been detected by differences in the ratios of the methyl resonances. No new resonances would be observed since the two methyl groups on the outer pyrrole rings in bilirubin IIIα and XIIIα are each identical to one of the methyl groups on the outer pyrrole rings of bilirubin IXα .

The results indicate that the variable amounts of bilirubin IIIα and XIIIα found in commercial bilirubin are artefacts formed during the isolation procedure. On the other hand, the later results indicate that the isomerisation could occur in vivo, although only when the bilirubin is free, since albumin - bound bilirubin appears not to disproportionate. [87]

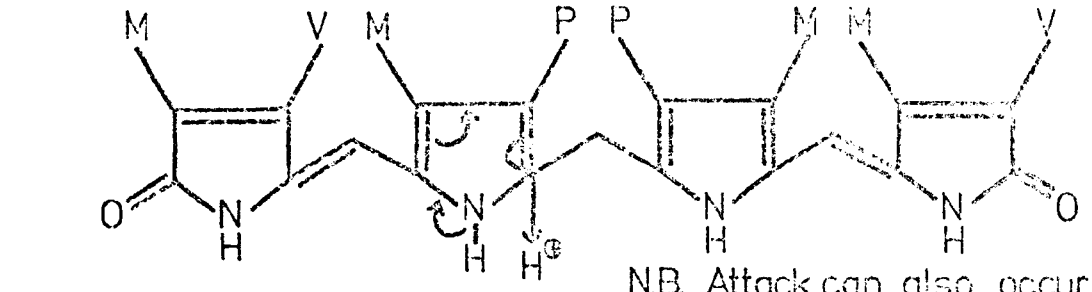
A possible mechanism for the isomerisation reaction involves electrophilic substitution of bilirubin and fragmentation at the central methylene bridge. Recombination of the dipyrrylmethene subunits produced would then lead to isomer formation. In acidic media, the initial step is probably the uptake of a proton while in mildly alkaline media, the initial step is probably the abstraction of a proton from a molecule of water. Such a scheme is outlined in Figure1.7. Attack by water at the exocyclic methylene group of the dipyrrylmethenes could occur to give hydroxymethyl derivatives, which could react as shown in Figure 1.8 to give the isomeric bile pigments.

The isomerisation reaction can occur with any bilirubin and therefore, the acid treatment of, say, a mixture of bilirubin IXα IXβ ,IXγ and IXδ would result in a multicomponent mixture of products. Thus any attempt to determine the presence of non IX configuration bilirubins under possible isomerising conditions[82, 83] can lead only to a qualitative result. At the same time, it is unlikely that any minor isomer present will be removed completely, since no one isomer is susceptible to preferential attack.

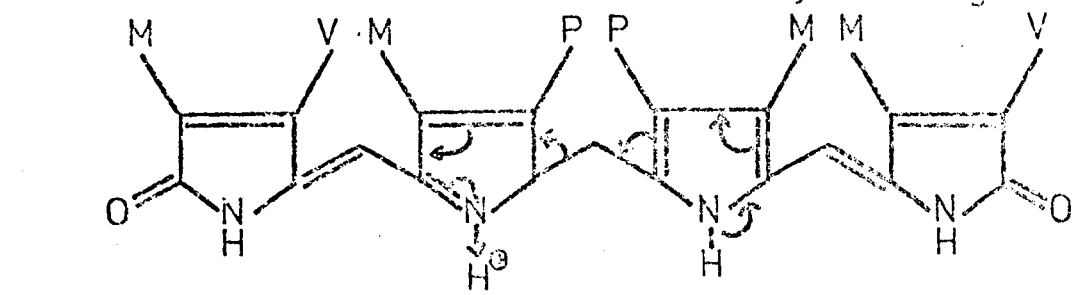
The two independent observations [82, 89] that two minor, non - IX configuration isomers (unidentified in one case [82] and shown to be IIIα and XIIIα in the other [89]) were found in the products from the sodium amalgam reduction of bilirubin, followed by a ferric chloride - acid oxidation, may now be explained. It was originally suggested that an isomerisation had occurred during alkaline reduction. It is now clear, that in addition to the probable presence of the IIIα and XIIIα isomers in the bilirubin used, isomerisation must have occurred during the acidic oxidation step.

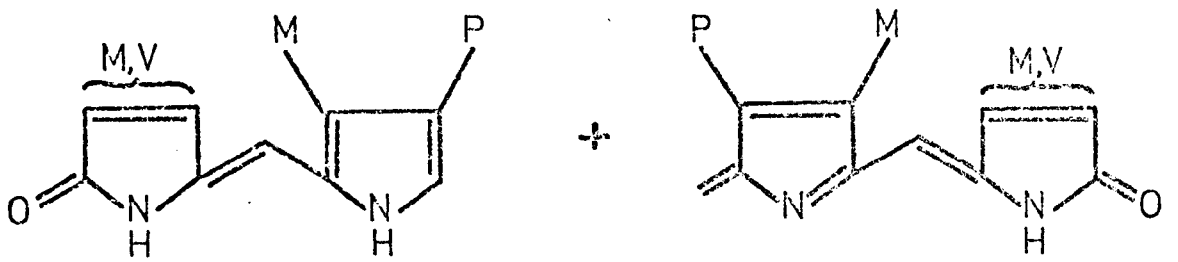
Chromic acid or chromate oxidations have been used in the structure determinations of bile pigments. [90, 91] However, since

FRAGMENTATION:

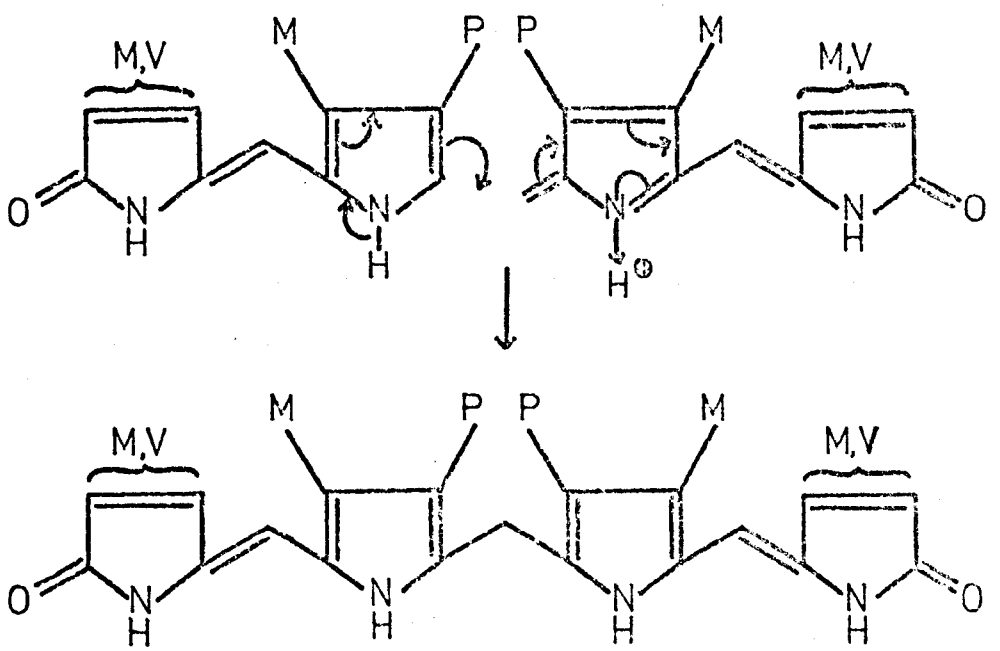


N.B. Attack can also occur at the other side of the central methylene bridge.



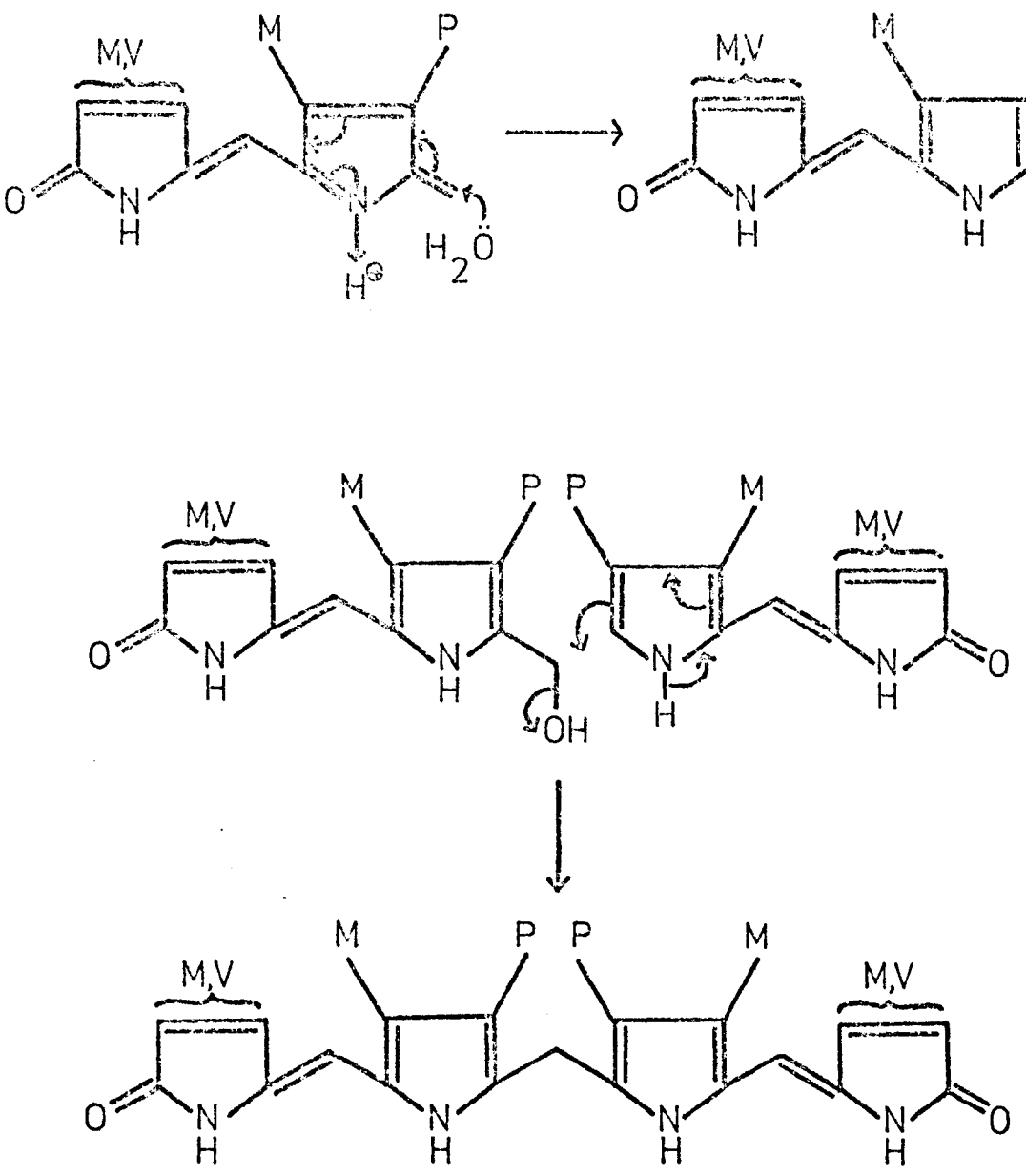


RECOMBINATION:



BILIRUBIN IIIα, IXα, XIIIα

FIGURE 1.7 THE DISPROPORTIONATION OF BILIRUBIN IXα



BILIRUBIN IIIα, IXα, XIIIα

FIGURE 1.8. AN ALTERNATIVE MECHANISM FOR THE RECOMBINATION PROCESS SHOWN IN FIGURE 1.7

the technique is usually used to determine the nature of the pyrrole rings present, rather than the absolute configuration (although in some circumstances the chromate oxidation will yield this information), any isomerisation occurring in the bilirubinoid - type pigments will not generally affect the final result.

Consequently, when interpreting any experimental result, particularly when the acid treatment of bilirubin is involved, allowance must be made for the possible presence of isomers other than of the IX configuration.

1.3. Jaundice and the Photochemistry of Bilirubin.

Although the topic of jaundice is not directly relevant to this thesis, it will be discussed briefly with reference to some recent work concerning the photochemistry of bilirubin. A full discussion of jaundice is given elsewhere. [10e, 92]

Jaundice is characterised by a yellow discolouration of the skin and organs, and is caused by the retention of bilirubin. Jaundice of the newborn has been treated by phototherapy, which reduces the high level of bilirubin present by converting it to colourless products. Since uncertainty exists over the structures and toxicities of the photodecomposition products, attempts have been made to identify them.

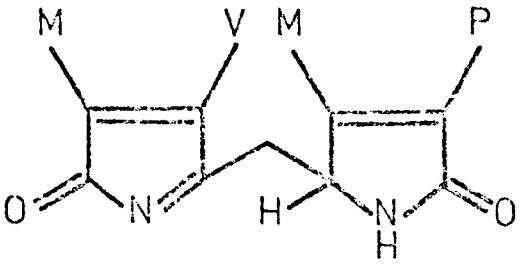
The photodecomposition, aerobically and in diffuse daylight, of a chloroform solution of bilirubin has been studied. [93] Most of the bilirubin decomposes to give dipyrrylmethenes (1.XXIII.- 1.XXV.), 3 - carboxyethyl -4- methyl pyrrole-2,5-dicarboxylic acid (1.XXVI.) and the maleimides (1.XXVII.- 1.XXXI.), while a small fraction decomposes with retention of the tetrapyrrolic structure to give, inter alia, biliverdin. All of these products are easily

derived from bilirubin by various oxidation and reduction reactions.

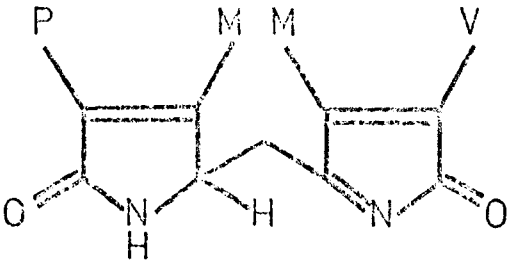
Irradiation (about 24 hours) of oxygen flushed solutions of bilirubin in dilute methanolic ammonia gives a complex mixture of products. In this way, the dipyrrylmethenes (1.XXXIIa.[94], 1.XXXIIIa.[94, 95]), haematinic acid [94] (1.XXX.) and methylvinylmaleimide [96, 97] (1.XXVII.) have been isolated. It is suggested that, in vivo, the products formed during phototherapy are the dipyrrylmethenes (1.XXXIIb, 1.XXXIIIb.), methylvinylmaleimide and haematinic acid. [94] Since the latter two compounds appear not to exhibit the usual enzyme toxicity associated with maleimide, it is likely that if these compounds are formed in vivo they would be non - toxic. [94]

The course of the photolysis reaction depends on the nature of the solvent. Thus, in chloroform, biliverdin is the main product of irradiation [95] whereas in methanolic ammonia, biliverdin is formed early during the photolysis but disappears on continued irradiation.[96] Biliverdin formation may involve radical abstraction at the central methylene carbon bridge of bilirubin, while the fragmentation reaction may be rationalised in terms of the photosensitised generation of singlet oxygen followed by its cycloaddition to bilirubin with subsequent solvolysis. [95] Evidence has been presented which suggests that bilirubin can sensitise its own destruction [98] and a possible reaction scheme is outlined in Figure 1.9.

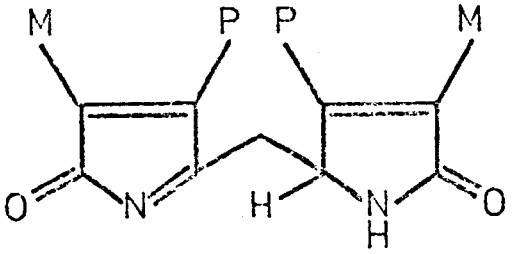
Some evidence, eg. the appearance and disappearance of biliverdin during long term photolysis [96] suggests that biliverdin may be intermediate in the photodecomposition process. However, this question remains unresolved, for while methylvinylmaleimide has been detected after the photolysis of biliverdin (although only after 40 - 130 hours photolysis),[99] it also appears that biliverdin dimethyl ester, at least, is not an oxygen sensitiser and inhibits bilirubin



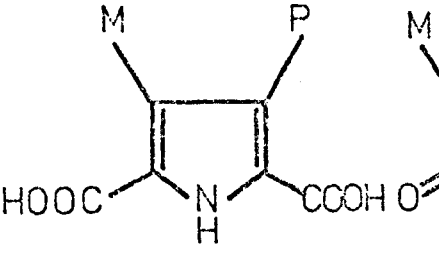
(1.XXIII)



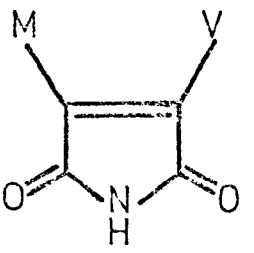
(1.XXIV)



(1.XXV)



(1.XXVI)



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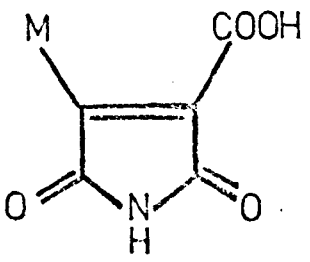

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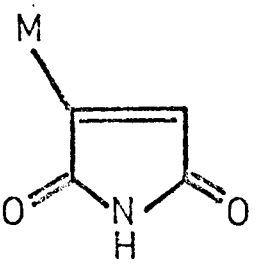

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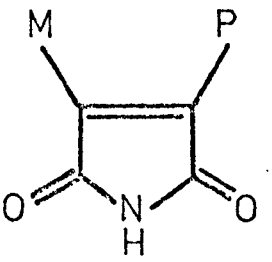

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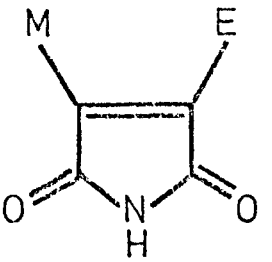
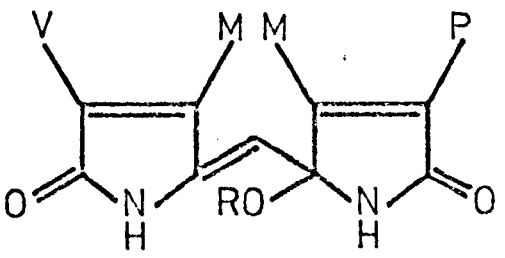
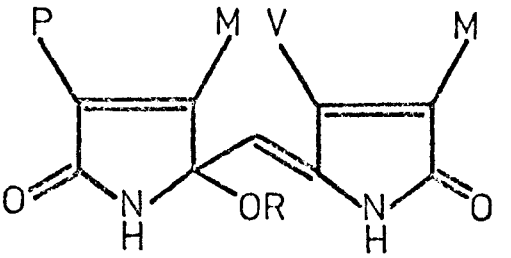


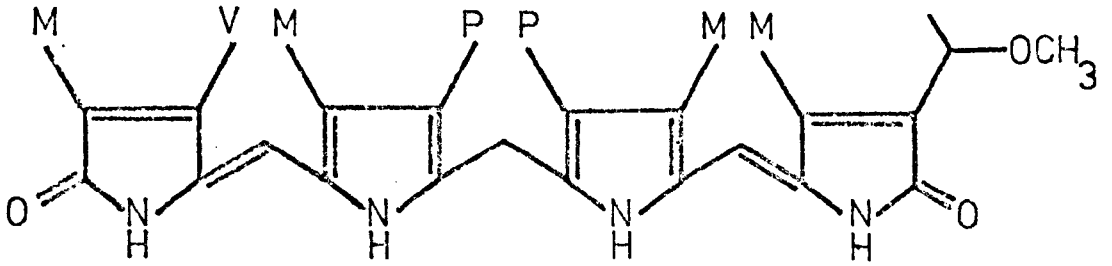
(1.XXXI)



(1.XXXIIa) R=M
(1.XXXIIb) R=H



(1.XXXIIIa) R=M
(1.XXXIIIb) R=H



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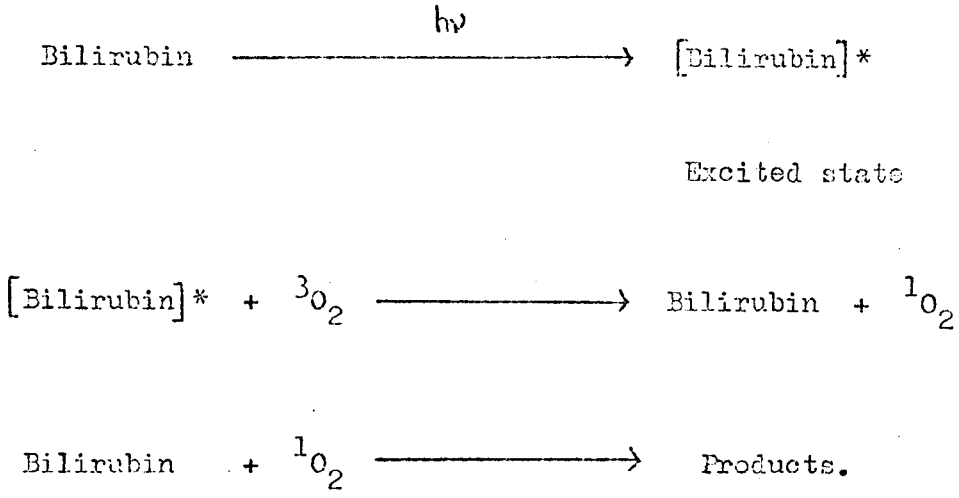


Figure 1.9. The Photochemical Reactions of Bilirubin.

photo oxidation.[100] On this basis, it is suggested that biliverdin is not an intermediate in the main pathway of bilirubin photo - decomposition.

Finally, irradiation of a 10% methanol in chloroform solution of bilirubin has been shown to form (1.XXXIV.) and it is suggested that bilirubin could react in a similar manner with nucleophilic substances in vivo during phototherapy.[101]

CHAPTER 2

TAUTOMERISM , STRUCTURE AND HYDROGEN BONDING IN BILIRUBIN.

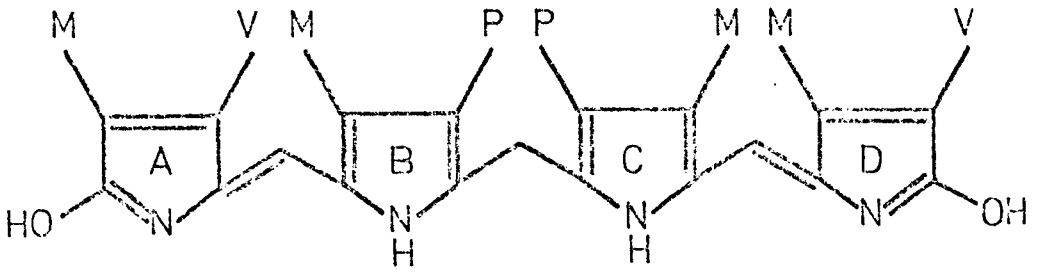
2. TAUTOMERISM, STRUCTURE AND HYDROGEN BONDING IN BILIRUBIN.

2.1. Introduction.

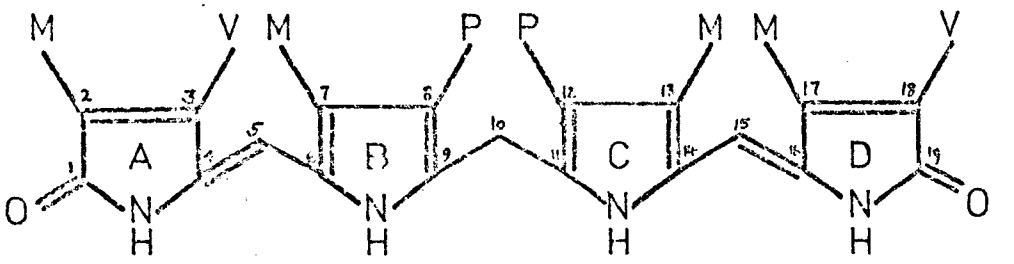
The structure of bilirubin has been established, by both degradative [4] and synthetic [6] methods, and it was suggested that the structural formula of bilirubin was (2.I.).[102] This represents the terminal pyrroles (A and D) as α- hydroxypyrroles thus giving the bislactim tautomer of bilirubin (2.I.). Largely on account of the weak basic character of bilirubin together with its apparent inability to form stable metal complexes, it was suggested that bilirubin tautomerised to the bislactam form (2.II.).[8c] In the lactim form, there are two tertiary nucleophilic nitrogen atoms (in rings A and D) which could coordinate with metal atoms whereas in the lactam form no similar nitrogen atoms are available. This tautomerism was subsequently investigated by several groups.

The simplest models for bilirubin are the hydroxypyrroles; α - hydroxypyrroles (2.III.) have been shown to exist as lactams[103] while β - hydroxypyrroles (2.IV.) appear to exist predominantly in the enol form. [104]

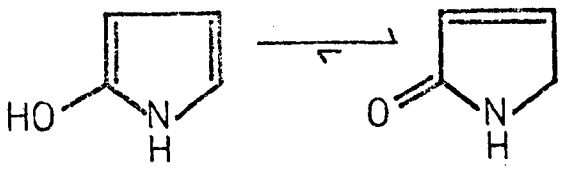
The spectrophotometric titration curves for four types of bile pigments (urobilins, violins, verdins, and rubins) (Figure 2.1.) have been determined. [105] On the basis of the relative basic strengths of these compounds, it was concluded that they were best represented as bislactams. If the series urobilins, violins, verdins is considered, the chromophores containing the tertiary nitrogen atom become more extensive, so that the lone pair electrons on this nitrogen atom become less available for donation to a proton. This results in the observed decreasing basic strengths in going from urobilins to verdins. If the rubins exist in the bislactam form, they ought to be the least basic compounds in the series, since no tertiary nitrogen is present. A predominantly acidic character



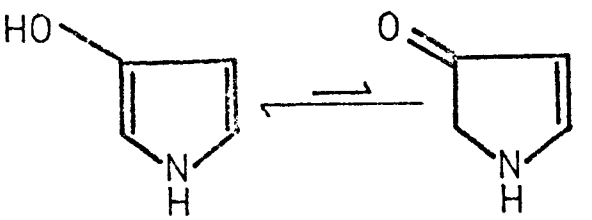
(2.I)



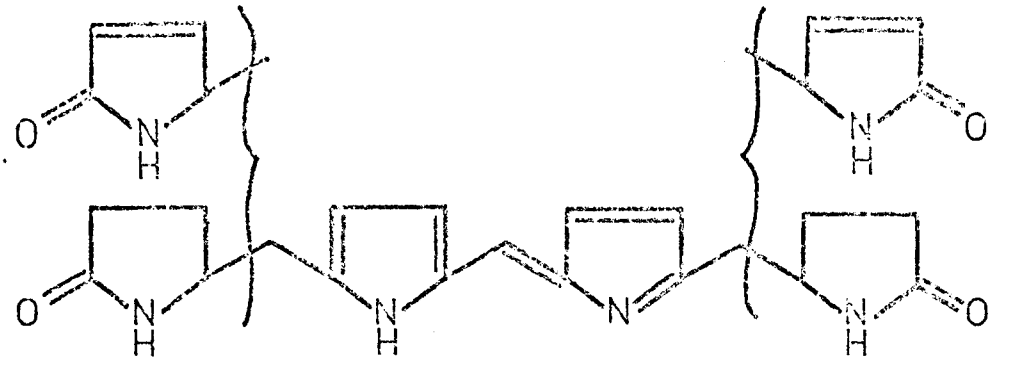
(2.II)



(2.III)

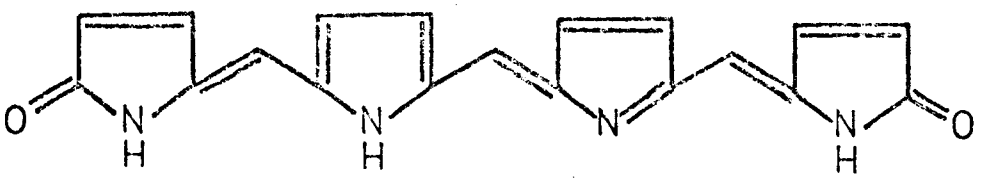


(2.IV)



UROBILINS

VIOLINS



VERDINS

RUBINS

FIGURE 2.1. FOUR CLASSES OF BILE PIGMENTS

for the rubins was found,[105] and this is readily explained if the dipyrrylmethene subunits each contain a carbonyl group. This is capable of accommodating the anionic charge resulting from the dissociation of either N-H group and would lead to stabilisation of the anionic form (Figure 2.2). All four protons in the bislactam structure should, therefore be more readily ionised than the corresponding acidic proton in pyrrole itself.[105]

Infra red spectral studies of bilirubin have been interpreted in several ways. Thus, the absorption band at 3,420 $cm.^{-1}$ has been attributed to the N-H stretching mode of a pyrrole, combined with that of a cyclic γ - lactam, implying that the terminal pyrrole rings are in the lactam form.[106] The absorption band at 1246 $cm.^{-1}$ has been attributed to the C-O stretching mode in the group HO—C=N-, thereby favouring the lactim tautomer.[107] The band observed in the region 1650 - 1630 $cm.^{-1}$ has been variously assigned to the C=C stretching mode,[107] the C=N stretching mode[108] and the C=O stretching mode of a lactam.[88]

Comparison of the visible and u.v. spectra of bilirubin with those of various dipyrrylmethenes has shown that bilirubin exists as a bislactam,[109] while a monolactam - monolactim form has also been proposed.[110]

Thus, the problem of the tautomeric forms of bilirubin is confused and the object of the current investigation was to clarify this. In a recent paper,[111] i.r., u.v., and ^{1}H n.m.r. studies of bilirubin were interpreted as indicating that bilirubin exists as the enol tautomer whereas bilirubin dimethyl ester exists as the lactam tautomer. This tautomerism on esterification was explained in terms of the loss of hydrogen bonding between the carboxyl groups of the propionic acid side chains which held the molecule in a conformation suitable for hydrogen bonding between the two terminal

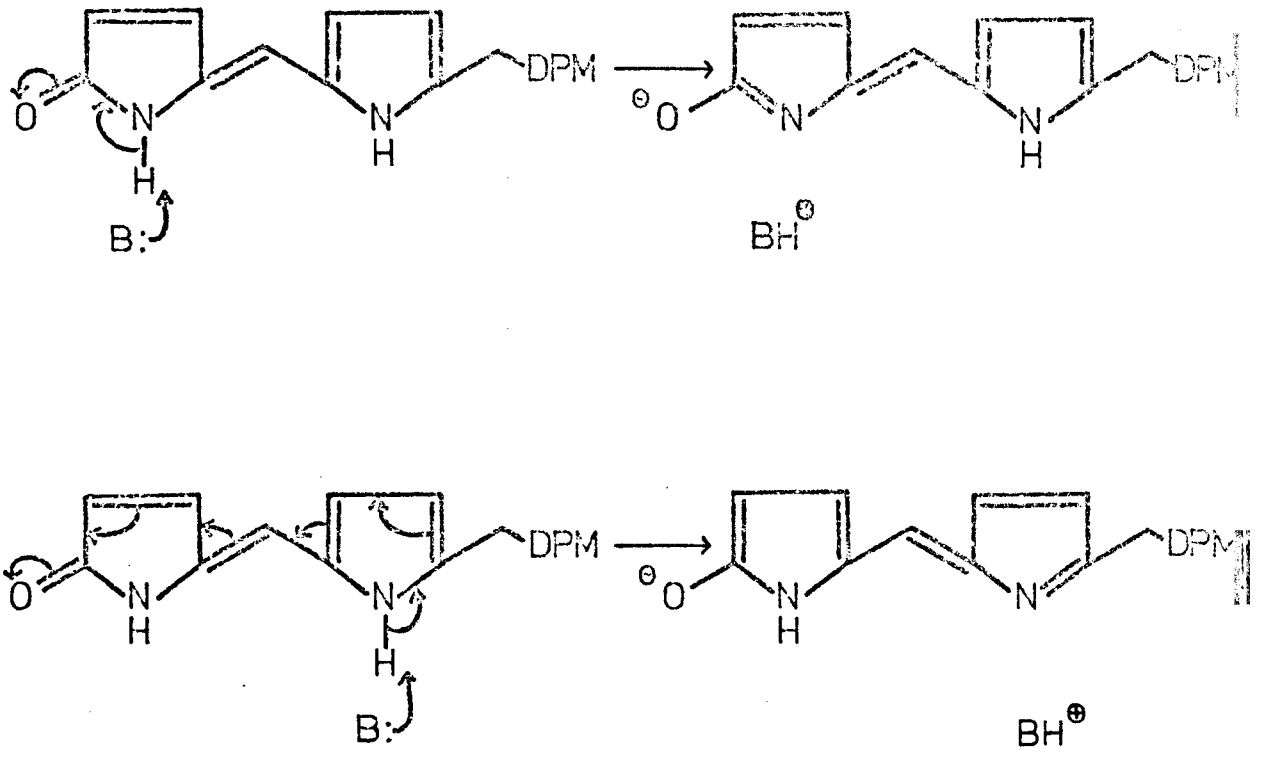
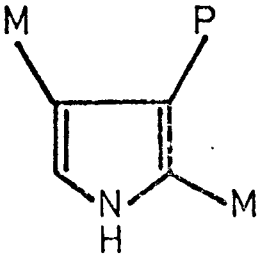
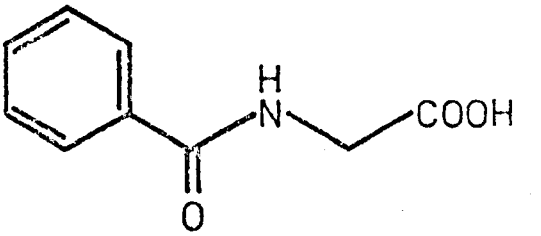


FIGURE 2.2. STABILISATION OF THE RUBIN ANION



(2.V)



(2.VI)

enolic hydroxyl groups.[111]

Since bilirubin contains two carboxyl acid groups, intramolecular hydrogen bonds can occur either between these carboxyl groups or between a carboxyl group and a N-H group of a pyrrole or lactam. Three possible hydrogen bonded structures have been proposed[106, 112] and it has been suggested that bilirubin adopts one hydrogen bonded form in the solid state and another in solution.[106] The evidence for the medium - dependent hydrogen bonded structures appears dubious in parts (eg. the appearance of O-H vibrational absorption bands in the i.r. spectrum of a compound in which all the labile protons have been exchanged with deuterons), so a reinvestigation was undertaken in this work.

2.2. Results and Discussion.

2.2.1. Tautomerism.

The published ^{1}H n.m.r. spectrum of bilirubin, using $^{2}H_{6}$-DMSO as solvent, shows, inter alia, three broad singlets at 0·12τ (1H), −0·01τ (1H) and −0.45τ (2H).[111] These are assigned to the two protons of the carboxylic acid groups (−0·45τ) and to the two N-H protons of the pyrrole rings B and C (−0·01τ and +0·12τ). A broad resonance observed at 4·75τ is assigned to the enolic O-H protons of the lactim tautomer. If the lactim tautomer is the predominant form in this solvent, then the observation of the enol O-H protons at such a high field is unusual. Normally, the resonance position would be expected to be at lower field because the O-H groups are deshielded by the anisotropy of the π-electron system of the C=N bond.[113a]

The ^{1}H n.m.r. spectrum of bilirubin, crystallised from chloroform/methanol, dissolved in $^{2}H_{6}$-DMSO, was recorded at 60MHz. The major differences observed compared with the published spectrum[111] are (i.) the absence of an absorption at 4·75τ, (ii.) the presence

of a broad absorption at 6·60τ due to water, and (iii.) the presence of a broad signal at −1.70τ, which, however, does not correspond to an integral number of protons. The spectra of several other freshly prepared samples, using solvent from the same source, show the same features, but as the resonance due to the presence of water in the solvent increases in intensity, so the intensity of the resonance at −1.70τ decreases. It would appear therefore, that the resonance at −1·70τ is water dependent.

Using carefully dried $^{2}H_{6}$ -DMSO, the ^{1}H n.m.r. spectrum of bilirubin, recorded at 100MHz (Figure 2.3.), shows broad singlets at −1.90τ (2H), −0.50τ (2H), −0·10τ (1H), and +0.45τ (2H). These six low - field protons can be assigned in two ways. Firstly, if bilirubin exists as the lactam tautomer in DMSO solution, then they correspond to four N-H protons and two carboxylic acid protons. Secondly, if the lactim tautomer predominates, they correspond to two N-H protons, two carboxylic acid protons and two enolic O-H protons. In order to distinguish these possibilities, exchange reactions were carried out using bilirubin and the model compounds 2,4 -dimethylpyrrole-3-propionic acid [114] (2.V.), which contains a pyrrole N-H group and a carboxylic acid group and resembles the B and C rings of bilirubin, and N-benzoylglycine (2.VI.) which contains a secondary amide N-H group.

Successive additions of deuterium oxide (10µl, 0.5 mmole) to a solution of bilirubin (40mg, 70µmole) in $^{2}H_{6}$ -DMSO (0·5ml) cause all the low field signals to collapse, whereas similar additions of water cause only the signal at −1·90τ to collapse (Figure 2.4.). The ^{1}H n.m.r. spectra of solutions of (2.V.) and (2.VI.) in $^{2}H_{6}$ -DMSO are also affected by the addition of water. In these cases, the signals due to protons attached to nitrogen persist, whereas those due to hydroxylic protons collapse. These results

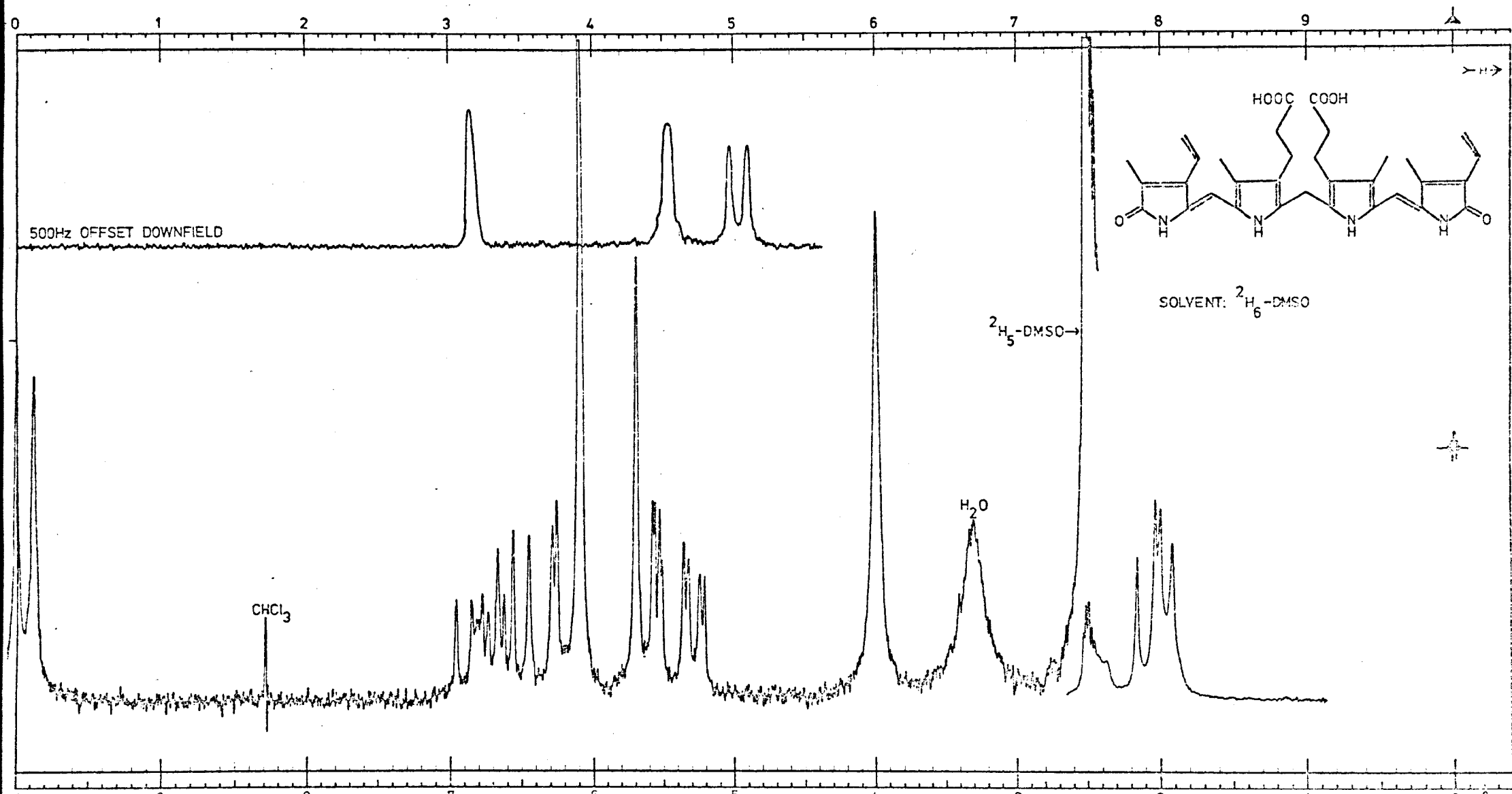


FIGURE 23. THE 100MHz ^{1}H N.M.R. SPECTRUM OF BILIRUBIN

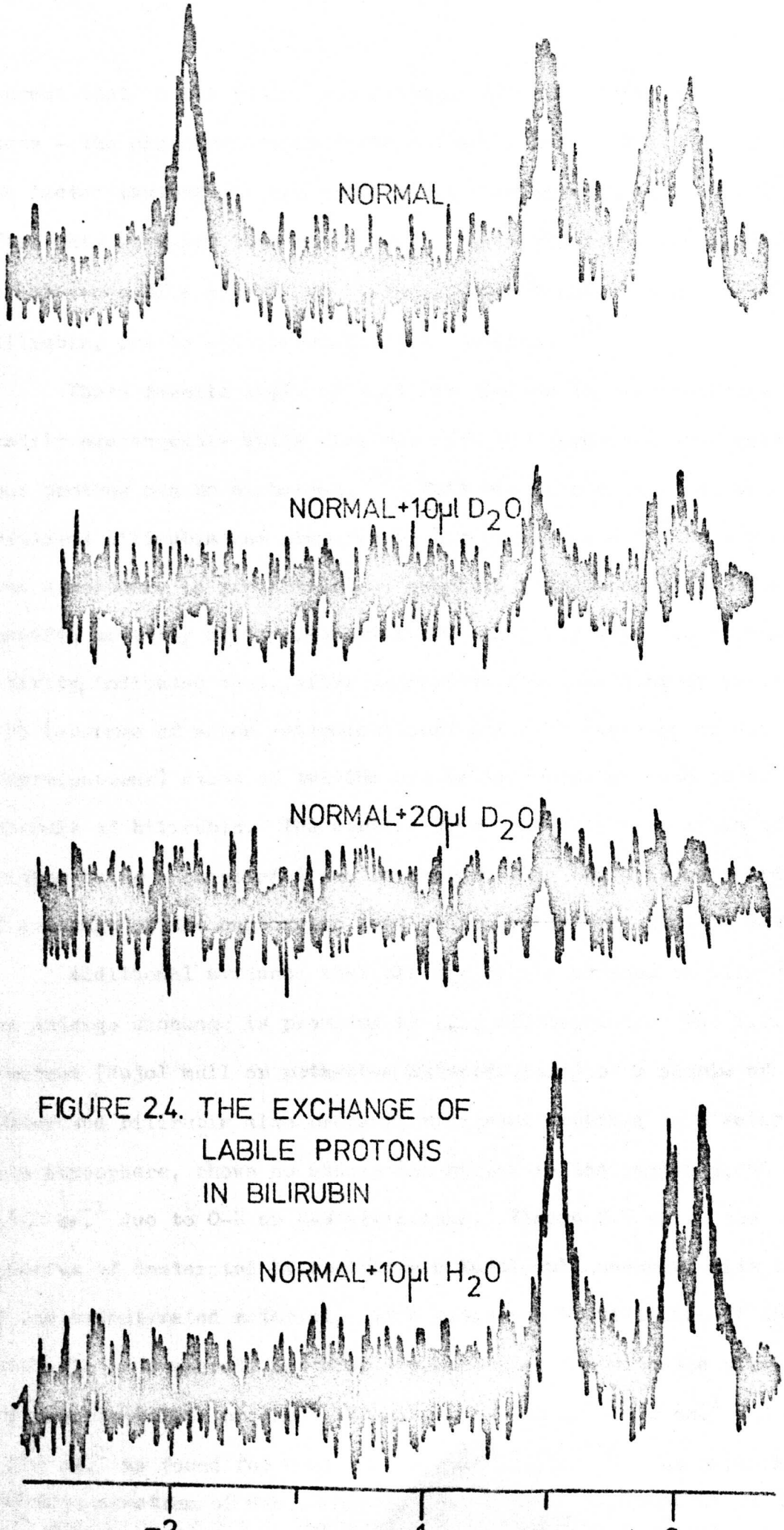


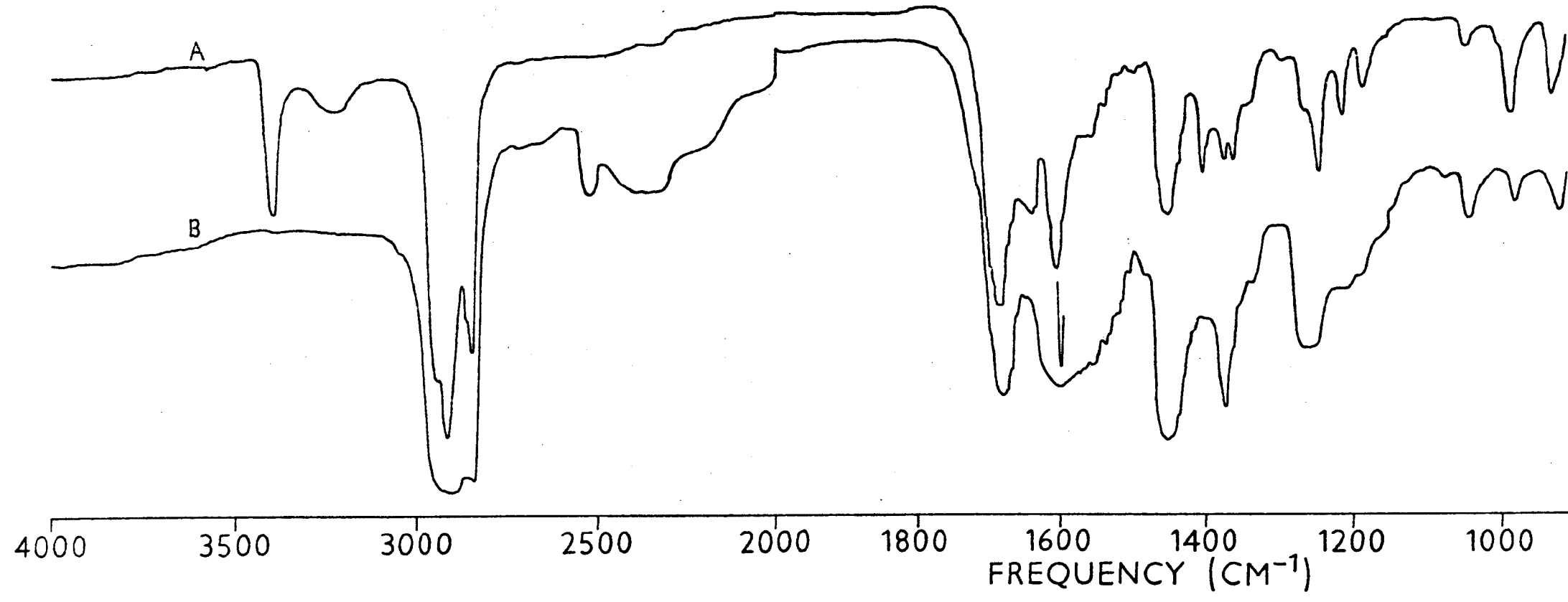
FIGURE 2.4. THE EXCHANGE OF LABILE PROTONS IN BILIRUBIN

suggest that in bilirubin, two protons only are attached to oxygen atoms - the carboxylic acid protons - and that, in dry $^{2}H_{6}$-DMSO, the lactam tautomer is the predominant form in solution. Shortly after this work had been completed, a paper was published confirming the presence of a singlet, at -1·89τ, in the ^{1}H n.m.r. spectrum of bilirubin, due to the carboxylic acid protons. [88]

These results indicate that six protons in bilirubin are readily exchangeable while previous work has suggested that only four protons can be exchanged.[106] This was reinvestigated, and tritiated bilirubin was prepared by direct exchange [106] in a water-free atmosphere (a precaution not observed previously [106]). The specific activity of two preparations, carefully dried to constant activity, indicated that, after correction for quenching by bilirubin, 5·95 (average of seven determinations) and 6·03 (average of six determinations) atoms of tritium can be incorporated into each molecule of bilirubin. The earlier report of only four atoms of tritium being incorporated may be explained by the extreme rapidity of exchange of the carboxylic acid protons with adventitious water.

Additional evidence that all six labile protons in bilirubin can undergo exchange is provided by i.r. spectroscopy. The i.r. spectrum (Nujol mull or potassium chloride disc) of a sample of deuterated bilirubin also prepared by direct exchange in a water - free atmosphere, shows no absorption maxima in the region 3,000 - 3,600 $cm.^{-1}$ due to O-H or N-H vibrations. Figure 2.5 shows the spectrum of deuterated bilirubin as a Nujol mull compared with that of the undeuterated material. If a potassium bromide disc of deuterated bilirubin is prepared, deuteron - proton exchange with the slightly hygroscopic matrix produces absorption maxima at 3,400 $cm.^{-1}$ and 3,280 $cm.^{-1}$ as found for undeuterated bilirubin. [108] The published KBr disc spectrum of deuterated bilirubin shows absorptions at 3,420

FIGURE 2.5. THE I.R. SPECTRA OF BILIRUBIN (A) AND DEUTEROBILIRUBIN (B) AS NUJOL MULLS

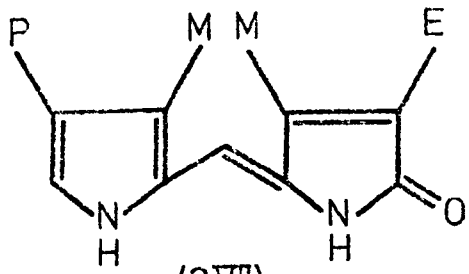


and 3,300 $cm.^{-1}$ due to N-H and O-H vibrations, whereas that in chloroform shows almost no absorption in the region 3,200 - 3,500 $cm.^{-1}$ [106] These results, taken in conjunction with the apparent exchange of only four protons, were interpreted as indicating that bilirubin exists in two hydrogen bonded forms, one in the solid state and the other in solution (see section 2.2.2. of this chapter). The presence of a large O-H group absorption in the spectrum of deuterated bilirubin is apparently ignored, even though it was suggested that the four protons exchanged were the two carboxylic acid protons and the two N-H protons of the lactam rings. The best explanation for these results is again deuteron - proton exchange in the KBr disc while the sample used for the solution spectrum, which was prepared by extraction of freshly precipitated deuterobilirubin with chloroform, would be wet with deuterium oxide, which could exchange with any adventitious water in the system.

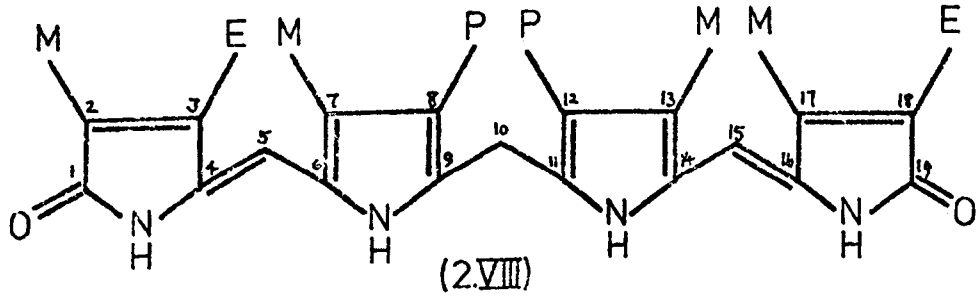
In proposing the lactim tautomer,[111] a comparison was drawn between the positions of the visible absorption maxima of bilirubin (λ_{max} 450 nm., solvent not specified) and isoneoxanthobilirubic acid (2.VII.) (λ_{max} 395 nm., presumably in the same solvent). It was suggested that this difference could not be accounted for by the absence of a vinyl substituent in (2.VII.) and must arise from a difference in the chromophores in each compound, ie. since (2.VII.) exists as the lactam tautomer, [105] then bilirubin must exist as the lactim tautomer. This comparison is dubious for several reasons. Firstly, the solvent is not specified and if the data for (2.VII.) is assumed to be taken from the literature, [105] then the solvent is aqueous buffer at a neutral pH, whereas that for bilirubin could be either chloroform or neutral aqueous buffer. Secondly, the two halves of bilirubin molecule do not have identical chromophores. Thus, in the A-B dipyrrylmethene, the vinyl substituent is cross

conjugated with the main chromophore whereas the one in the C - D dipyrrylmethene is linearly conjugated with the main chromophore. Therefore, there ought to be a difference in the positions of the visible absorption maxima of the two dipyrrylmethenes and the absorption maximum of bilirubin can be considered to be the sum of the two individual maxima. However, in mesobilirubin (2.VIII.), the two chromophores are the same as that in isoneoxanthobilirubic acid so, a priori, there ought to be very little difference between the positions of the absorption maxima of these compounds. In neutral aqueous conditions, mesobilirubin has an absorption maximum at about 410 nm [105] (same conditions as for (2.VII.)). The difference (15 nm) between mesobilirubin and (2.VII.) may be due to interactions between the dipyrrylmethene subunits in mesobilirubin, interactions which are not possible in (2.VII.). Hence the difference between the positions of the absorption maxima of bilirubin and (2.VII.) is probably due to the absence of a vinyl group in (2.VII.) and so no information regarding the lactam - lactim tautomerism can be gained in this way.

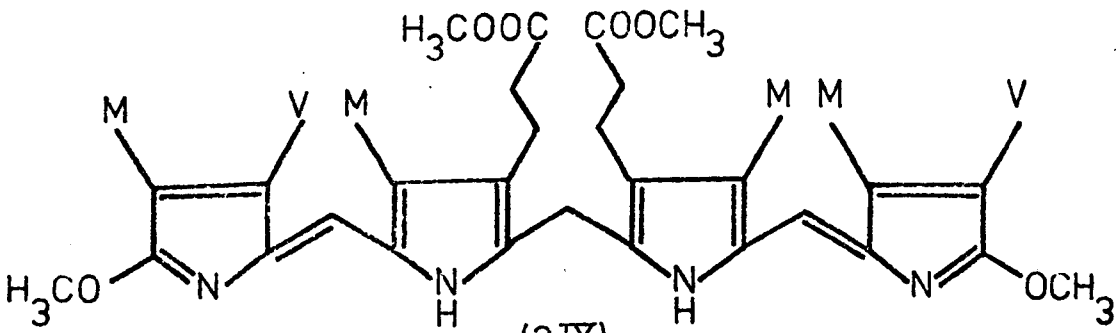
Bilirubin will react with diazomethane giving predominantly the dimethyl ester together with a small amount of α,α' - dimethoxybilirubin dimethyl ester [102] (2.IX.). In this compound, tautomerism is impossible and the chromophore is fixed in the lactim form. Comparison of the visible spectra of bilirubin and (2.IX.), a more valid comparison than between bilirubin and (2.VII.), suggests that bilirubin, in chloroform solution, exists as the lactam tautomer. If it is assumed that the absorption maximum of the lactim tautomer of bilirubin (2.I.) in chloroform would differ negligibly from that of the enol ether (2.IX.) in chloroform (λ_{max} 418 nm), then the observed absorption maximum of bilirubin in chloroform at 454 nm must indicate that the lactam tautomer predominates in this solvent. The formation of (2.IX.) shows that a small proportion of bilirubin



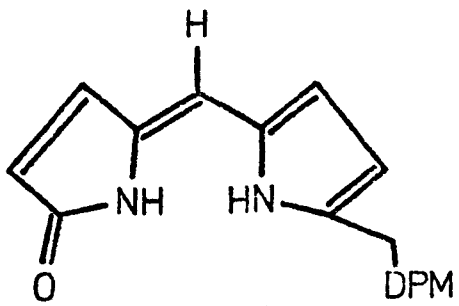
(2.VII)



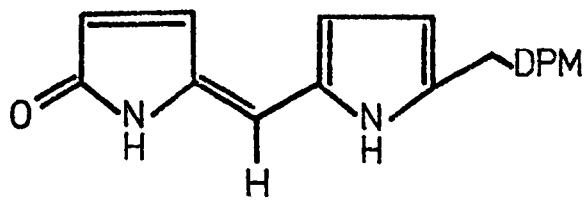
(2.VIII)



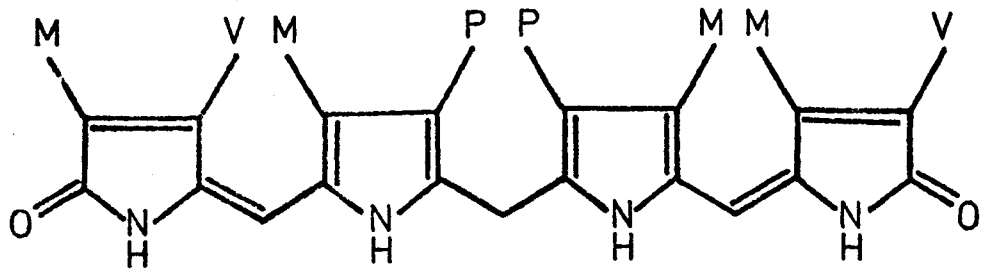
(2.IX)



(2.X)



(2.XI)



(2.XII)

in solution must be present as the lactim tautomer. Prolonged treatment of bilirubin in chloroform or DMSO with diazomethane does not enhance the yield of (2.IX.).[88] No explanation has yet been offered to account for these observations, but it has been suggested that they are possibly the result of some altered interaction between the carboxylic acid groups and the heterocyclic systems of bilirubin.[83] Such an interaction might result from intramolecular hydrogen bonding in bilirubin.

2.2.2. Structure and Hydrogen Bonding.

Before discussing the various hydrogen bonded structures for bilirubin which have been proposed, mention must be made of the possible cis - trans isomerism in bilirubin itself. Bilirubin is formed from haem and therefore should contain one of the basic steric requirements of haem, namely that the hydrogen atoms of the methine bridges are trans with respect to the nitrogen atoms of the adjacent lactam rings (2.X.) and not cis (2.XI.).

By convention, bilirubin is generally represented as the cis isomer (2.XII.). In theory, bilirubin does possess the capacity to isomerise in this way. The central methylene bridge protons, being adjacent to an extended conjugated system containing a carbonyl group onto which a negative charge may be delocalised, are perhaps rather more acidic than a normal alkyl methylene function. Thus enolisation via the loss of one of these protons or the N-H proton of either pyrrole ring B or C, rotation about the C(4) - C(5) and C(15) - C(16) carbon - carbon bonds and re - ketonisation produces the cis isomer (Figure 2.6.).

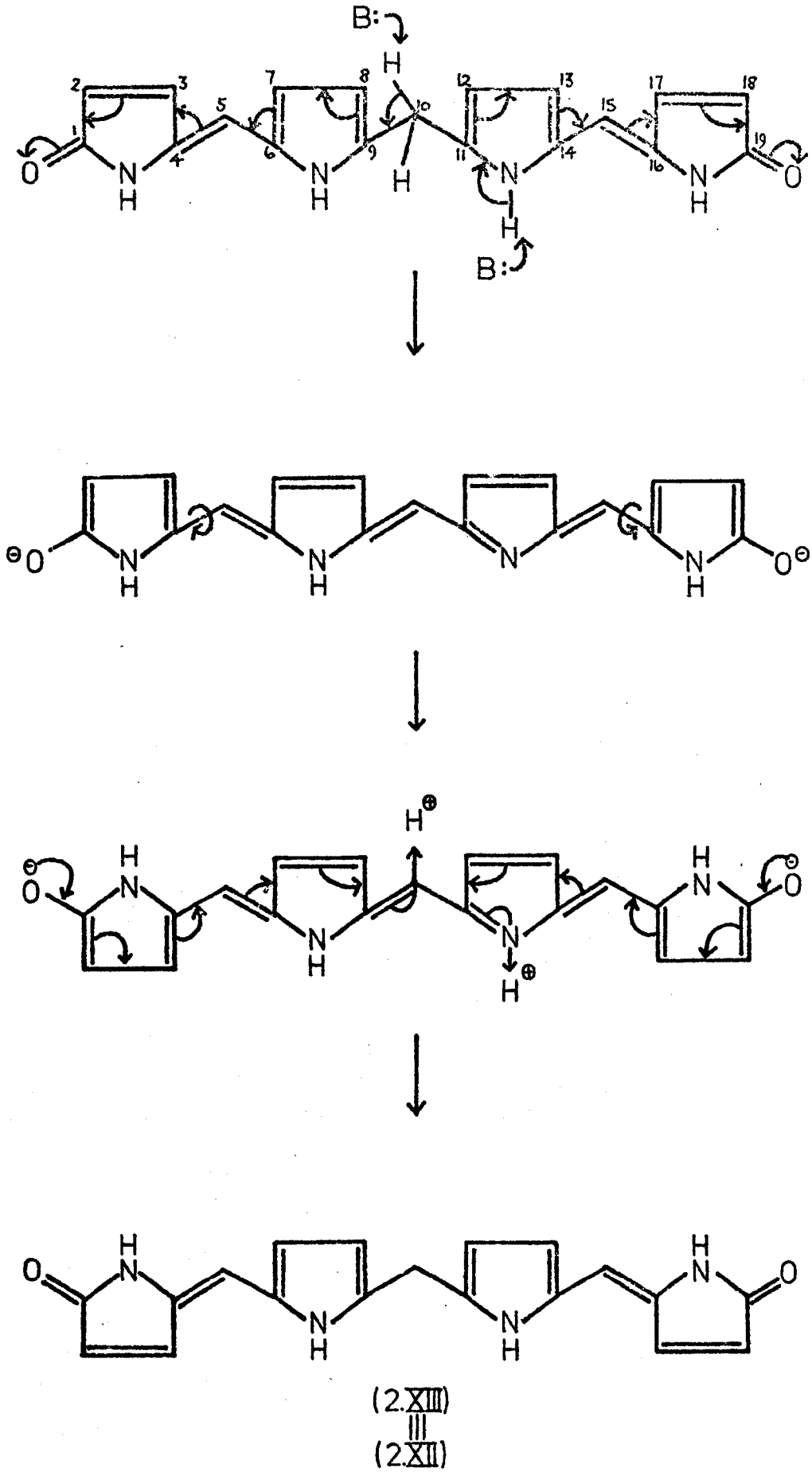
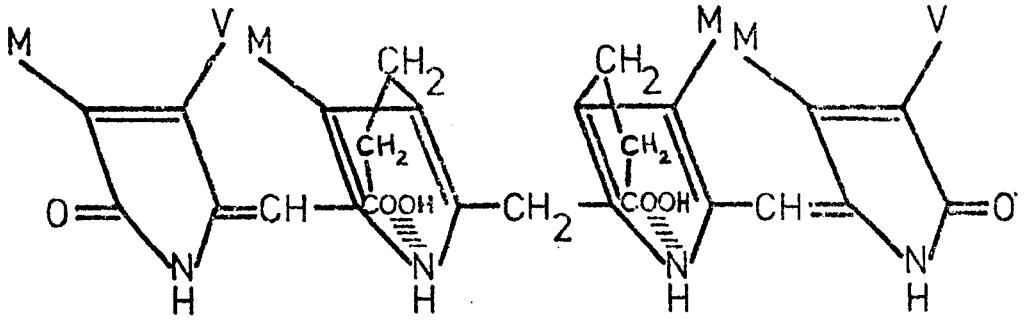


FIGURE 2.6. THE ISOMERISATION OF BILIRUBIN. Both mechanisms are shown on the same molecule.

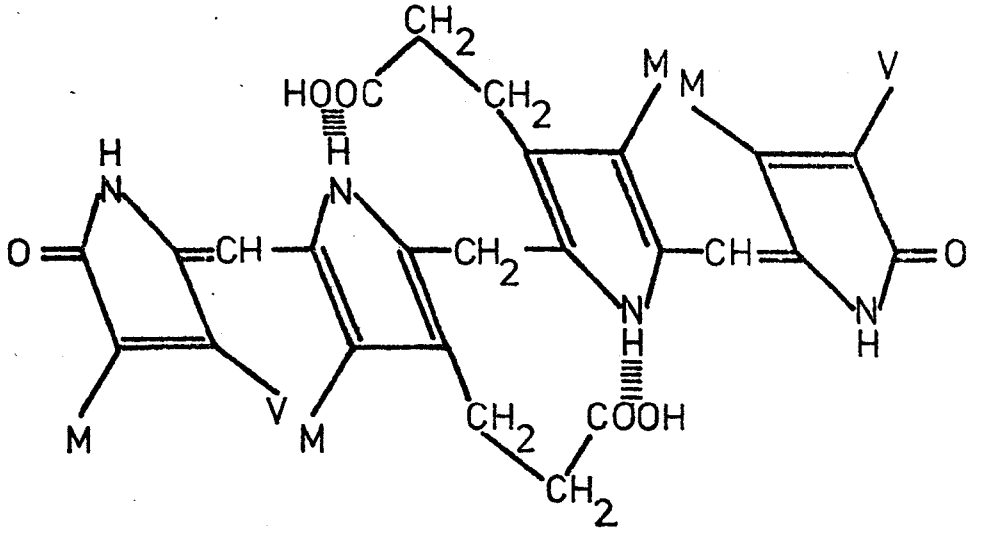
Whether or not this is a feasible process *in vivo*, because *in vitro* the reaction could require extreme alkaline conditions, it is important to recognise that the isomerism does in theory, at least, exist. When reading published literature concerning hydrogen bonding in bilirubin, it is apparent that erroneous conclusions have been deduced through the use of both *cis* and *trans* isomers.

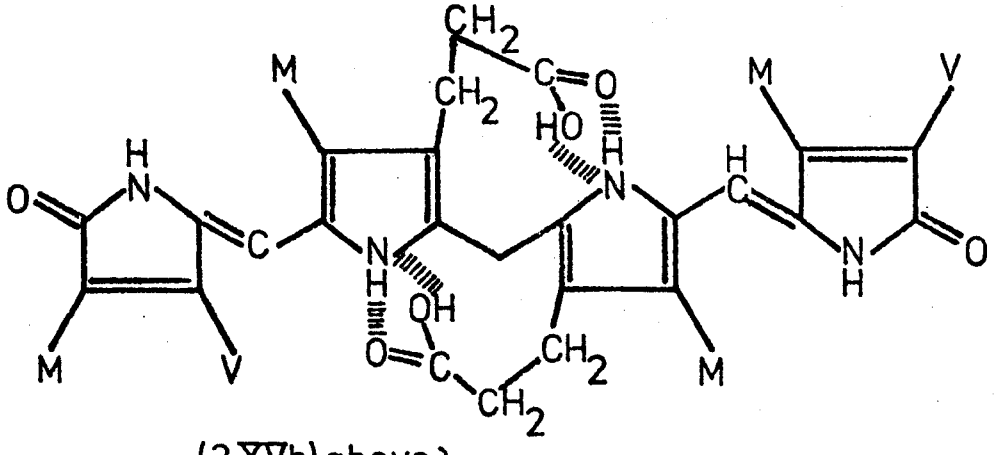
Four fundamental hydrogen bonded structures for bilirubin have been proposed (2.XIV. - 2.XVII.) and three of these (2.XIV. - 2.XVI.) are reproduced here in the exact forms in which they were published. Structure (2.XIV.) appears impossible on steric grounds alone, although this structure has been considered likely in a recent paper.[93] A model of (2.XIV.) indicates that it is impossible to form hydrogen bonds between the carboxylic carbonyl group and the pyrrole N-H group without severely distorting the molecule. Structure (2.XVII.) in which the hydroxyl group of one of the carboxylic acid functions forms a hydrogen bond to the π-electron system of either pyrrole ring B or C has also been considered.[88] This can be considered as being "half way" towards structure (2.XIV.). However, evidence from the i.r. spectrum of bilirubin (Figure 2.5.) suggests that this structure is not adopted. *A priori*, one would expect the stretching frequency of the carbonyl groups to be affected to a much lesser extent in (2.XVII.) than in the other cases cited, because they are not directly involved in the hydrogen bonding. The observed carbonyl frequency at 1690 $cm.^{-1}$ indicates that the carbonyl groups participate in strong hydrogen bonding, since saturated aliphatic carboxylic carbonyl groups absorb at 1700 - 1725 $cm.^{-1}$ in the absence of hydrogen bonding.[115a]

Structures (2.XVa,b.) and (2.XVI.) are more feasible. It has been suggested, on the evidence of isotope exchange experiments (see section 2.2.1. of this chapter), that (2.XVI.) occurs in the solid

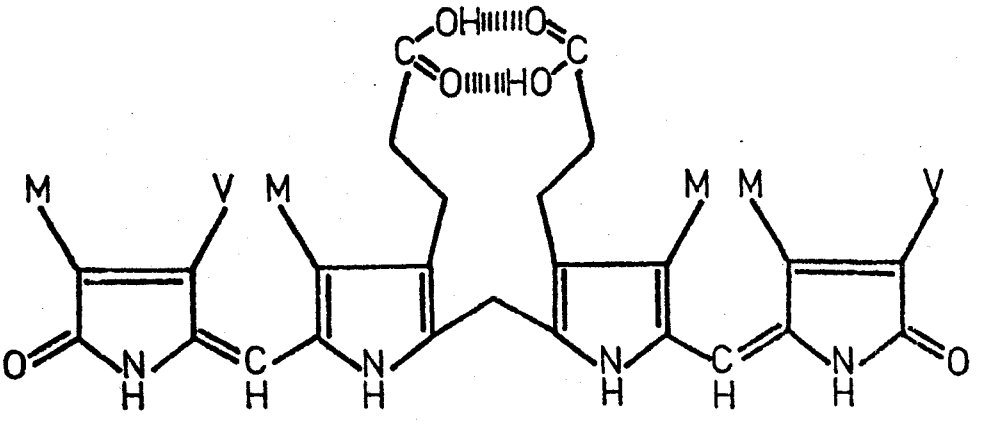


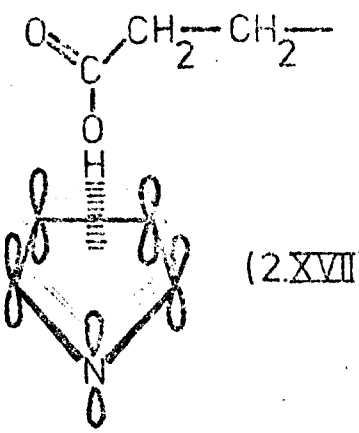
(2.XIV) above
(2.XVa) below
} (From ref.112)



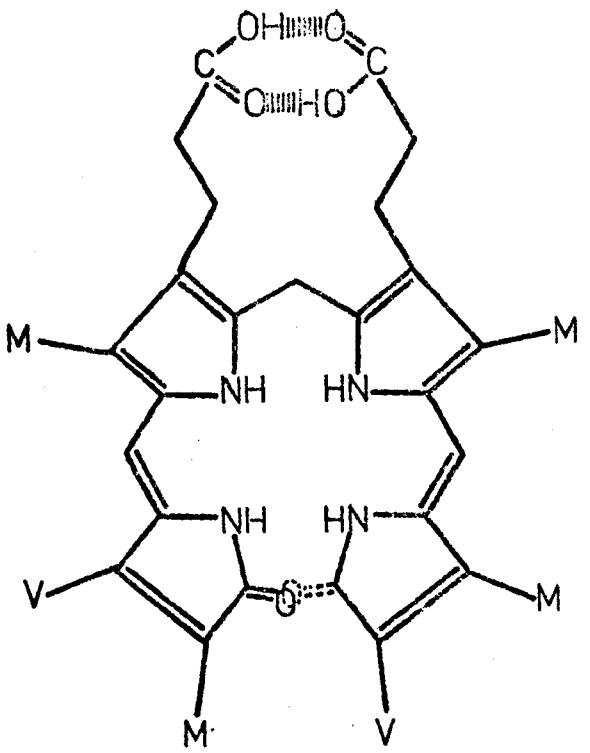


(2.XVb) above
(2.XVI) below
} (From ref.106)

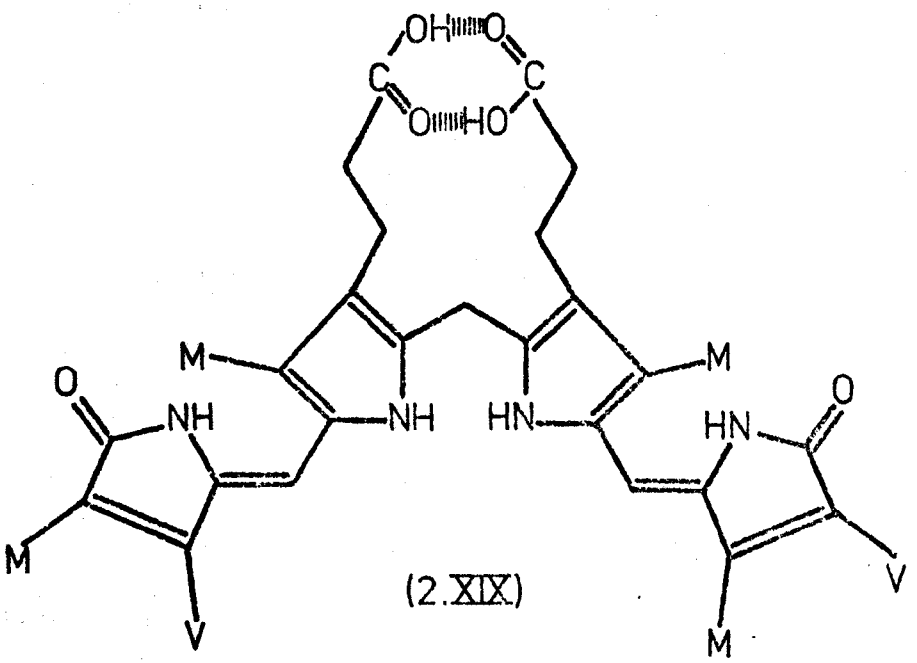




(2.XVII) (From ref.88)



(2.XVIII)



(2.XIX)

state while (2.XVb.) occurs in chloroform solution.[106] Models constructed of the two forms indicated that each was free from strain[106] and if the diagrams are to be interpreted rigorously then (2.XVb.) shows the trans isomer while (2.XVI.) shows the cis isomer. Obviously the two forms are not now immediately interconvertible in going from the solid state into solution without the enolisation process, described above, occurring first - a process unlikely to occur in the neutral medium of a chloroform solution. Whichever isomers it was intended to depict, it is possible for the trans isomer to adopt the structure (2.XVIII.) which is similar to (2.XVI.). The subsequent discussion will concentrate solely on hydrogen bonded forms of the trans isomer.

If one form is prevalent in the solid state while another prevails in solution eg. (2.XVIII.) and (2.XVb.), one might expect the carboxylic groups to absorb at slightly different i.r. frequencies in the two forms, since different groups are involved in the hydrogen bonding. In the present work, the i.r. spectra of bilirubin were recorded in different media and it was found that the position of the carboxylic carbonyl absorption does not alter whether the sample is prepared as a Nujol mull, potassium bromide disc, in chloroform solution or in dioxan solution (Figure 2.7.). In contrast, the i.r. frequency at which the carboxylic carbonyl group in 2,4- dimethylpyrrole -3- propionic acid (2.V.) absorbs is strongly dependent on the medium and ranges from 1690 $cm.^{-1}$ (Nujol mull) to 1735 $cm.^{-1}$ in dioxan solution (Figure 2.8.). These results indicate not only that the carbonyl groups of the propionic acid side chains participate in strong intramolecular hydrogen bonds (v.supra), but that the hydrogen bonding does not alter with the medium. Thus, bilirubin can exist only as (2.XVb.) (or as seems more likely, a closely related structure) or (2.XVIII.).

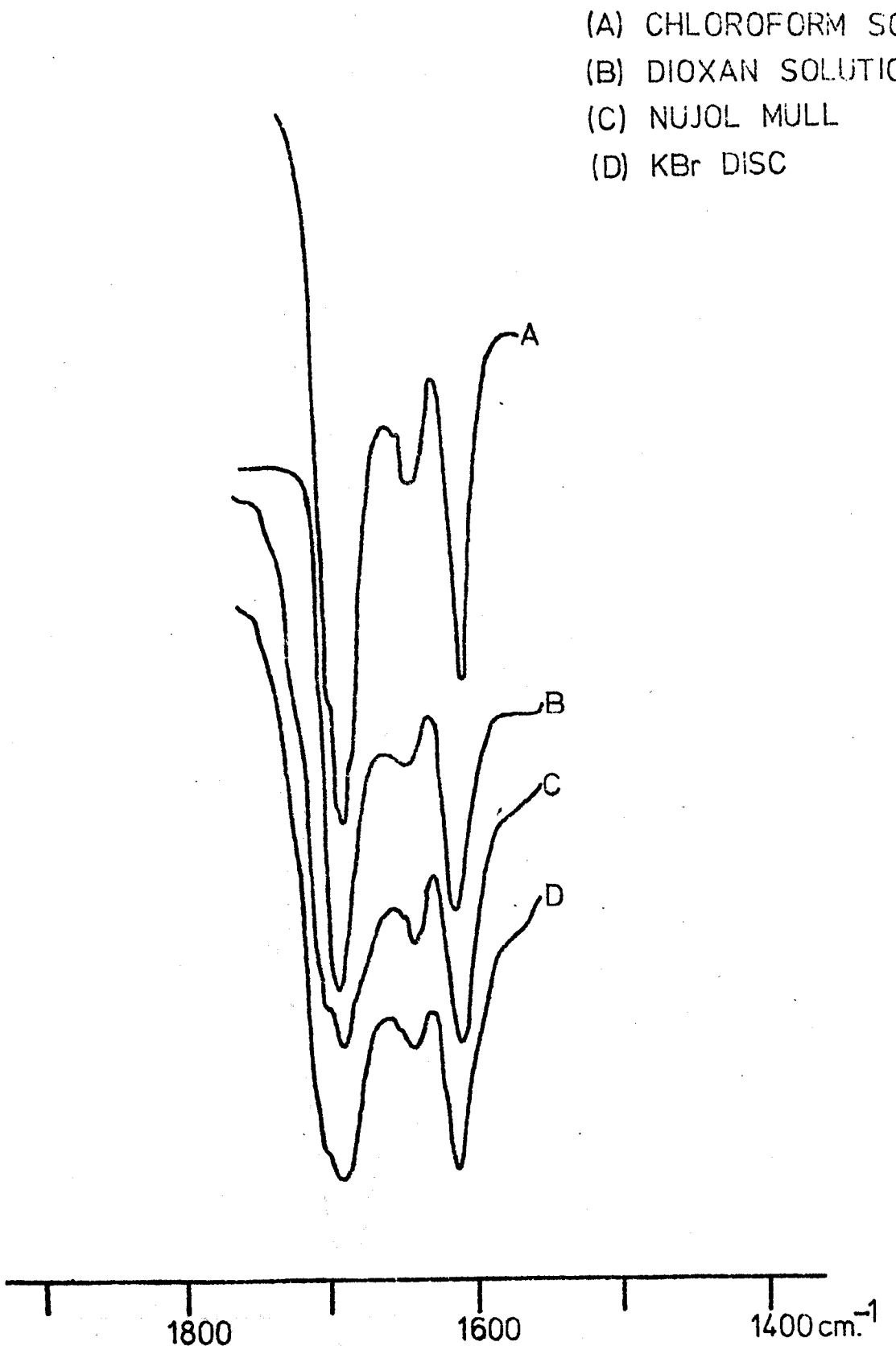


FIGURE 2.7. THE CARBONYL REGIONS OF THE I.R. SPECTRA OF BILIRUBIN IN DIFFERENT MEDIA

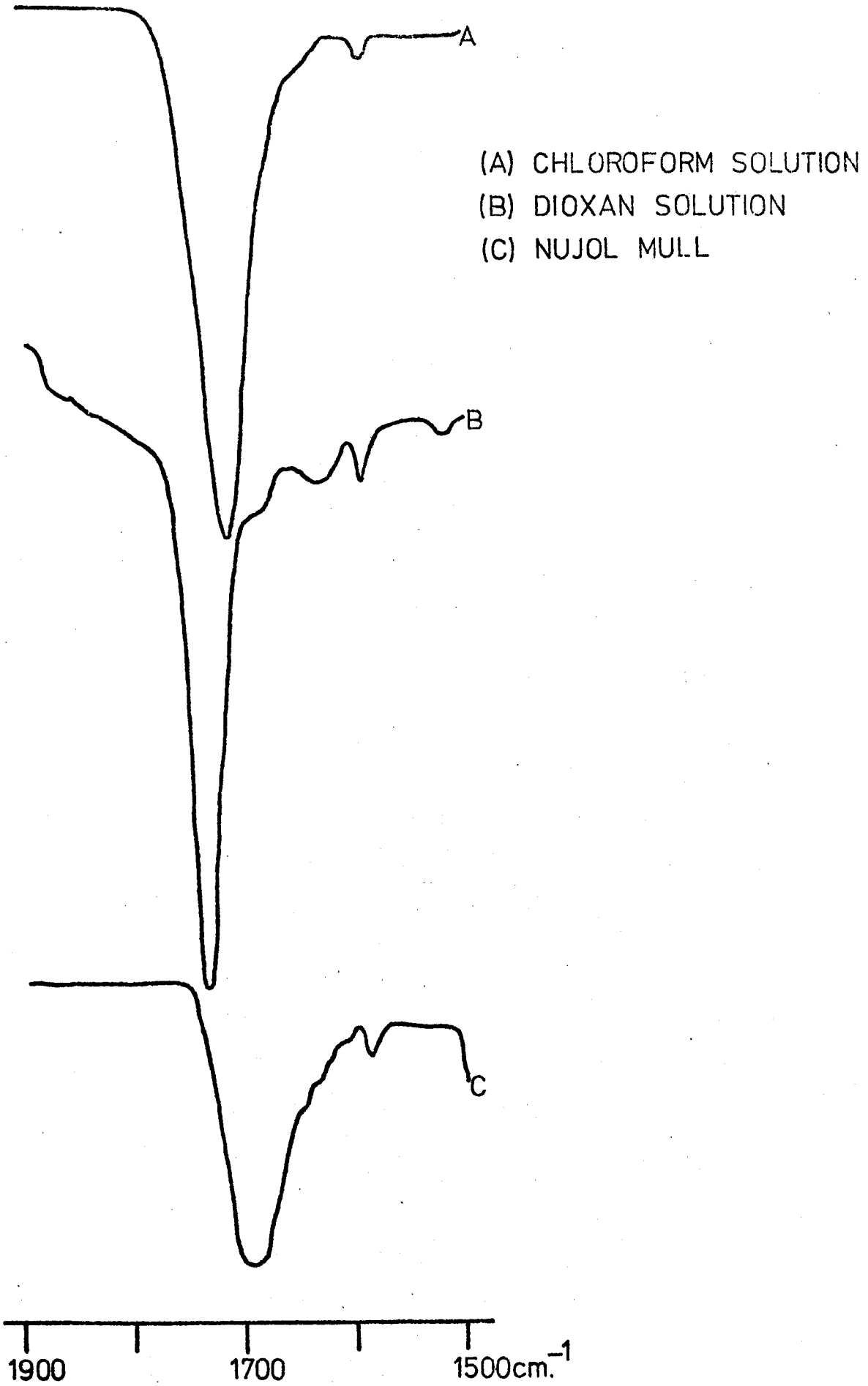


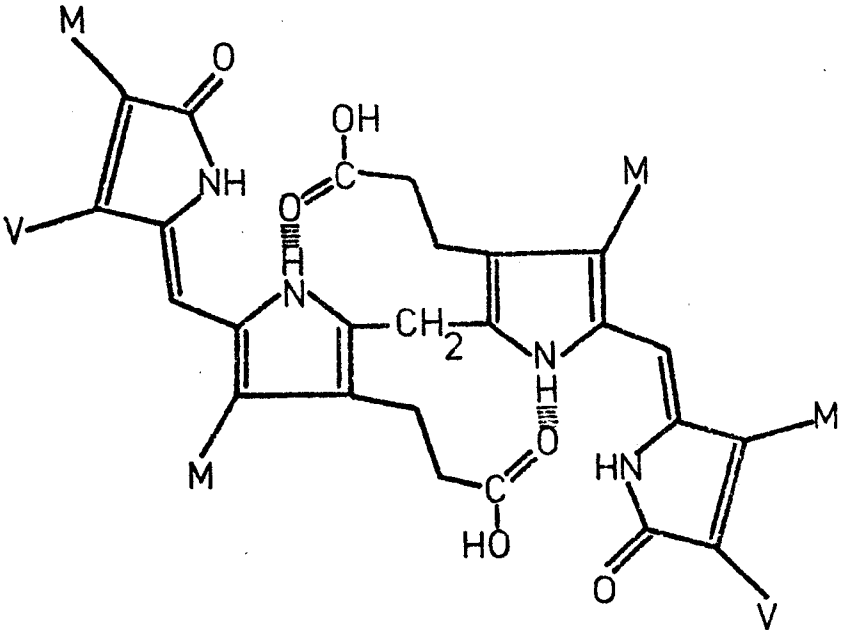
FIGURE 2.8. THE CARBONYL REGIONS OF THE IR SPECTRA OF (2.V) IN DIFFERENT MEDIA

In (2.XVIII.), a planar, protoporphyrin - like structure is impossible because of steric crowding between the carbonyl groups of the terminal lactam rings. (In going from haem to bilirubin a single carbon atom has been replaced by two oxygen atoms). Therefore the molecule can either adopt a three dimensional "V" shape with the two arms of the molecule held apart by steric repulsion between the lactam rings or alternatively, and to be preferred, can adopt a planar "V" shape by allowing rotation about the C(5) - C(6) and C(14) - C(16) carbon - carbon bonds to take the lactam rings away from each other as in (2.XIX.).

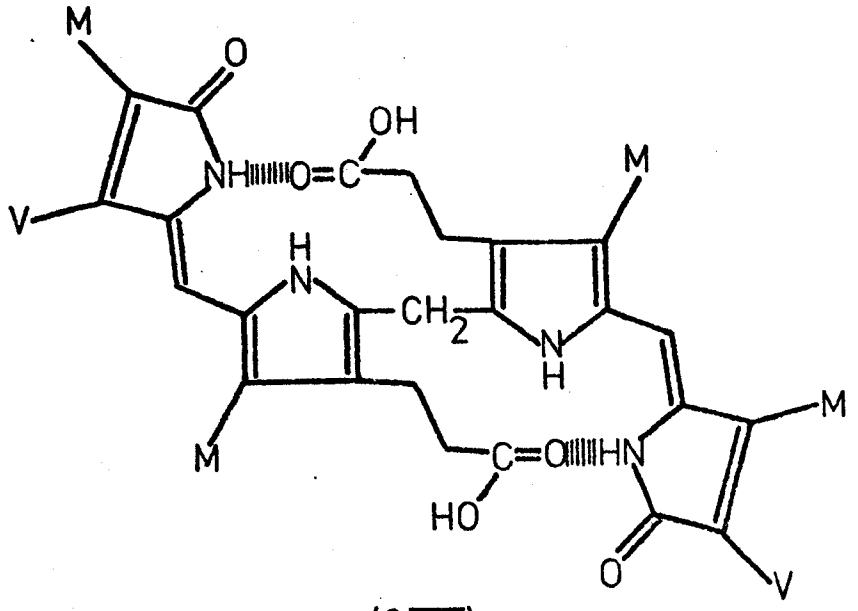
The possibility of intermolecular hydrogen bonded dimers and polymers based on the carboxyl - carboxyl hydrogen bonded structure has been suggested.[106] In the solid state, one cannot distinguish these forms from the intramolecularly bonded monomer by i.r. spectroscopy, but in dilute solution, any intermolecular hydrogen bonds should be broken down. Therefore the i.r. spectrum of a dilute solution should show the presence of a non - hydrogen bonded carboxylic carbonyl group, unless the molecule adopts an intramolecularly hydrogen bonded structure based on the same system. Since bilirubin is soluble in chloroform only to the extent of 1mg/ml, giving a $1{\cdot}7 \times 10^{-3}$ molar solution, it might be expected that at this concentration, all intermolecular hydrogen bonds would be broken. Nevertheless, in solution, the carbonyl frequency is still observed at 1690 $cm.^{-1}$ indicating the hydrogen bonding is unchanged. Dilution experiments to determine if a change in hydrogen bonding occurs are not practical because of the poor solubility of bilirubin. The change of intermolecular to intramolecular hydrogen bonding on dilution is unlikely, since the molecule ought to form and retain the hydrogen bonded structure which is energetically most favourable, which in this case ought to be the intramolecular form.

When suggested originally,[106] structure (2.XVb.) was thought to be strain free. However models indicate three related structures which are less strained than (2.XVb.). These are (2.XX.), (2.XXI.) and (2.XXII.) which is intermediate between the first two structures.

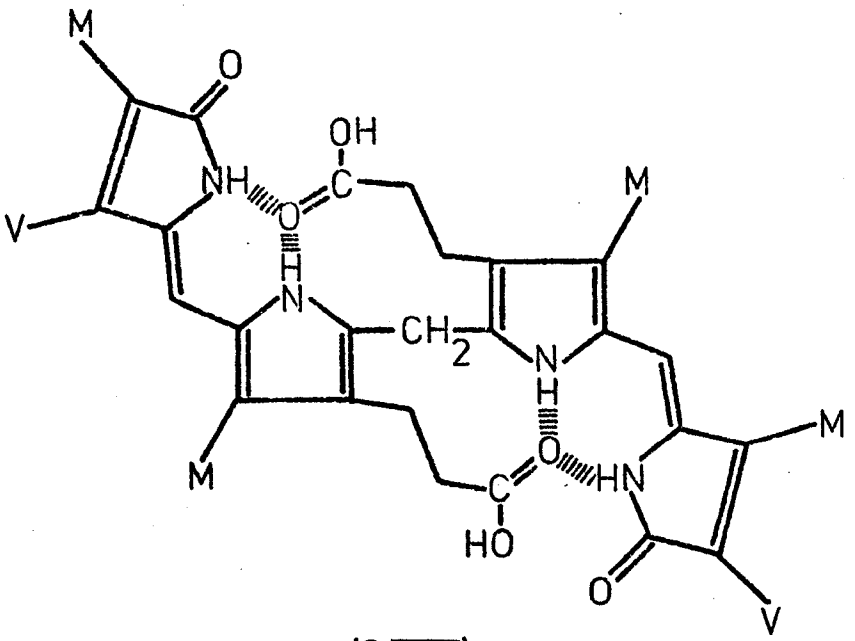
How are the four most likely structures (2.XIX.) - (2.XXII.) to be distinguished? The i.r. spectrum of bilirubin (Figure 2.5.) shows a sharp absorption at 3,410 $cm.^{-1}$ and a broad absorption at 3,230 $cm.^{-1}$ with no other significant absorption between 2,800 and 1,700 $cm.^{-1}$ The absorption at 3,410 $cm.^{-1}$ can be assigned to the non-hydrogen bonded N-H stretching vibration of a cyclic lactam,[115b] but it is rather too low for the non - hydrogen bonded N-H vibration of a pyrrole which usually appears at about 3,500 $cm.^{-1}$,[115c] and is also rather too low for the free O-H vibration of a carboxylic acid group, which is usually found between 3500 - 3560 $cm.^{-1}$[115a] The band is rather too sharp to contain any hydrogen bonded O-H or N-H vibrations. The band at 3,230 $cm.^{-1}$ can be assigned to hydrogen bonded N-H vibrations of the pyrroles and the lactams since hydrogen bonded O-H vibrations generally occur between 2,500 - 2,700 $cm.^{-1}$[115a] The absence of any other significant absorption bands in this region suggests the absence of any hydrogen bonded O-H groups in bilirubin. Thus (2.XVb.) and (2.XIX.) are eliminated. There is additional evidence for discounting (2.XIX.) since this form contains the hydrogen bonded structure of a carboxylic acid dimer, which generally gives rise to a broad absorption region with many sub - maxima between 3,000 and 2,500 $cm.^{-1}$[115a] In free protoporphyrin, it is expected that intramolecular hydrogen bonding occurs readily between the carboxyl groups of the propionic acid side chains, and in a series of deuteroporphyrin free acids, eg.(2.XXIII.) deuteroporphyrin itself, broad bands near 3,100 and 2,600 $cm.^{-1}$, characteristic of dimeric carboxylic acids are found.[116] Since (2.XIX.) is the direct


M
O
NH
V
OH
C
O
H
N
CH2
N
H
M
M
O
C
HO
HN
M
O
V

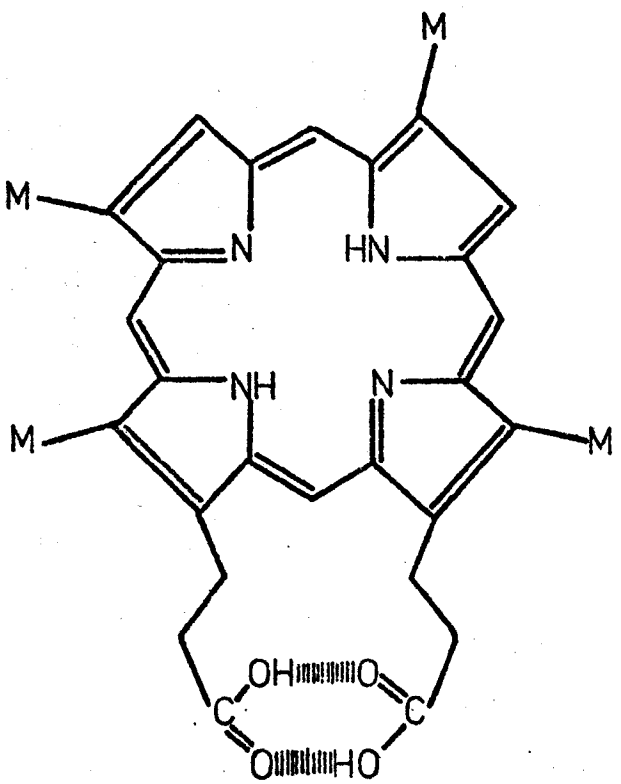
(2.XX)


M
O
NH
O=C
OH
V
H
N
CH2
N
H
M
M
C=O
HN
HO
M
O
V

(2.XXI)



(2.XXII)



(2.XXIII)

equivalent of these porphin diacids, the absence of any like bands in the i.r. spectrum of bilirubin (even as a KBr disc, which shows the region of the spectrum obscured by Nujol) tends to suggest that bilirubin does not adopt a form like (2.XIX.).

Even though no free N-H groups are present in (2.XXII.), this structure is compatible with the spectral evidence if the band at 3,410 $cm.^{-1}$ is assigned to the O-H vibration which must then be weakened by the effect of the hydrogen bonding of the N-H groups to the carbonyl group.

Structures (2.XX.) and (2.XXI.) are also compatible with the i.r. spectrum if the band at 3,410 $cm.^{-1}$ is assigned to the free O-H and N-H vibrations, while that at 3,320 $cm.^{-1}$ is assigned to the bonded N-H vibration in each case.

Consequently any one of either (2.XX.), (2.XXI.) and (2.XXII.) seems to be a plausible hydrogen bonded structure for bilirubin. Models of these three forms are shown in Plates 2.1 - 2.3 .If it is assumed that the hybridisation state of the lactam nitrogen atoms approaches sp^2 rather than sp^3 then the models of forms (2.XX.) and (2.XXI.) indicate that structure (2.XXII.) would follow directly. While these three cannot be distinguished as yet, there is a slight overall preference for the form (2.XXII.).

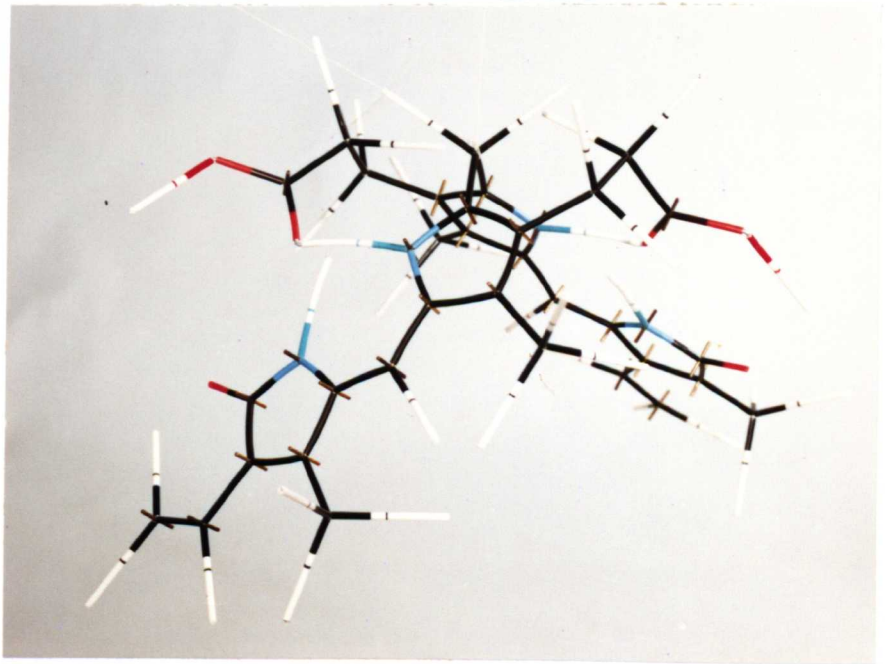


PLATE 2.1.

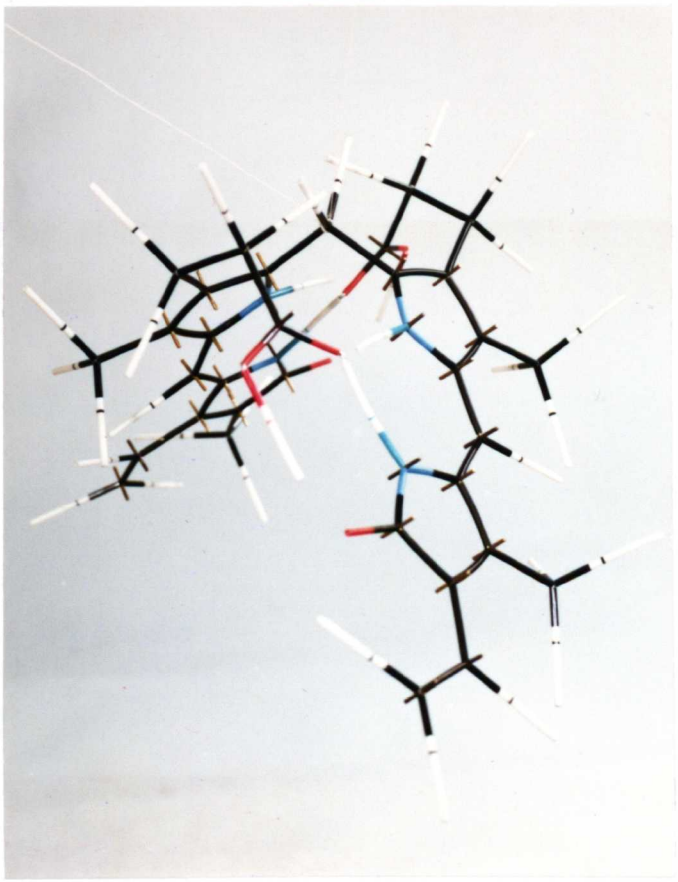


PLATE 2.2.

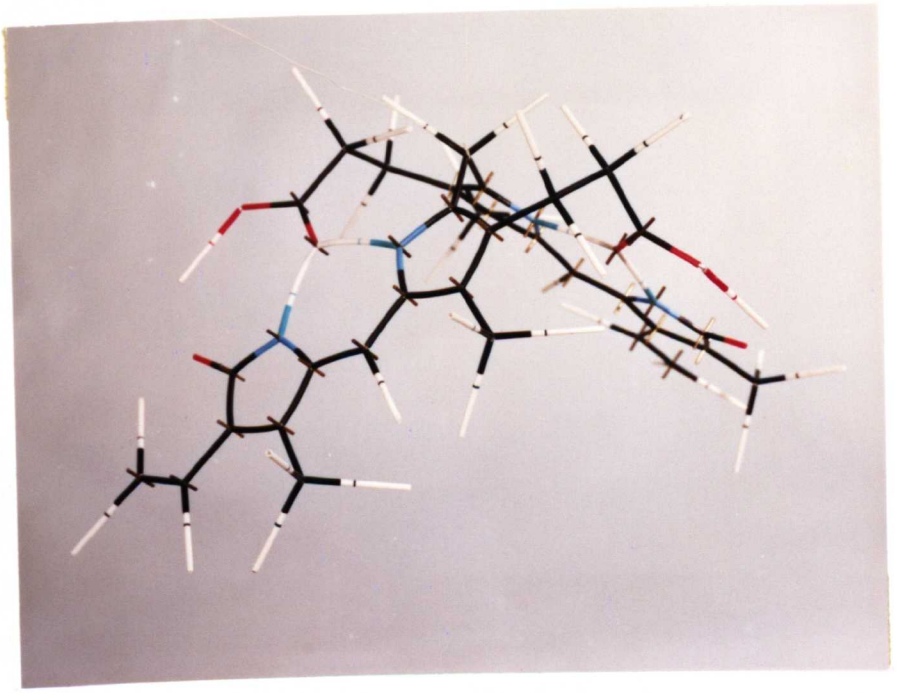


PLATE 2.3.

2.3. Experimental.

Materials

Bilirubin was obtained from BDH Chemicals Ltd., and crystallised from chloroform - methanol before use.[111]

u.v. (Chloroform) : λ_{max} 454 nm (ε 59, 400). Lit.,[88] λ_{max} 453nm (ε 58,800).

Analysis (S) : Found C, 67·87; H, 6·02; N, 9·20%.
$C_{33}H_{36}N_4O_6$ requires C, 67·81; H, 6.16; N, 9·59%.

$^2H_6$-DMSO was dried over freshly heated 4A molecular sieve, in a well - stoppered vial, for one week before use.

Methods.

2,4 - Dimethylpyrrole -3- propionic Acid (2.V.).- This was prepared according to the reaction sequence shown in Figure 2.9.

Methyl 4 - acetyl -5- oxohexanoate (2.XXIV.).- This was prepared as described[117] using methyl acrylate (50g, 1·2 mole). Distillation yielded methyl -4-acetyl -5- oxohexanoate (28g., 30%.), b.p.134 - 135°C /20 mm Hg.

i.r. (Liquid film) ν_{max} : 1735(s), 1700(s) $cm.^{-1}$

^1H n.m.r.(60 MHz, $CDCl_3$) τ : -6·85(s), +6·05(s)(together 1H), 6·31(s)(3H), 7.76(s), 7.81(s), 7.30 - 8.00(m),(together 10H).

Analysis (S) : Found C, 58.00; H, 7.62%.
$C_9H_{14}O_4$ requires C, 58.06; H, 7.53%.

Mass Spectrum (Hull) m/e : 186(M^+,2), 144(31), 113(34), 112(32), 111(9), 97(6), 84(14), 74(10), 71(19), 69(7), 59(7), 55(17), 43(B^+, 100).
Accurate mass of molecular ion = 186·08926.
$C_9H_{14}O_4$ requires 186.08920.

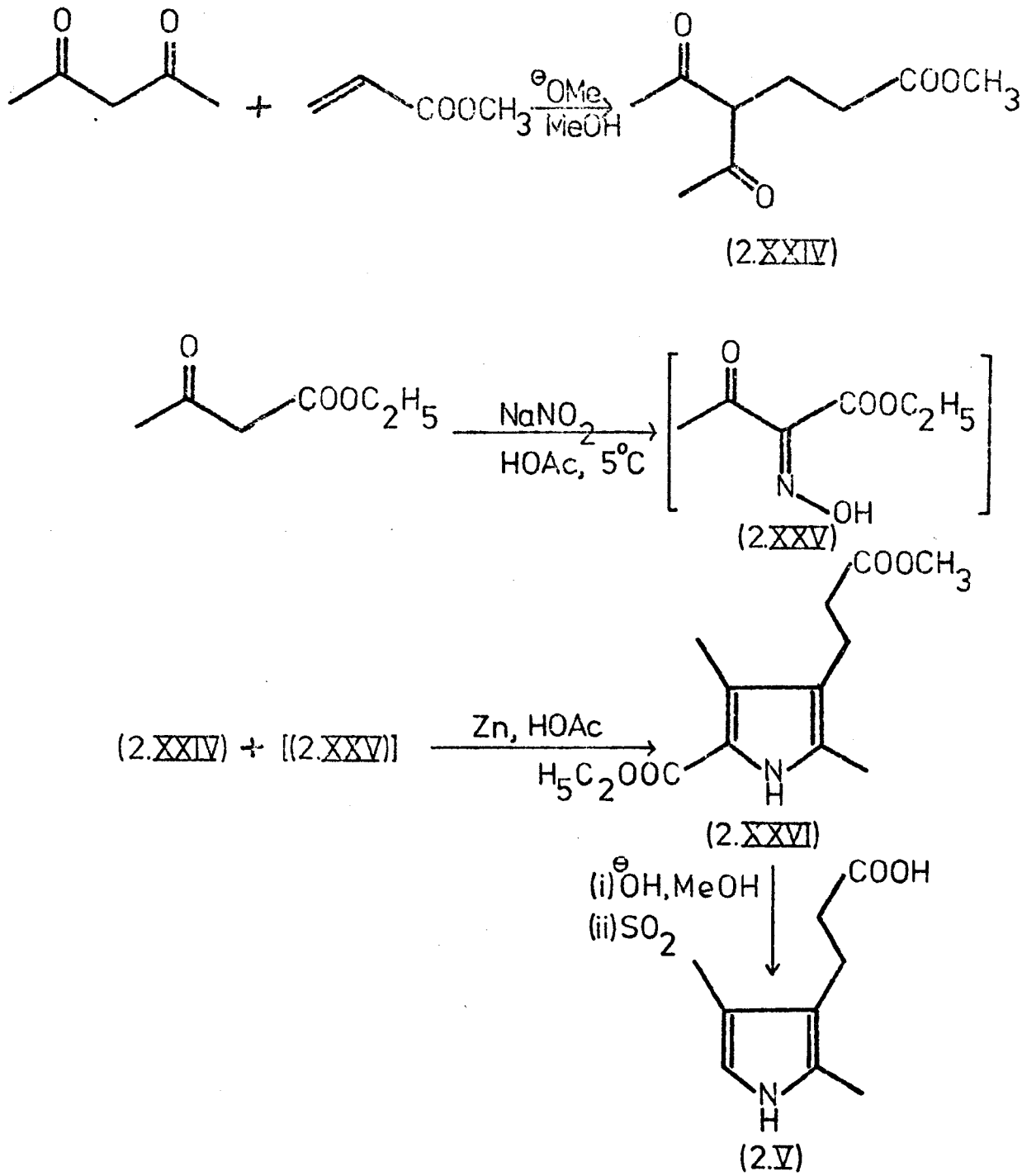


FIGURE 2.9. PREPARATION OF 2,4-DIMETHYLPYRROLE-3-PROPIONIC ACID

Ethyl 4-2'- methoxycarbonylethyl-3,5-dimethylpyrrole-2-carboxylate (2.XXVI.).- Ethyl acetate (13g, 0·1 mole) was nitrosated as already described,[117] using the sodium nitrite - glacial acetic acid method, to yield the intermediate (2.XXV.). This was allowed to react with methyl 4-acetyl-5-oxohexanoate (18·6g, 0·1 mole) as already described [117] to give (2.XXVI.). Crystallisation from absolute ethanol (charcoal) yielded colourless plates, (12g, 48%), m.p. 106·5 - 107°C.

i.r. (Nujol mull) ν_{max} : 3310(s), 1730(s), 1660(s) cm^{-1}

u.v. (Methanol) : λ_{max} 280nm (ε 17,900), 245nm(sh).

^1H n.m.r.(60 MHz, $CDCl_3$) τ : 0·60(br,s,1H), 5·65(q,J=7Hz, 2H), 6·31 (s,3H), 7·40(m,4H), 7·70(s,3H), 7·75(s, 3H), 8·62(t,J= 7Hz,3H).

Analysis (B) : Found C,61.59; H, 7.53; N,5.48%.

$C_{13}H_{19}NO_4$ requires C,61.66; H,7.51;N,5.53%.

Mass Spectrum(Hull) m/e : 254(8), 253(M^+, 44), 208(18), 181(15), 135(10), 134(86), 133(10), 120(8), 98 (5), 97(5), 96(10), 95(6), 94(7), 93(7), 91(7), 79(7), 78(5), 77(11), 66(6), 65(11), 53(7), 42(7).

Accurate mass of molecular ion = 253.1320.

$C_{13}H_{19}NO_4$ requires 253.1314.

2,4-Dimethylpyrrole-3-propionic acid (2.V.).- The diester (2.XXVI.) was hydrolysed and decarboxylated by a modification of the method already described. [114] (2.XXVI.) (5g, 0·02mole) was heated under gentle reflux under nitrogen with water (25ml), methanol (7.5 ml), and sodium hydroxide (5g, 0.125mole) for three hours. After fractional distillation of the methanol, sulphur dioxide was passed into the remaining hot liquid until effervescence had ceased and a precipitate

had formed. The slurry was cooled, extracted with ether (3 x 50ml), the ether dried over anhydrous Na_2SO_4 and the solvent removed in vacuo to leave a pale yellow solid. Crystallisation from chloroform - petroleum ether (b.p. 60 - 80°C) (charcoal) yielded pale yellow plates, (2.14g, 50%), m.p. 145 - 146°C, (Lit.,[118] 139 - 140°C.).

i.r.(Nujol mull) ν_{max} : 3,600(s), 1700(br,s) $cm.^{-1}$

u.v.(Methanol) : End absorption only.

^1H n.m.r.(60 MHz, $CDCl_3$) τ : -0.90 (br,s,1H), 2.40(br,s,1H), 3.55(s,1H), 7.35(m,4H), 7.80(s,3H), 7.92(s,3H).

Analysis (S) :Found C, 64.45; H, 7.82; N, 8.37%. $C_9H_{13}NO_2$ requires C, 64.68; H, 7.78; N, 8.38%.

Mass Spectrum(Hull) m/e : 167(M^+,24), 122(7), 120(6), 109(10), 108(B^+,100), 107(14), 106(12), 94(6), 80(5), 78(8), 68(5), 67(5), 66(6), 54(6), 52(6), 46(8), 42(9), 40(12), Accurate mass of molecular ion = 167.0941. $C_9H_{13}NO_2$ requires 167.0946.

N-Benzoylglycine (2.VI.).- This was prepared as already described.[118a]

Mesobilirubin (2.VIII.).- Bilirubin was hydrogenated as already described,[88] except that the crystallisation was from chloroform at -20°C.

u.v.(Chloroform) : λ_{max} 432nm (ε56,600). Lit.,[88] λ_{max} 434nm (ε57,800).

Analysis (S) :Found C,66.87; H,6.70; N,9.44%. $C_{33}H_{40}N_4O_6$ requires C,67.32; H,6.85; N, 9.52%.

Mass Spectrum (PCMU) : Accurate mass of molecular ion = 588.2936. $C_{33}H_{40}N_4O_6$ requires 588.2948.

The cracking pattern is shown and discussed in Chapter 5.

α,α'- Dimethoxylbilirubin Dimethyl Ester (2.IX.).- Bilirubin was methylated as already described.[102] Crystallisation from light petroleum ether (b.p. 40 - 60°C) yielded plates, m.p. 153 - 154°C, (Lit., m.p. 150 - 152°C,[111] 156°C [102]). Insufficient material was obtained for an elemental analysis.

u.v. (Chloroform) : λ_{max} 418nm (ε50,300). Lit.,[88] λ_{max} 418nm (ε 49,900).

Mass Spectrum (PCMU) : Accurate mass of molecular ion = 640.3255. $C_{37}H_{44}N_4O_6$ requires 640.3261.

The cracking pattern is shown and discussed in Chapter 5.

Deuterated Bilirubin.- All operations were carried out in a water free atmosphere. Bilirubin (73mg, 0.125mmole) was shaken with a solution of sodium (35mg, 1.5mmole) in deuterium oxide (10ml, 99.8 atom % D) for one minute in the dark. Deuterium chloride (0.2ml of a 38% solution in D_2O, 99.5 atom % D) was added and the precipitate filtered and dried over phosphorus pentoxide at 20 mmHg.

u.v.(Chloroform) : λ_{max} 450nm (ε58,300).

The preparation of Nujol mulls and KBr discs for i.r. spectroscopy was carried out as far as possible in a water - free atmosphere.

Experiments with Radioisotopes.

Scintillation Fluid.- A peroxide - free dioxan - based medium was used containing naphthalene (60g/l), 2,5 - diphenyloxazole(PPO) 4g/l) and 1,4 - bis - (5 - phenyloxazol -2-yl) benzene (POPOP) (0.1g/l). The dioxan was previously dried overnight over sodium wire and distilled, in subdued light, from sodium borohydride.

10ml aliquots of this medium were used per sample vial.

Construction of Quenching Curve.- Bilirubin has a light absorption maximum at 454nm and the absorption profile is such that a considerable proportion of the fluorescence of both PPO, at 363nm, and particularly POPOP, at 430nm, is absorbed by the bilirubin. Thus at bilirubin concentrations greater than 0.25mg/10ml scintillation fluid, there is greater than 50% quenching, and above 0.1mg/10ml, the quenching is almost 100%.

A quenching curve was constructed in the following manner:- Aliquots (0.1ml - 0.6ml) of a solution of cold bilirubin in scintillation fluid (0.05mg/ml) were made up to 10ml with scintillation fluid. Tritiated water (10 μl, activity ~3 x 10^4cpm/10 μl) was added to each. The apparent activity was determined and compared with the activity of a similar sample lacking bilirubin. The results of 21 determinations are shown graphically in Figure 2.10., with the line of least squares analysis drawn in.

Tritiated Bilirubin.- All operations were carried out in a water - free atmosphere. Bilirubin (73mg, 0.125mmole) was shaken with a solution of sodium hydroxide (60mg, 1.5mmole) in tritiated water (5ml, activity 5.5 x 10^9cpm/ml) for one minute in the dark. Hydrochloric acid (0.2ml conc.HCl diluted with tritiated water(1ml)) was added and the precipitate filtered and dried to constant activity over phosphorus pentoxide at 20mm Hg.

u.v.(Dioxan) : λ_{max}453nm (ε 59,200).

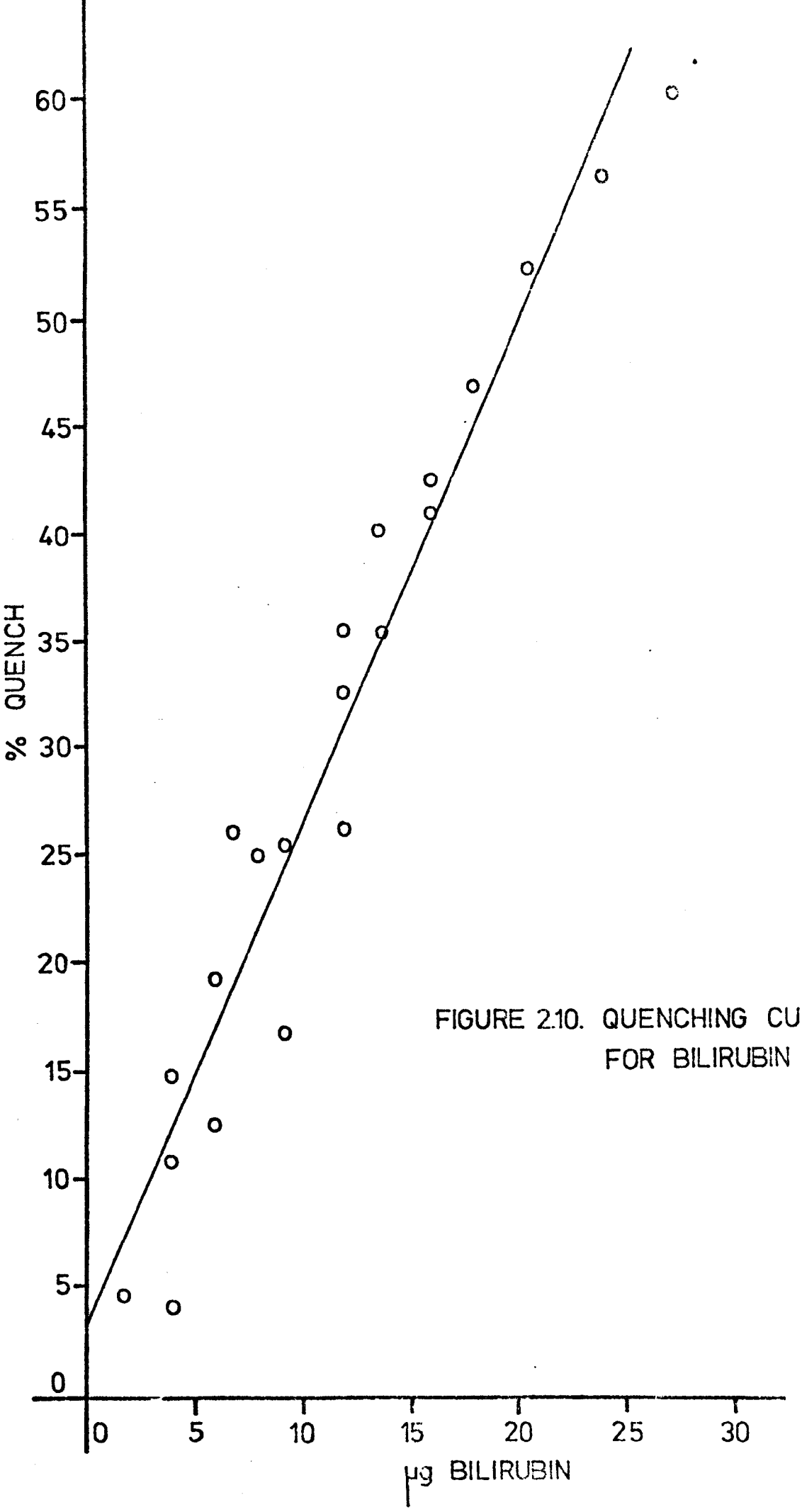


FIGURE 2.10. QUENCHING CURVE FOR BILIRUBIN

The activity of the filtrate was determined and found to be 9.59×10^7 cpm/mmole, thus if n atoms of hydrogen are exchangeable per molecule of bilirubin, then the tritiated bilirubin should have a specific activity of $9.59n \times 10^7$ cpm/mmole.

The specific activity of the tritiated bilirubin was determined as follows:- Aliquots (0.1ml - 0.6ml) of a solution of tritiated bilirubin in dioxan (about 0.03mg/ml, the exact concentration being determined spectrophotometrically) were made up to 10ml with scintillation fluid and the samples counted. After correction for quenching, it was shown that, for two preparations, 5.95 (average of seven determinations) and 6.03 (average of six determinations) atoms of tritium are incorporated into each molecule of bilirubin.

CHAPTER 3

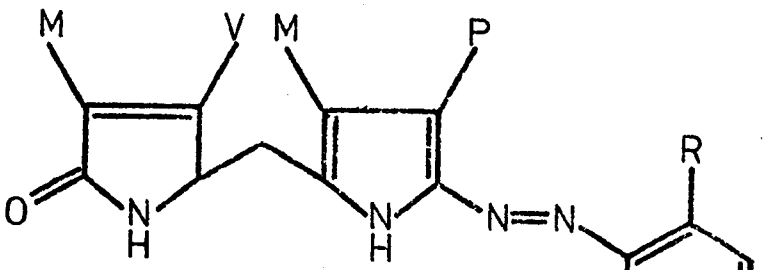
THE DIAZO REACTION.

3. THE DIAZO REACTION.

3.1. Introduction.

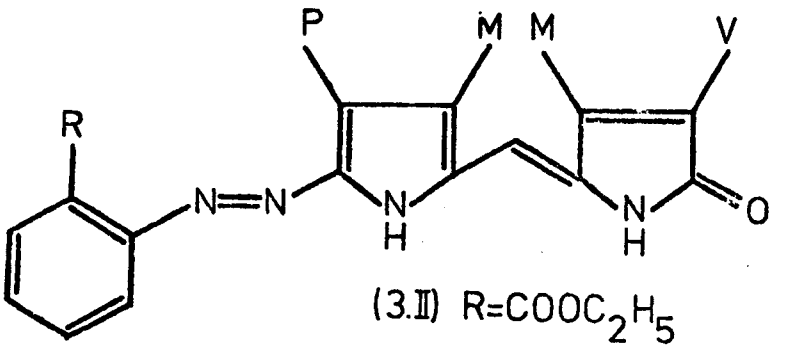
Bilirubin reacts with many diazotised aromatic amines [119] and is cleaved at the central methylene bridge to form two isomeric azopigments eg. (3.I.) and (3.II.). The reaction between bilirubin and diazotised sulphanilic acid - the van den Bergh reaction [120] - is used in the quantitative determination of bilirubin and its conjugates in sera. Two types of reaction have been identified, the "indirect" reaction, given by unconjugated bilirubin only in the presence of a "promoter" (usually methanol) - which may serve to disrupt the hydrogen bonds in bilirubin - and the "direct" reaction, given by conjugated bilirubin which requires no added promoter.

The dipyrrylmethene azopigments had been shown to be products of the diazo reaction,[102] but only recently have the isomers been separated and identified. [121,122] The pairs of isomers (3.I.), (3.II.) and (3.III.),(3.IV.) have been identified from the reactions of bilirubin with diazotised ethyl anthranilate [121,122] and diazotised aniline [122] respectively. It will be seen, that all the carbon atoms in bilirubin are accounted for in the azopigments save one, that of the central methylene bridge. The diazo reaction is thought to occur in two steps,[123,124] and it has been postulated that the central methylene bridge carbon atom is released as formaldehyde during the second step of the reaction. [123, 124] However, no formaldehyde could be detected in the distillate from the van den Bergh reaction using 3 - methyl -2- benzothiazolone hydrazone (MBTH) (3.V.). [125] MBTH reacts with aliphatic aldehydes in the presence of oxygen and dilute acid to form the intense blue cation (3.VI.). [126] In the absence of other reported attempts to detect any formaldehyde renewed attempts were made in the current investigation to determine if the aldehyde is formed during the course of the diazo reaction.



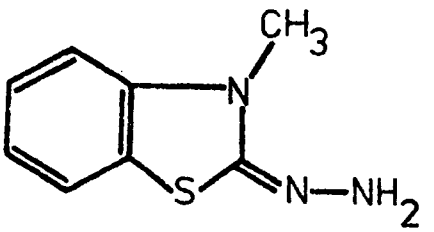
(3.I) R=$COOC_2H_5$

(3.III) R=H

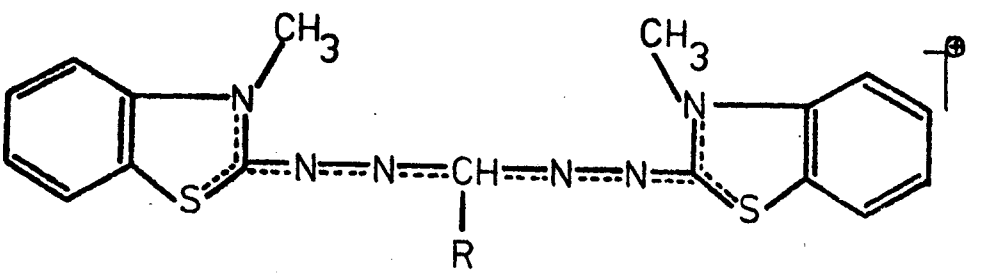


(3.II) R=$COOC_2H_5$

(3.IV) R=H



(3.V)

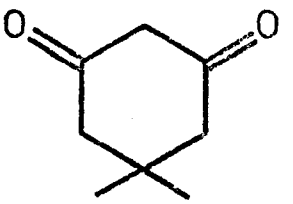


(3.VI) R=H or Alkyl

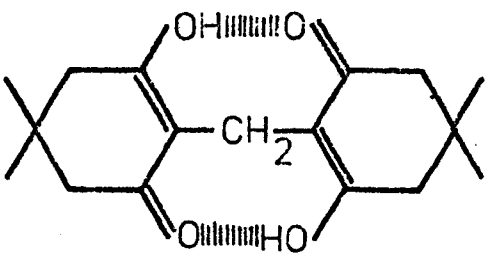
3.2. Results and Discussion.

Two approaches were used to detect the formaldehyde:- firstly, as the free aldehyde and secondly, to trap it chemically and then identify the derivative so formed.

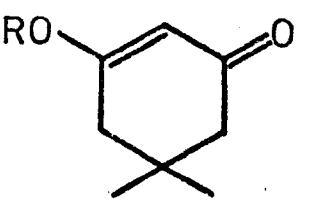
Formaldehyde can be readily detected by GLC using a column with pentaerythritol tetraacetate as the stationary phase.[127] In this work, using 6ft and 12 ft columns, the limit of detection of formaldehyde was found to be 0.05% w/v formaldehyde in methanol/water. Theoretical considerations of the diazo reaction show that 0.33mmole of bilirubin (195mg) will yield only 10mg of formaldehyde, then if the reaction is carried out in a total volume of 100ml, this represents a maximum concentration of formaldehyde of 0.01% w/v. Apart from the low concentration of formaldehyde involved, it was found unpractical to inject a sample of the reaction mixture directly into the gas chromatograph because of the presence of involatile materials, while distillation of the formaldehyde with the solvents would serve only to reduce the formaldehyde concentration still further. For these reasons this method was abandoned in favour of trapping the formaldehyde chemically with 5,5 - dimethylcyclohexa-1,3-dione (dimedone) (3.VII.) to form formaldehyde dimethone(3.VIII.). With free bilirubin, the diazo reaction can be readily carried out in a chloroform - methanol medium. Since both these solvents contain C_1 units that could give rise to formaldehyde, this solvent system was rejected and a 1,2 - dichloroethane - tert. butanol medium used instead, even though the reaction is much slower in this medium. Ethanol was tried, with a reaction rate similar to that observed with methanol, but this shows the disadvantage of the formation of a troublesome by - product - 5,5 - dimethyl -3- ethoxycyclohex -2- enone (dimedone ethyl ether) (3.IX.) - during the slightly acid conditions of the last stage of the reaction sequence. A corresponding similar by - product (3.X.)



(3.VII)



(3.VIII)



(3.IX) R=CH_2CH_3

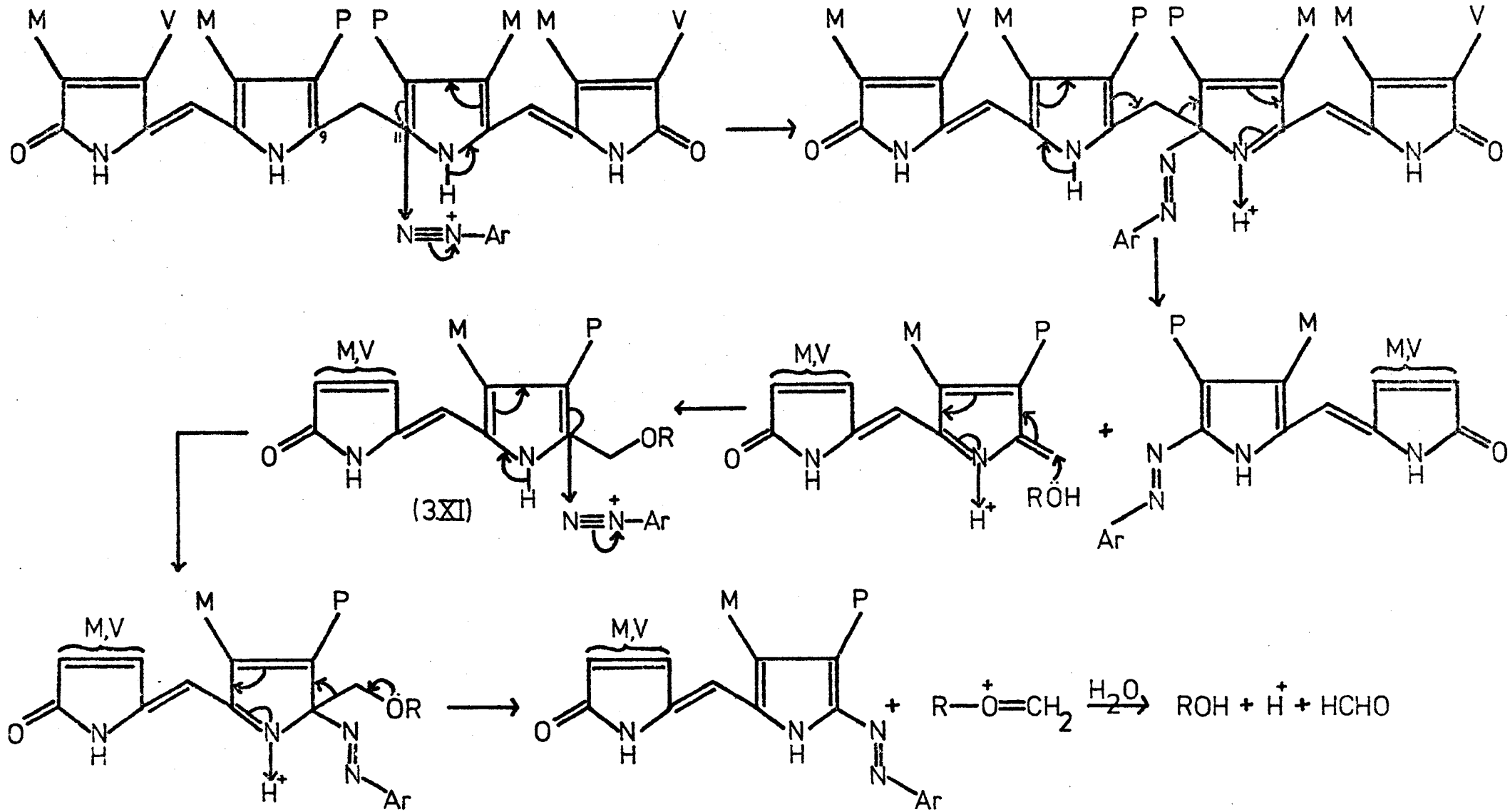
(3.X) R=$C(CH_3)_3$

is unlikely in the case of tert. butanol because of the ready cleavage of the $(CH_3)_3C$-O bond in acid conditions.

The diazo reaction was carried out in a 1,2 - dichloroethane-tert. butanol medium and the solvents distilled into a solution of dimedone. After work-up, formaldehyde dimethone, identical in all respects with an authentic sample, could be isolated, thus showing that formaldehyde is indeed formed during the diazo reaction. No formaldehyde dimethone was obtained from two control reactions, one in which the diazonium compound was omitted and the other in which the bilirubin was omitted.

A possible mechanism for the reaction is shown in Figure 3.1.; it should be noted, however, that the initial attack by the diazonium cation can occur at either C(9) or C(11). The general reaction scheme is similar to that already reported [123] for which confirmatory kinetic evidence has been presented [123, 124, 128] and which must follow since all the main products of the reaction are now known (ignoring any side reactions). The dipyrrylmethene (3.XI., R =H) has already been postulated as an intermediate in the reaction [123, 124] and has been claimed to have been isolated [123] and to react with diazotised aromatic amines. [123] Therefore, an elegant way to confirm that formaldehyde is formed during the second step of the reaction is to isolate (3.XI.) and allow it to react with a diazonium compound and determine any formaldehyde produced as formaldehyde dimethone. However, all attempts, in this work, to repeat the isolation of (3.XI., R = H), as described, [123] failed. No other methods of isolation were attempted.

FIGURE 3.1. A SUGGESTED MECHANISM FOR THE FORMATION OF FORMALDEHYDE FROM BILIRUBIN



3.3. Experimental.

Materials.

Bilirubin was obtained from BDH Chemicals Ltd., and was purified before use.[129]

u.v.(Chloroform) : λ_{max} 454nm (ε 59,400). Lit.[88], λ_{max} 453nm (ε 58,800).

Analysis (B) : Found C,67.04; H,6.16; N,9.45%.
$C_{33}H_{36}N_4O_6$ requires C,67.81; H,6.16; N,9.59%.

1,2 - Dichloroethane, Tert.Butanol and Petroleum Ether (b.p. 60 - 80°C) were distilled before use.

Diethyl Ether (A.R.) and Dimedone were used without further purification.

Methods.

Gas Chromatography.- Two columns were prepared, 6' x 3/8" o.d. and 12' x 3/8" o.d. using 20% w/w pentaerythritol tetraacetate on PhasePak Q.[127] An F & M Gas Chromatograph was used with the column temperature at 95°C and a helium flow rate of 25ml/min. at 25p.s.i.

Detection and Isolation of Formaldehyde as Formaldehyde Dimethone (3.VIII.)

a) Using 1,2 - Dichloroethane and Tert.Butanol.- Bilirubin (195mg, 0.33mmole) was suspended in a mixture of 1,2 - dichloroethane (50ml) and tert. butanol (50ml). A solution of benzenediazonium chloride (2mmole)[130] was added and the mixture stirred in a closed flask, in the dark, at room temperature, for 30 minutes. A further quantity of benzenediazonium chloride (2mmole) was added and the solution stirred for 30 minutes more. The reaction mixture was then evaporated to dryness, the solvents being distilled into a solution of dimedone (200mg) in tert. butanol (50ml), with the receiver

connected to a Dreschel bottle containing a similar solution of dimedone (200mg) in tert. butanol (50ml). The dimedone solutions were combined and heated under gentle reflux for 30 min. After cooling and removal of the solvents <u>in vacuo</u>, TLC (chloroform) of the residue showed the presence of formaldehyde dimethone, R_F = 0.44. The formaldehyde dimethone was isolated by elution from a silicic acid column (100 mesh, 14cm x 3cm o.d.) with petroleum ether (b.p. 60 - 80°C) - diethyl ether (3:1, v/v). This material was used without further purification. Recovery was up to 20mg (20%), m.p. 186 - 187°C Lit.,[131] 191 - 191·5°C.

i.r. (Chloroform) ν_{max} : 1610(s), 1580(s) $cm.^{-1}$

u.v. (Qualitative, Methanol) : λ_{max} 257nm increasing to 288nm on the addition of base.

^1H n.m.r.(60 MHz, $CDCl_3$) τ : 6.80(s,2H), 7.70(s,8H), 8.95(s,12H)
The enol hydroxyl protons were not observed.

Mass Spectrum(Hull) m/e : 293(5), 292(M^+,20), 191(5), 180(5), 166(5), 165(22), 161(5), 153(8), 152 (23), 149(8), 147(6), 145(6), 141(7), 140(21), 137(14), 125(11), 124(13), 123 (11), 121(10), 112(19), 111(11), 110(10), 109(19), 107(13), 105(11), 98(11), 97(27), 96(11), 95(27), 93(14), 91(16), 85(17), 84(25), 83(B^+,100), 82(18), 81(27), 79(17), 77(12), 73(13), 71(23), 70(23), 69(42), 68(23), 67(27), 60(13), 57(46), 56(73), 55(96), 54(19), 53(21), 51(11), 50(12), 45(14), 44(27), 43(54), 42(23), 41(89), 40(23).
Accurate mass of molecular ion = 292.1677.
$C_{17}H_{24}O_4$ requires 292.1674.

The material was identical in all respects to an authentic sample of formaldehyde dimethone.[131, 132]

When 1,2 - dichloroethane (50ml) and tert. butanol (50ml) containing formaldehyde (10mg, equivalent to 195mg of bilirubin) were distilled as above the formaldehyde dimethone recovered never exceeded 20% of the theoretical amount (100mg).

b) Using 1,2 - Dichloroethane and Ethanol.

The diazo reaction was carried out exactly as described above using ethanol in place of tert. butanol, but TLC (chloroform) of the product obtained showed a large amount of a product with R_F = 0.22. This material was isolated by PLC (chloroform) and found to be dimedone ethyl ether (3.IX.), identical with an authentic sample prepared as described below.

i.r.(Liquid film) ν_{max} : 1660(s), 1610(s) $cm.^{-1}$

u.v.(Qualitative, Methanol) : λ_{max} 253nm.

^1H n.m.r.(60 MHz, CCl_4) : 4.75(s,1H), 6.05(q,J=7Hz, 2H), 7.75(s,2H), 7.90(s,2H), 8.60(t,J=7Hz, 3H), 8.90(s,6H).

Mass Spectrum(Hull) m/e : 169(7), 168(M^+,48), 154(5), 153(16), 140 (6), 113(10), 112(87), 98(12), 86(12), 85 (11), 84(B^+,100), 83(10), 69(60), 68(76), 67(8), 57(8), 56(9), 55(16), 53(6), 42(7), 41(20), 40(15).

Accurate mass of molecular ion = 168.1146.

$C_{10}H_{16}O_2$ requires 168.1150.

Dimedone Ethyl Ether.- This was prepared as already described[118b] using dimedone (7g, 0.05mole) and diethyl sulphate (6.6ml, 7.7g, 0.05mole) Distillation of the final product yielded dimedone ethyl ether (5g, 60%), b.p. 130°C/9mm.

i.r.(Liquid film) ν_{max} : 1660(s), 1610(s) $cm.^{-1}$

u.v.(Qualitative, Methanol) : λ_{max} 253nm.

^{1}H n.m.r.(60 MHz, CCl_4) : 4.75(s,1H), 6.10(q, J=7Hz, 2H), 7.75(s,2H), 7.91(s,2H), 8.62(t, J=7Hz, 3H), 8.91(s,6H).

Mass Spectrum(Hull) m/e : 169(11), 168(M^+, 58), 153(14), 140(12), 125(6), 113(10), 112(B^+,100), 111(5), 97(7), 85(9), 84(93), 83(27), 70(5), 69 (50), 68(58), 67(8), 57(8), 56(17), 55 (20), 53(6), 43(27), 42(6), 41(21), 40(12).

Accurate mass of molecular ion = 168.11645.

$C_{10}H_{16}O_2$ requires 168.11502.

CHAPTER 4.

METAL COMPLEXES OF BILIRUBIN.

4. METAL COMPLEXES OF BILIRUBIN.

4.1. Introduction.

The apparent inability of bilirubin to form stable metal complexes was explained by assuming that bilirubin existed as the bislactam [8c] rather than the bislactim form. The latter contains two tertiary nitrogen atoms which would coordinate to a metal ion, whereas these nitrogen atoms are not present in the bislactam and so, this form should not form complexes. In contrast, biliverdin and other tertiary nitrogen - containing tetrapyrroles, readily form metal complexes. [10f, 133] However, it appears that bilirubin will also form metal complexes, particularly with zinc ions. Thus, the addition of a 2% solution of zinc acetate in methanol to a solution of bilirubin in chloroform - methanol (1:1) induces a bathochromic shift in the absorption spectrum from 452nm to 476nm.[134] This is explained by a reversible complex formation with the zinc ions, since the addition of acid causes decomposition of the complex and the absorption maximum returns to 452nm. [134] Mesobilirubin will also form a complex with a bathochromic shift from 430nm to 451nm. [134] Similar effects were observed when aqueous zinc acetate was added to a solution of bilirubin in ethanolamine, the complex being decomposed on the addition of a chelating agent (EDTA).[135]

The presence of zinc ions in an aqueous alkaline solution of bilirubin has been shown to cause an increased rate of decomposition of bilirubin. [136, 137] The complexes formed between bilirubin and transition and rare earth metals in chloroform - ethanol have also been examined, and it was found that transition metals that formed strong, square planar complexes caused rapid bilirubin degradation. Of the lanthanide elements, only samarium (III) showed any tendency to form covalent bonds. [110]

Recently, the complexes between the biladiene (4.I.) and the transition metals Co(II), Ni(II), Cu(II), and Zn(II) have been isolated and characterised.[138, 139] All the complexes have a ligand : metal ratio of 1:1, but those of Co(II) and Zn(II) contain two tetrapyrrole residues with tetrahedral coordination about the metal atom, while those of Cu(II) and Ni(II) contain one tetrapyrrole residue with square planar coordination. However, while (4.I.) is a biladiene - ac, it is unlike bilirubin in that it lacks the lactam carbonyl groups, and as a result, contains two tertiary nitrogen atoms. If bilirubin forms similar complexes, it must presumably first enolise to the bislactim.

It has been suggested that zinc may be important in liver function[140] and bilirubin is known to inhibit human and equine liver alcohol dehydrogenase,[141] and equine liver glutamate dehydrogenase.[142] Both of these enzymes require zinc ions for their activity and it has been suggested that, inter alia, the mechanism of inhibition by bilirubin may be due to the formation of a complex between the bilirubin and zinc ions.

In view of these observations an examination of some zinc complexes was undertaken with a view to the elucidation of their structures.

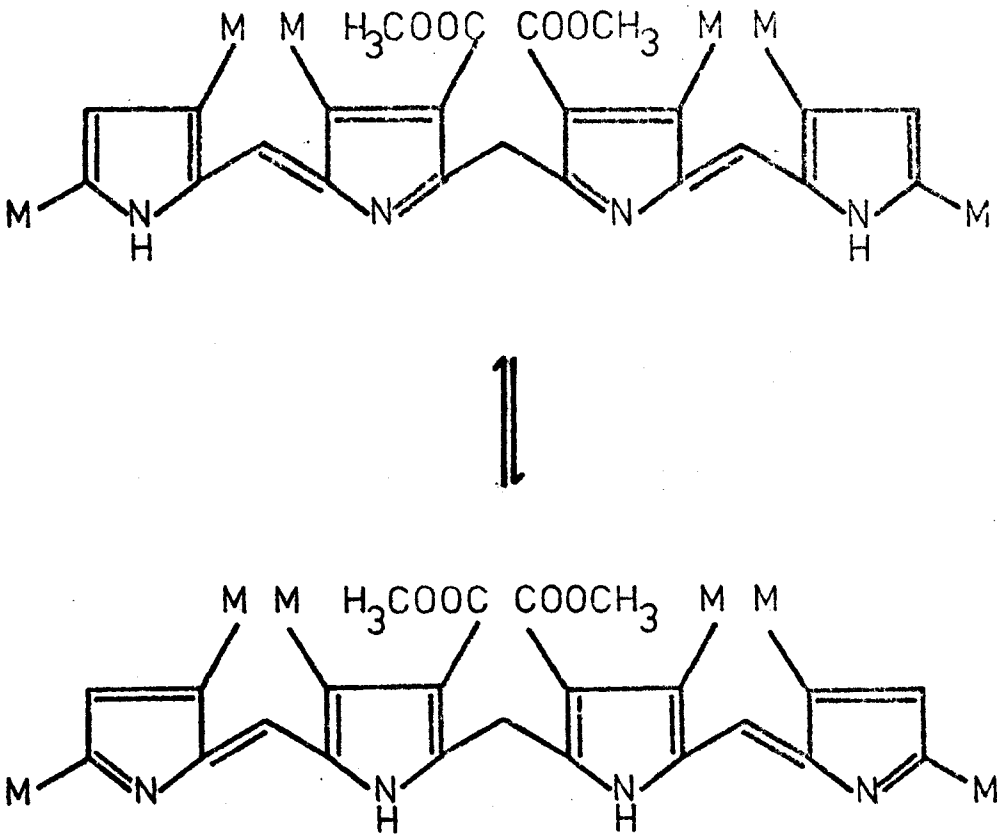
4.2. Results and Discussion.

4.2.1. Bilirubin - Zinc Complexes.

4.2.1.1. Preliminary Observations.

The metal complexes of bilirubin have been examined using chloroform,[110] chloroform - methanol,[110, 134] chloroform - ethanol,[110] ethanolamine,[135] tetrahydrofuran,[110] DMSO,[110] and potassium cyanide - DMF[110] as solvents. In this work, the complexes formed between bilirubin and zinc acetate in DMF and DMSO were examined and will be discussed.

When solutions of zinc acetate and bilirubin in anhydrous DMF,



(4.I)

DMSO or N,N - dimethyl acetamide (DMA) are mixed, a bathochromic shift from 454nm to 526 nm is observed in the absorption spectrum and the solution becomes dark red (Figure 4.1a-c.). N,N -Diethylformamide was also examined as a solvent, but a large excess of zinc acetate was required for any appreciable complex formation to occur. Corresponding complexes are also formed between mesobilirubin and zinc acetate under identical conditions with a bathochromic shift from 420nm to 482nm (Figure 4.1d.). Similar spectral shifts are observed when the zinc salts of formic, propionic, n -butyric, and n -valeric acids are used in the place of zinc acetate. However, for the same degree of complex formation in each case, more zinc formate is required than any other zinc alkyl carboxylate tested. On the other hand, the zinc salts of the strong acids hydrochloric, sulphuric and trichloracetic are not conducive to complex formation in the dipolar - aprotic solvents used. Thus it would appear that on complex formation protons are liberated, which if not removed as the undissociated acid, (as in the case of acetic - n -valeric acids) effectively cause the decomposition of the complex. Indeed the addition of water or acid to, e.g. the complex with zinc acetate causes decomposition of the complex.

The complexes in DMF are fairly stable towards oxygen, the stability increasing in the presence of an excess of zinc acetate, whereas in DMSO, the complexes are stable only in the absence of oxygen. In the presence of oxygen, the colour due to the complex rapidly fades but the identities of the presumed oxidation products have not been determined.

Spectrophotometric examination of the addition of aliquots of a solution of zinc acetate in DMF solution to a solution of bilirubin in DMF shows a family of absorption curves (Figure 4.2.) with a

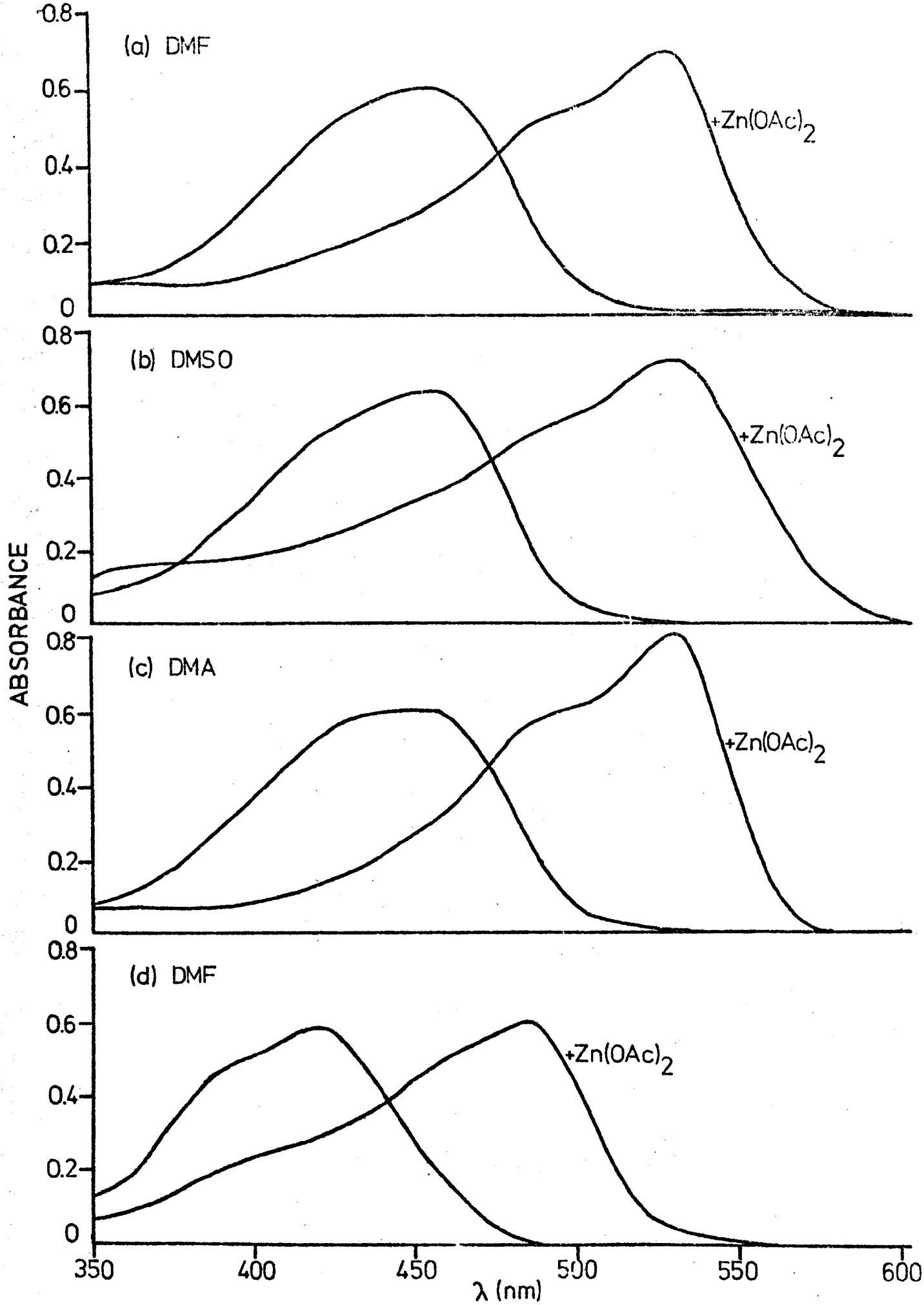
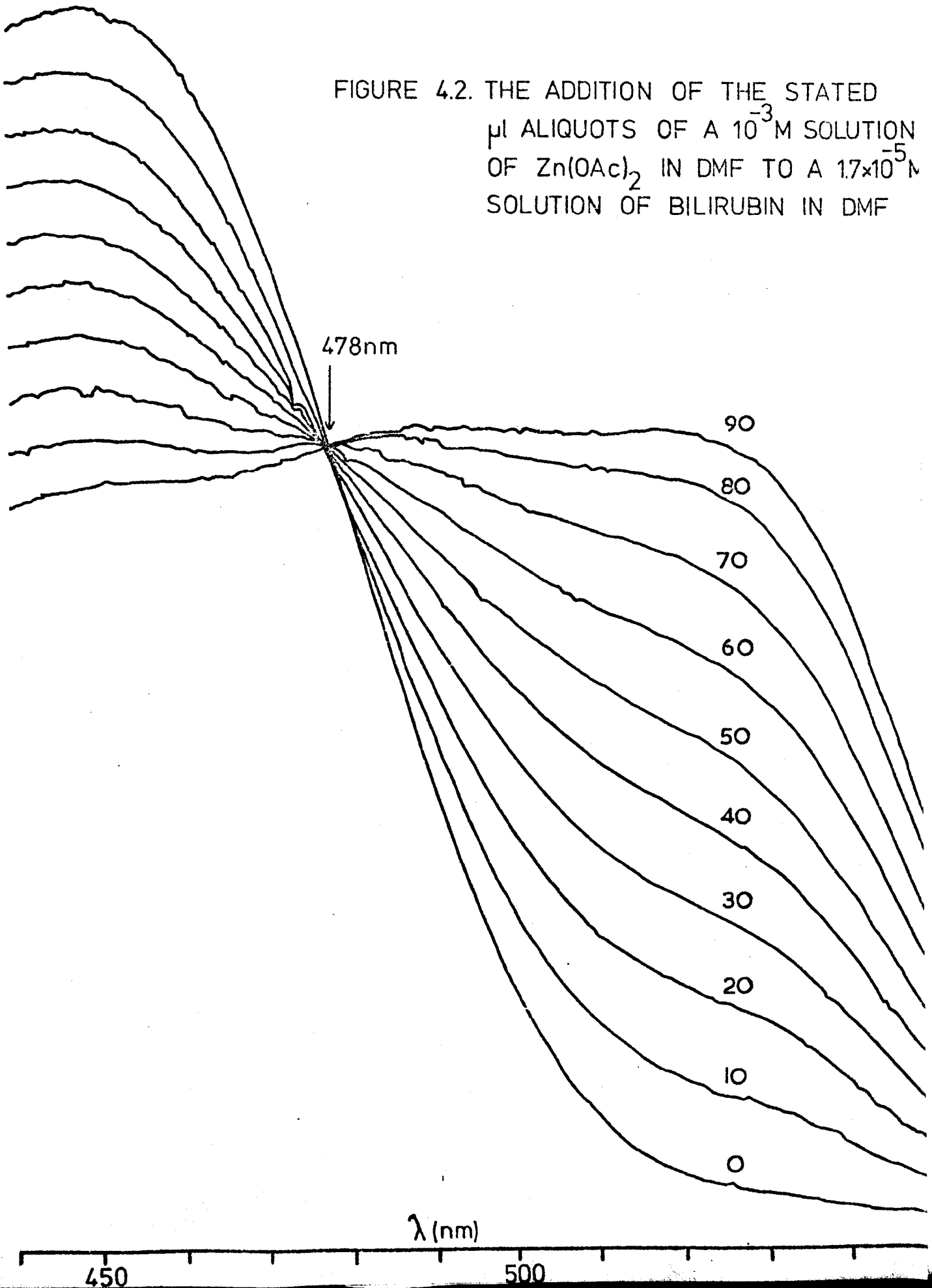


FIGURE 4.1. THE ADDITION OF 3 EQUIVALENTS OF $Zn(OAc)_2$ TO SOLUTIONS OF BILIRUBIN (a-c) AND MESOBILIRUBIN (d)

FIGURE 4.2. THE ADDITION OF THE STATED μl ALIQUOTS OF A 10^{-3}M SOLUTION OF $Zn(OAc)_2$ IN DMF TO A 1.7×10^{-5}M SOLUTION OF BILIRUBIN IN DMF



single isobestic point at 478nm indicating that the changes in concentration of the absorbing species (bilirubin and the complex) are related linearly and that the overall reaction has one rate determining step.[143] The complex nature of the reacting system precluded the calculation of a meaningful stability constant of the complex. Examination of the organic material recovered from the samples of the complexes (in DMF and DMSO) decomposed by water showed it to be identical to bilirubin in all respects, indicating that the complex formation is indeed a reversible process.

In view of the instability of the zinc complex in DMSO solution, compared with that in DMF, most of the quantitative determinations were carried out using the complex in the latter solvent.

4.2.1.2. Stoichiometry and Possible Structures of the Zinc Complexes.

Using the method of continuous variations,[144] it was shown that in both DMF and DMSO, the zinc : bilirubin ratio in the complexes is 3 : 1. Concentration of a solution of bilirubin and four equivalents of zinc acetate in DMF yields an orange precipitate and elemental analysis of two separate preparations indicated a nitrogen : zinc ratio of 2 :1. Taking these results together and assuming that the complex contains one tetrapyrrole residue, then there must also be two DMF molecules present in the complex. Samples of the DMF complex prepared from ^{14}C - labelled zinc acetate had specific activities, after correction for quenching, consistent with the presence of two acetate ions. The complex therefore has a bilirubin : zinc : acetate ion : DMF ratio of 1:3:2:2.

Isolation of the complex from DMSO solution, in an analogous manner yields a dark red solid, but with variable Zn : N and Zn : S

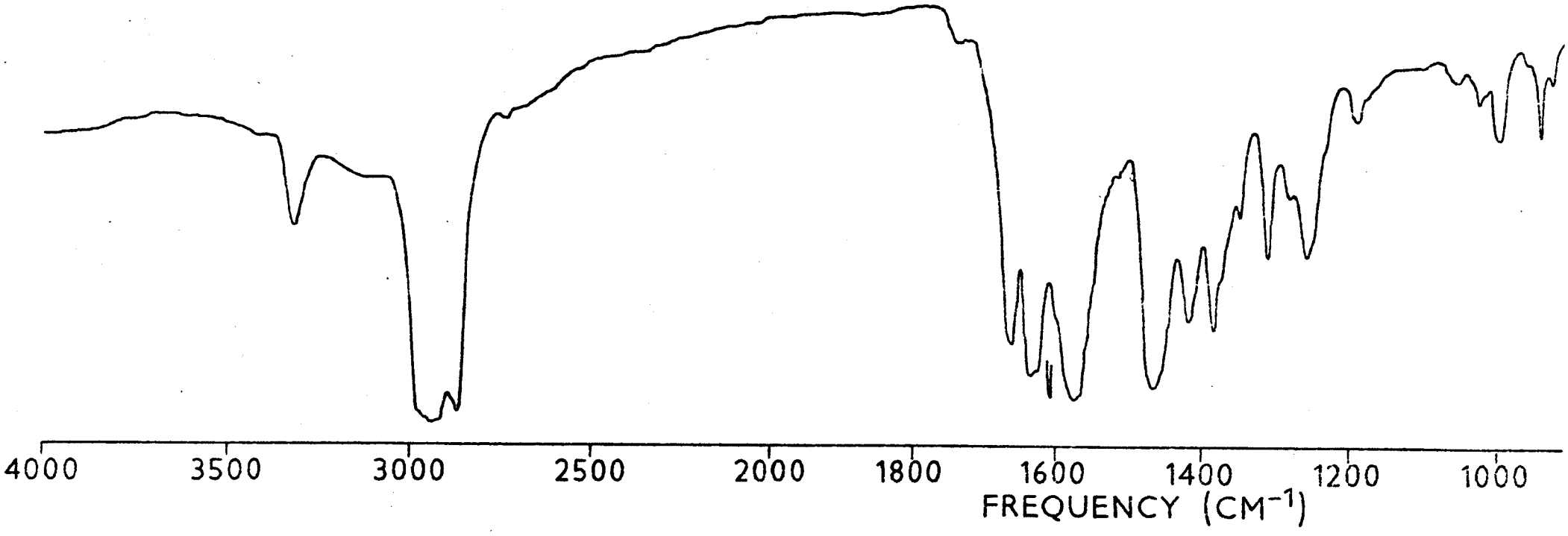
ratios for several samples isolated. Presumably, this complex undergoes some decomposition during the isolation procedure.

The i.r. spectrum of the isolated DMF complex as a Nujol mull (Figure 4.3.) shows a band at 3,300cm.$^{-1}$ indicating the presence of O-H or N-H groups, [115d] while the carbonyl region shows bands at 1660, 1630 and 1575cm.$^{-1}$ in contrast to that of bilirubin under the same conditions (Figure 2.5.).

Investigation of the isolated DMF complex by mass spectrometry shows that, on raising the temperature of a sample which had been directly inserted into the spectrometer, DMF is evolved at 50°C followed by acetic acid at 100 - 120°C. When the complex prepared from zinc perdeuteroacetate is similarly analysed, DMF and CD_3COOH are evolved. However, it is not possible to distinguish the cracking patterns of DMF, CH_3COOH or CD_3COOH because of the presence of decomposition fragments from the bilirubin ligand. This suggests that the DMF and acetate ions are present as ligands. The presence of free DMF is unlikely since the sample was maintained at a pressure of 0.1mm Hg at room temperature for 24 hours. Also the presence of free zinc acetate is unlikely, for while the hydrated salt does evolve acetic acid in the mass spectrometer under the conditions used above, it does so less readily than do the complexes.

It is not possible to record the ^1H n.m.r. spectrum of the DMF complex, since, once isolated, it is very insoluble. However, it is possible to record the spectrum of the DMSO complex since a concentrated sample can be prepared <u>in situ</u> using $^2H_6$-DMSO and zinc perdeuteroacetate. As both the DMF and DMSO complexes have the same zinc : bilirubin ratio and similar absorption spectra, it is assumed that the ^1H n.m.r. spectrum of the DMF complex would be similar to that observed for the DMSO complex. The major difference between

FIGURE 4.3. THE I.R. SPECTRUM OF THE DMF COMPLEX AS A NUJOL MULL



the spectra of bilirubin and the complex in $^{2}H_{6}$ - DMSO (Figure 4.4.) is the collapse of the signal due to the methine bridge protons at 3.91τ in the spectrum of bilirubin. The ^{1}H n.m.r. spectrum of mesobilirubin in $^{2}H_{6}$ -DMSO (Figure 4.5.), obtained in an analogous manner demonstrates more clearly the changes occurring on complex formation. Thus, the methine bridge protons, resonating as a singlet at 4.06 τ in mesobilirubin, resonate as a group of three singlets at 3.98τ, 3.88τ and 3.82τ in the DMSO complex, with the intensities of the two lower field resonances being equal. Integration of all three resonances suggests that, combined, they correspond to two protons. A possible explanation is that if the complex is in equilibrium with free bilirubin, the resonance at 3.98τ is due to free bilirubin while the remaining resonances arise from the complex. Why the complexation of zinc should cause this effect is uncertain, but while it suggests that the zinc ions are coordinated by the nitrogen atoms of bilirubin and mesobilirubin, the Zn(II) ion has a d^{10} configuration of electrons and is therefore diamagnetic and so should influence the n.m.r. spectrum very little. The attempted titration of the methine proton resonance with Zn(II) failed because whenever less than four equivalents of Zn(II) were added to the sample tube, the complex precipitated out.

All attempts to obtain the DMF complex in a macrocrystalline form failed, the complex being obtained instead as an apparently amorphous orange powder. X-Ray powder photographs of several samples of the complex show a few, reproducible diffraction bands differing from those obtained from crystalline zinc acetate or bilirubin alone. This indicates that the complex is microcrystalline and a definite chemical entity.

Before a structure is proposed certain assumptions must be made. There are no ligand field stabilisation effects in the Zn(II) ion

FIGURE 4.4. THE 100MHz ^{1}HN.M.R. SPECTRA OF BILIRUBIN (a) AND ITS ZINC(II) COMPLEX (b) IN $^{2}H_{6}$-DMSO

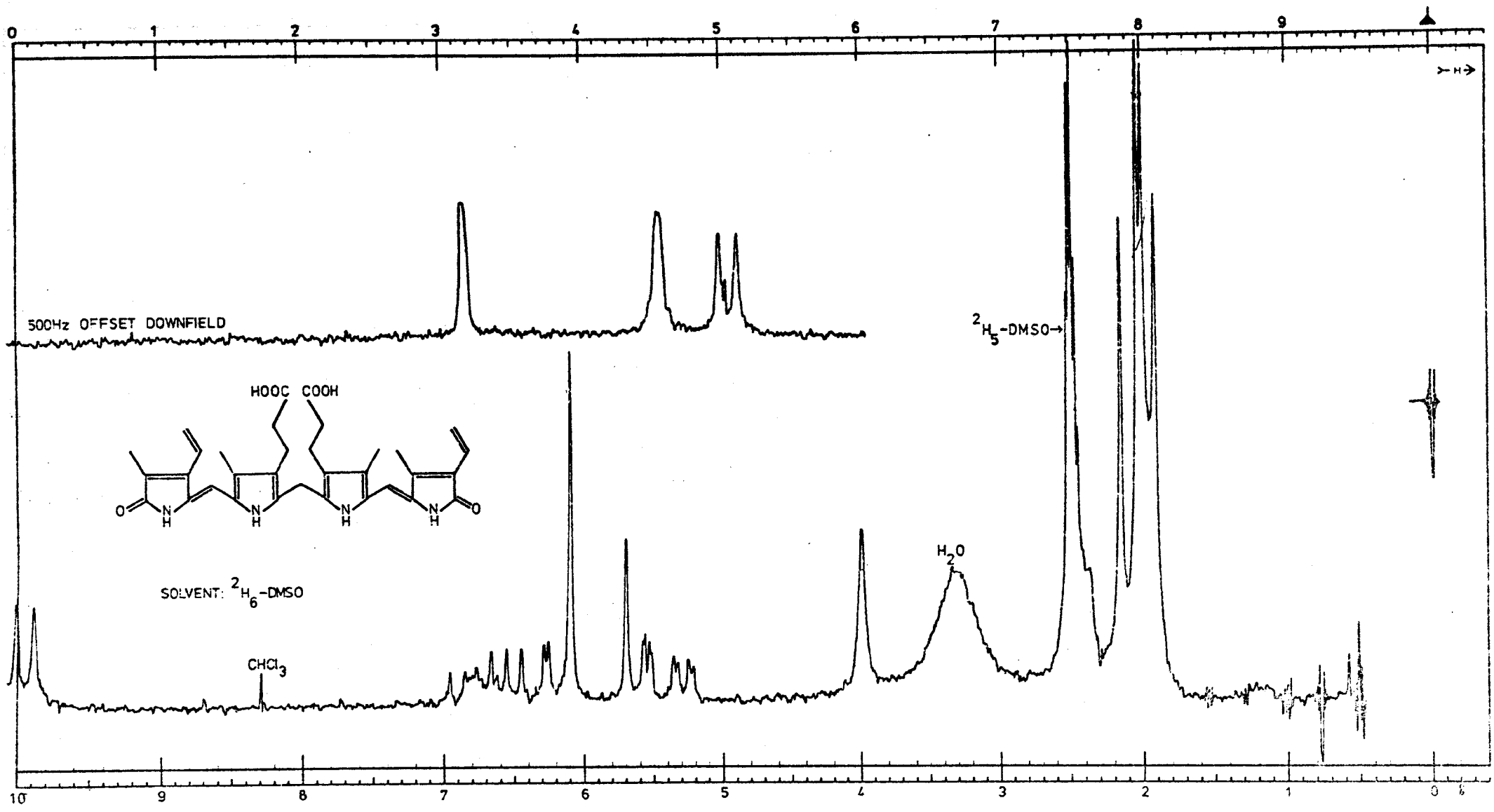

500Hz OFFSET DOWNFIELD
HOOC COOH
SOLVENT: $^2H_6$-DMSO
$CHCl_3$
H_2O
$^2H_5$-DMSO→
δ

FIGURE 4.4a.

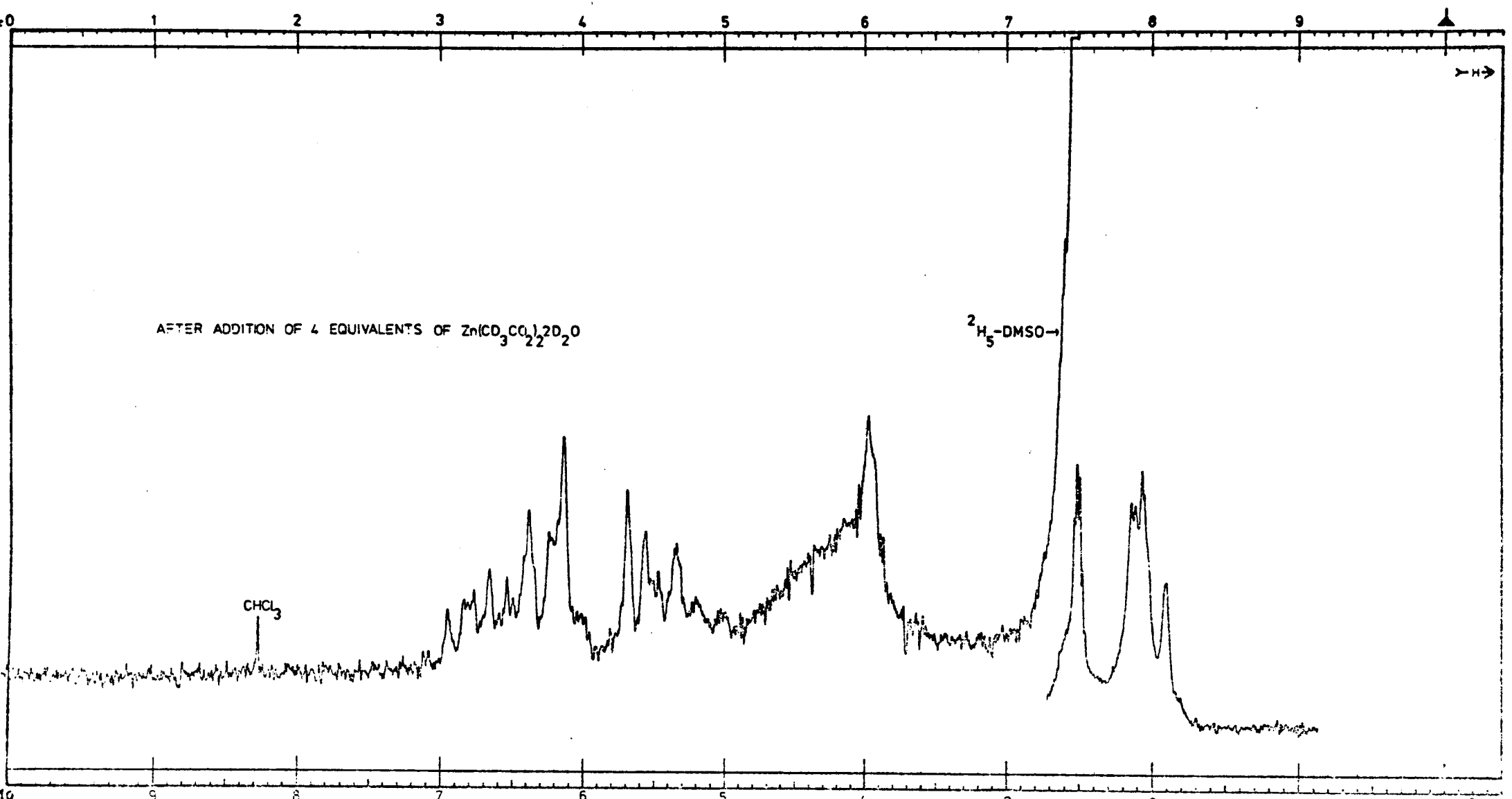

AFTER ADDITION OF 4 EQUIVALENTS OF $Zn(CD_3CO_2)_2 2D_2O$
$^2H_5$-DMSO→
$CHCl_3$
→H→

FIGURE 4.4b.

FIGURE 4.5. THE 100MHz ^{1}H N.M.R. SPECTRA OF MESOBILIRUBIN (a) AND ITS ZINC(II) COMPLEX (b) IN $^{2}H_{6}$-DMSO

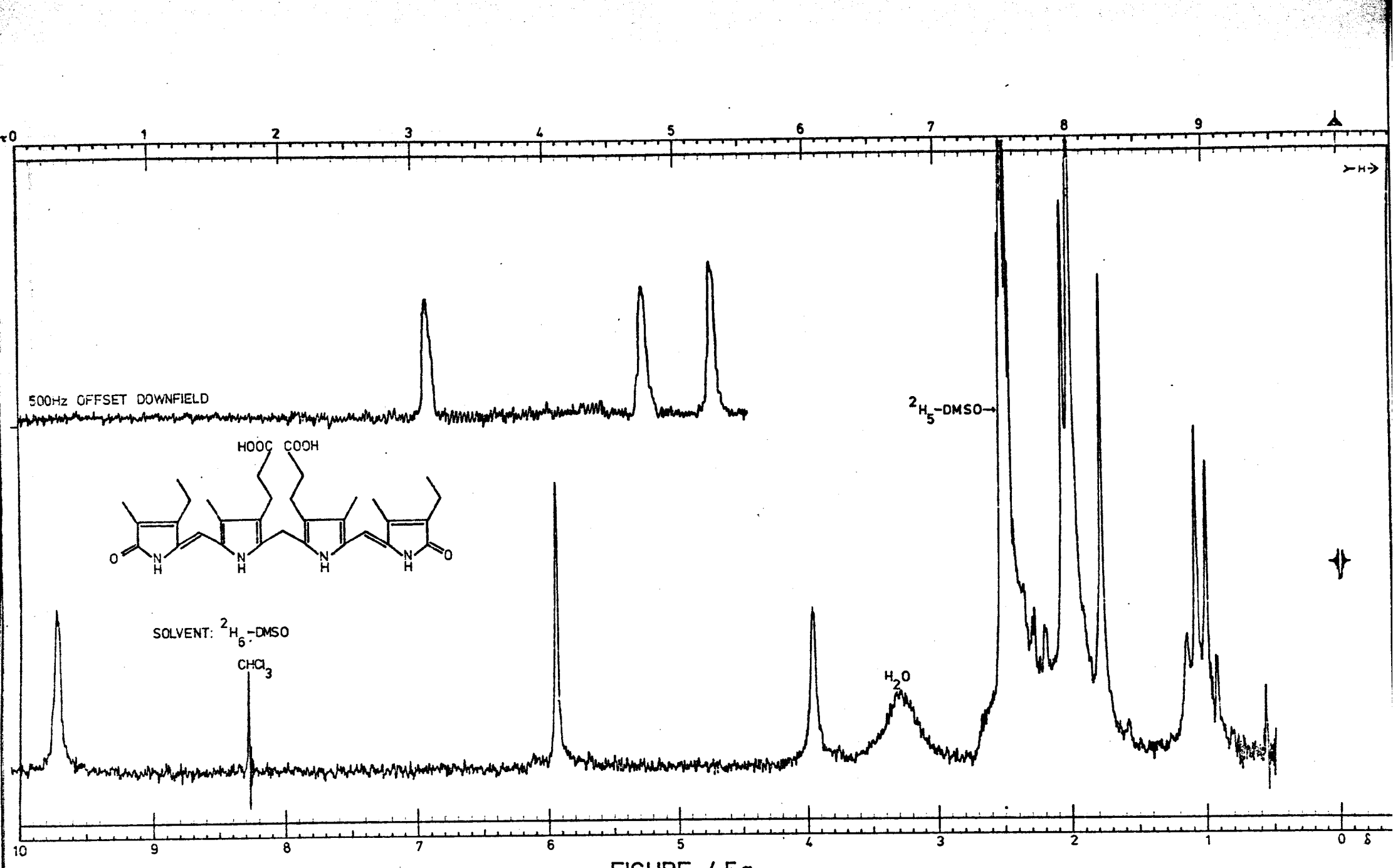

τ0
1
2
3
4
5
6
7
8
9
H
500Hz OFFSET DOWNFIELD
HOOC COOH
O
N H
N H
N H
O
N H
SOLVENT: $^{2}H_{6}$-DMSO
$CHCl_3$
H_2O
$^{2}H_5$-DMSO
10
9
8
7
6
5
4
3
2
1
0
δ

FIGURE 4.5a.

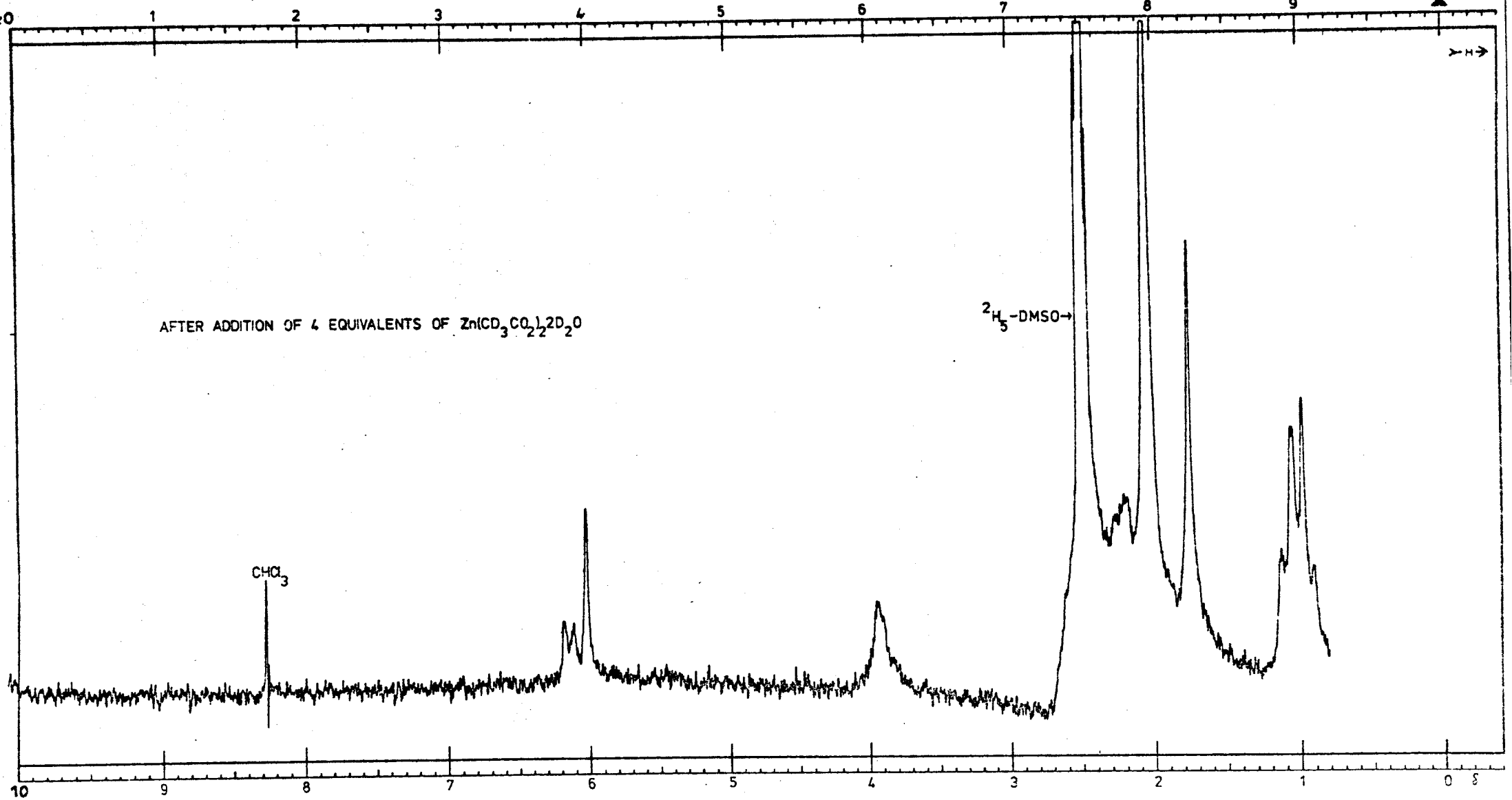
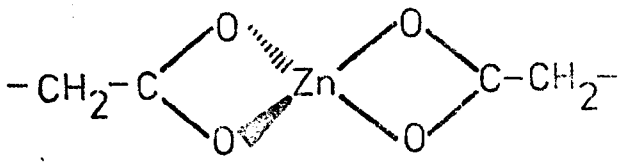

τ0
1
2
3
4
5
6
7
8
9
AFTER ADDITION OF 4 EQUIVALENTS OF $Zn(CD_3CO_2)_2 2D_2O$
$^2H_5$-DMSO→
$CHCl_3$
10
9
8
7
6
5
4
3
2
1
0
δ

FIGURE 4.5b.

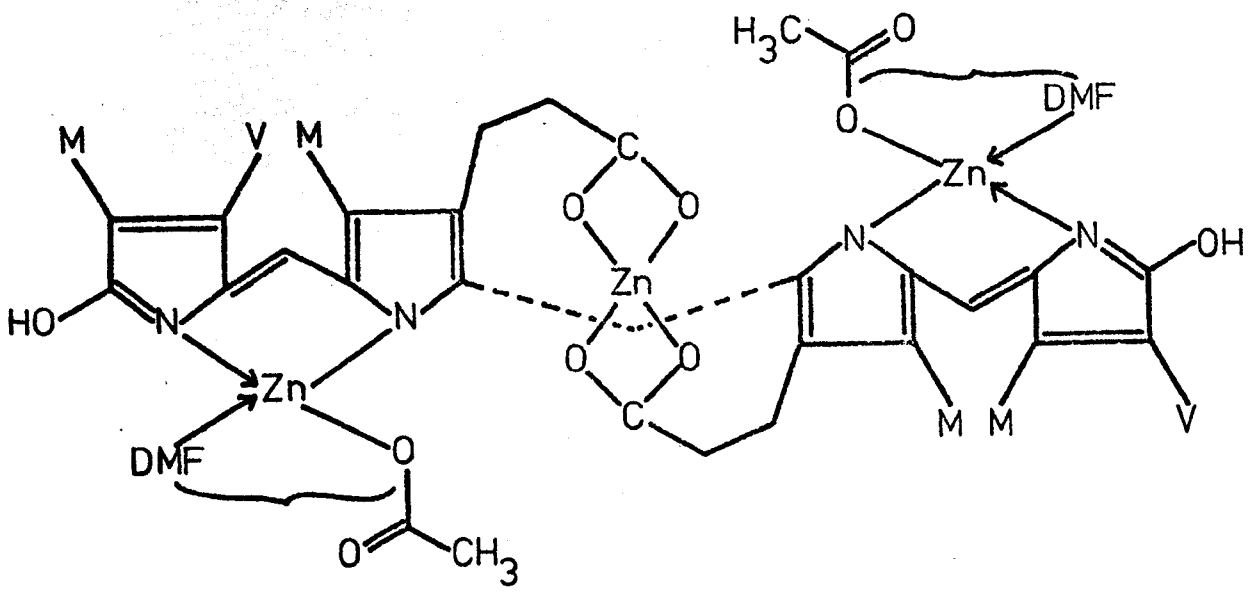
because of the completed d shell hence the stereochemistry of Zn(II) complexes is determined solely by considerations of the size, electrostatic forces and covalent bonding forces of the ligands. Therefore, Zn(II) generally adopts a coordination number of four with tetrahedral coordination of the ligands. [145a]

Assuming the complex to contain one tetrapyrrole residue, it is proposed that one of the zinc ions is coordinated by the carboxylate anions of the two propionic acid side chains, in a manner similar to that found in zinc acetate dihydrate. [146] However, unlike $Zn(OAc)_2.2H_2O$, which is octahedral with the acetate groups equatorial, it is suggested that the carboxylate-coordinated zinc ion in the DMF complex is tetrahedral, in the absence of any extra ligands which might have otherwise forced it to adopt an octahedral structure. This centre is therefore electrically neutral with the stereo - chemistry as shown by (4.II.). Only if the bilirubin enolises to the bislactim form, to give two tertiary nitrogen atoms can the other two zinc ions be coordinated in a manner in keeping with the normal bonding in metal complexes of pyrrolic systems. [133, 147] It is then possible that the enolisation is the rate determining step in the formation of the complexes, referred to above. The complete structure is shown by (4.III.) with the two vacant coordination sites on each of the pyrrole - coordinated zinc ions occupied by a uni - dentate acetate ion and a DMF molecule coordinated via the nitrogen atom. It will be seen that the molecule is electrically neutral.

A model of (4.III.), shown in Plate 4.1., indicates little strain or steric crowding. This appears to be a plausible structure - a structure based on the hydrogen bonded structure of bilirubin (2.XVIII.) is rejected because of even greater steric crowding than in the case of free bilirubin. If the insolubility of the DMF complex



(4.II)



(4.III)

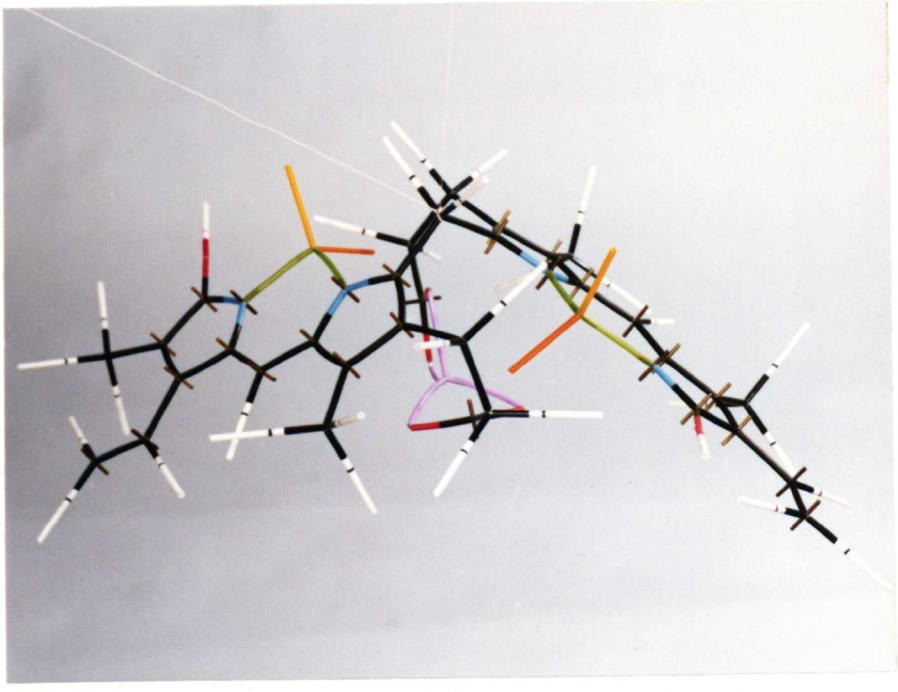


PLATE 4.1.

is significant then it is possible that the complex could be polymeric. Polymers, based on (4.III.), are possible with the units linked by the coordination of propionate groups from two different bilirubin molecules to a zinc ion. Unfortunately, the nature of the reaction mixture does not allow an estimate to be made of the molecular weight of the complex when it is formed initially in solution. Furthermore, the insolubility of the isolated complex does not permit an accurate determination of its molecular weight by cryoscopic, ebullioscopic or osmometric methods.

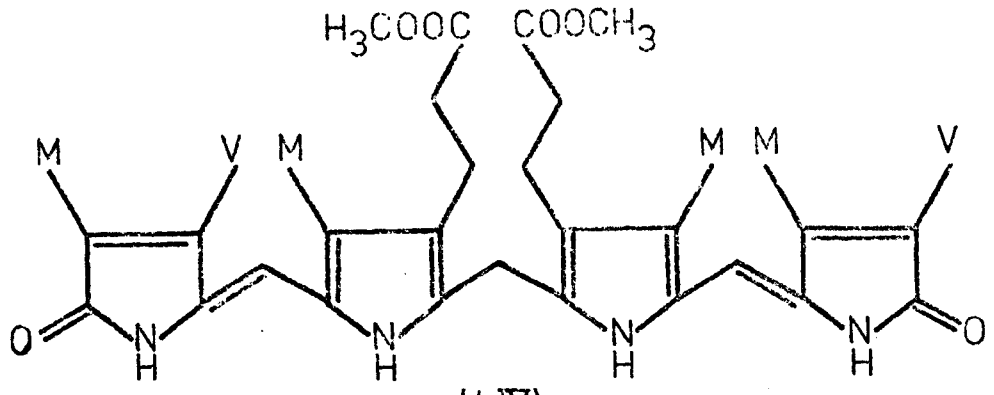
The i.r. spectrum of the complex is compatible with the structure (4.III.). If the band at 3,300cm.$^{-1}$ is assigned to the stretching mode of the enolic O-H group, the bands at 1660, 1630, and 1575cm.$^{-1}$ may be assigned in the following way. The carboxylate ions show two CO stretching bands in the i.r. spectrum, whether it is free or coordinated to a metal ion.[148] Theoretically, these bands (for symmetrical and asymmetrical stretching modes) should be further apart when the carboxylate group is a unidentate ligand than when it is a bidentate ligand. By comparison with examples already published,[148,149] the broad band at 1575cm.$^{-1}$ in the complex is assigned to the asymmetric stretching mode in the bidentate propionate and the unidentate acetate ligands. The band observed at 1415cm.$^{-1}$ is assigned to the symmetrical stretching mode of the unidentate ligands while that of the bidentate ligands is presumed to be obscured by the Nujol absorption band at 1465cm.$^{-1}$ The absorption at 1660cm.$^{-1}$ is assigned to the carbonyl stretching mode in the DMF ligand while that at 1630cm.$^{-1}$ is probably due to C=C and C=N stretching modes in the bilirubin ligand.

4.2.2. Zinc Complexes of Bilirubin Dimethyl Ester and α,α'- Dimethoxybilirubin Dimethyl Ester.

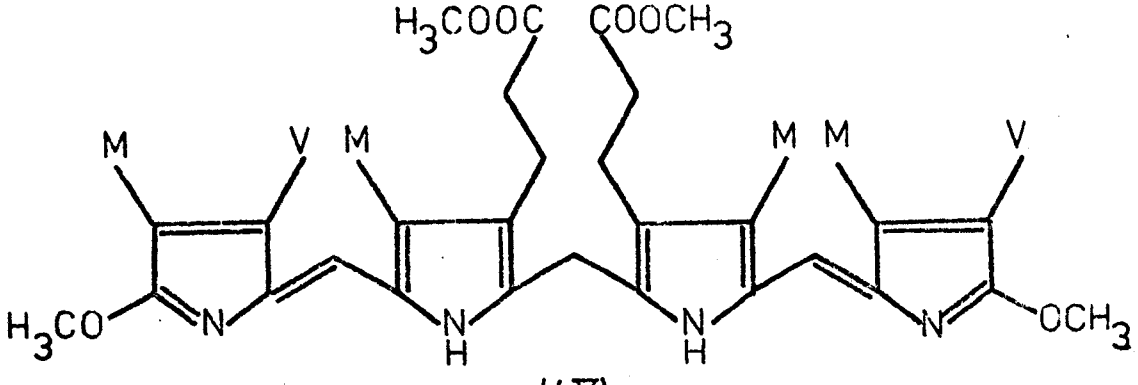
Both bilirubin dimethyl ester (4.IV.) and α,α'- dimethoxybilirubin dimethyl ester (4.V.) will form complexes with zinc acetate in DMF solution. In each case the complex is slower in forming than the corresponding complex of bilirubin, that of (4.IV.) taking 20 - 30 minutes at room temperature while that of (4.V.) takes 1 - 2 hours. This latter observation is unusual since (4.V.) is fixed in the bislactim form so the rate of complex formation ought to be equal to or greater than the rate observed for bilirubin itself unless the structures of the two complexes differ.

Using the method of continuous variations,[144] the Zn : (4.V.) ratio in the α,α'- dimethoxybilirubin dimethyl ester complex was found to be 2 : 1. Unfortunately this method cannot be used to determine the stoichiometry of the bilirubin dimethyl ester complex since the absorption spectrum of this complex varies with the amount of zinc added.

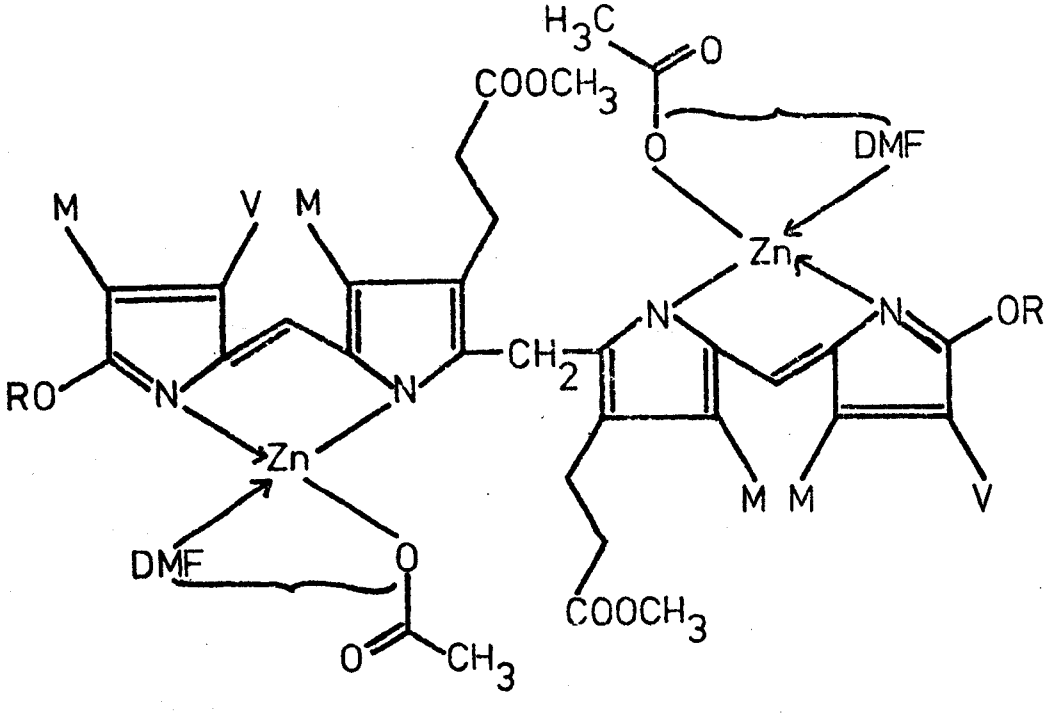
Figure (4.6.) shows the variations, with time, in the absorption spectra when two(a) and four(b) equivalents of zinc ions are added to a solution of bilirubin dimethyl ester in DMF. The corresponding absorption spectra obtained when two equivalents of zinc ions are added to a solution of α,α'- dimethoxybilirubin dimethyl ester are shown in Figure4.7a, while Figure 4.7b shows the effect of adding aliquots of two equivalents of zinc ions and recording the spectrum immediately after each addition. The appearance of an isobestic point in each case indicates a linear relationship between two components. However, in Figure 4.7a, the isobestic point (at 460nm) does not coincide with the absorption spectrum of the α,α'- dimethoxybilirubin dimethyl ester whereas the isobestic point in Figure 4.7b (at 455nm)



(4.IV)



(4.V)



(4.VIa) R=H

(4.VIb) R= CH_3

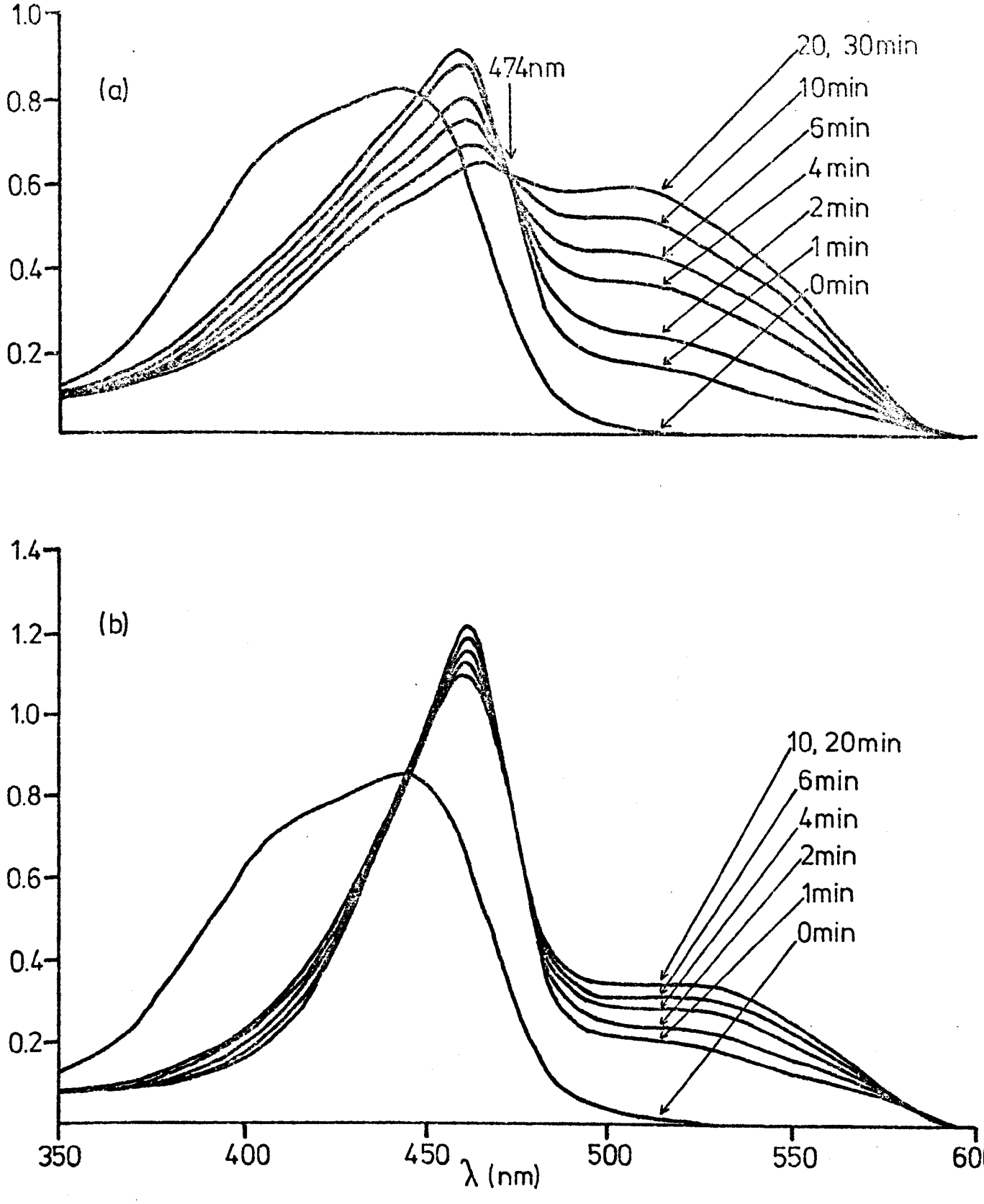


FIGURE 4.6. THE FORMATION, WITH TIME, OF COMPLEXES AFTER THE ADDITION OF (a) 2 AND (b) 4 EQUIVALENTS OF ZINC ACETATE TO BILIRUBIN DIMETHYL ESTER IN DMF

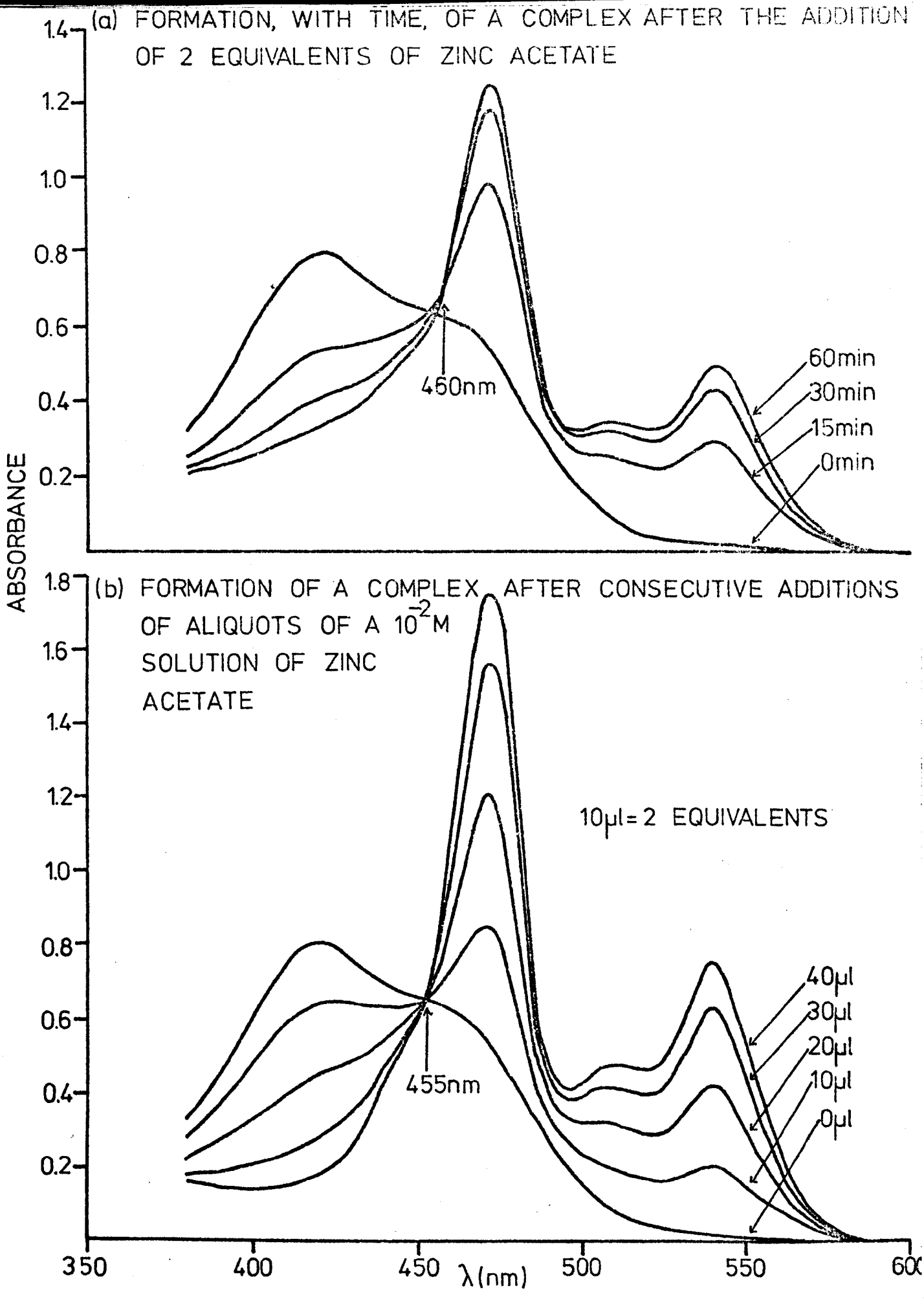


FIGURE 4.7. THE ADDITION OF ZINC ACETATE TO α,α'-DIMETHOXYBILIRUBIN DIMETHYL ESTER IN DMF

does. This may indicate that the initial complex (as in Figure 4.7b.) rearranges with time to give a complex with a Zn : (4.V.) ratio of 2 : 1. Possible structures for the complexes with (4.IV.) and (4.V.) are shown by (4.VIa.) and (4.VIb.) respectively, although the possibility of a binuclear complex for bilirubin dimethyl ester as found for (4.1.) cannot be ruled out. The behaviour of the bilirubin dimethyl ester complex may possibly be due to interaction between the π-electron systems of the dipyrrylmethenes and any zinc ions in excess of those required for complex formation.

In a manner similar to that described for bilirubin complexes, bilirubin dimethyl ester, identical in all respects to an authentic sample, can be recovered from the decomposition of the complexes formed with two and four equivalents of zinc ions in DMF solution. However, in the case of α,α' - dimethoxybilirubin d imethyl ester, insufficient material was available for the similar examination of the tetrapyrrole obtained from the decomposition of this complex.

4.2.3. Complexes Between Bilirubin and Other Metal Acetates.

Qualitative absorption spectra were obtained by adding aliquots of either a M/10 or saturated solution of the metal acetate in DMF to a 10^{-5}M solution of bilirubin in DMF. Not unexpectedly, no significant changes are observed when the acetates of Li(I), Na(I), K(I), Ca(II), Ba(II), Ag(I), Hg_2(II), and Tl(I) are added. The additions of Be (as $BeO(OAc)_6$) and Mg(II) produce hypsochromic shifts from 454nm to 414nm. The additions of Mn(II), Ni(II), Cu(II), and Pb(II) cause no shifts in the position of the absorption maximum but do cause decreases in the extinctions. These observations are rather surprising since one might expect, by analogy with porphyrin systems, for these cations to form complexes. Perhaps this reflects the

the inability of bilirubin to conform to the stereochemical requirements of these metals.

Only when the acetates of Co(II) and Cd(II) are added are bathochromic shifts, similar to those for Zn(II), observed. The absorption spectra of these complexes are shown in Figure 4.8, with that of the zinc complex, under the same conditions, included for comparison. The Co(II) and Zn(II) ions have very similar radii and it is suggested that the preferred stereochemistry in bilirubin - metal complexes is tetrahedral since it is known that Co(II) forms more tetrahedral complexes than any other transition metal ion.[145b] If bilirubin can form only tetrahedral complexes, then this may explain the absences of any complex formation with other metals as suggested above. Although the Cd(II) ion is larger than the Zn(II) ion, the formation of a cadmium complex is not unexpected since zinc and cadmium are group IIb metals. The third member of this group, mercury, when added as Hg(II) causes rapid oxidation of the bilirubin to a green pigment, presumably a verdin.

4.3. Experimental.

Materials.

Bilirubin (B.D.H. Chemicals Ltd.) was purified as described in Chapter 3, Section 3.3.

N,N -Dimethylformamide was dried over anhydrous barium oxide for several days, decanted, distilled under reduced pressure and redistilled under reduced pressure from, and onto, freshly heated molecular sieve(4A) before use.

Dimethyl Sulphoxide was dried over calcium hydride for several days and then distilled as described for DMF.

Zinc Acetate Dihydrate (A.R.) was used without further purification.

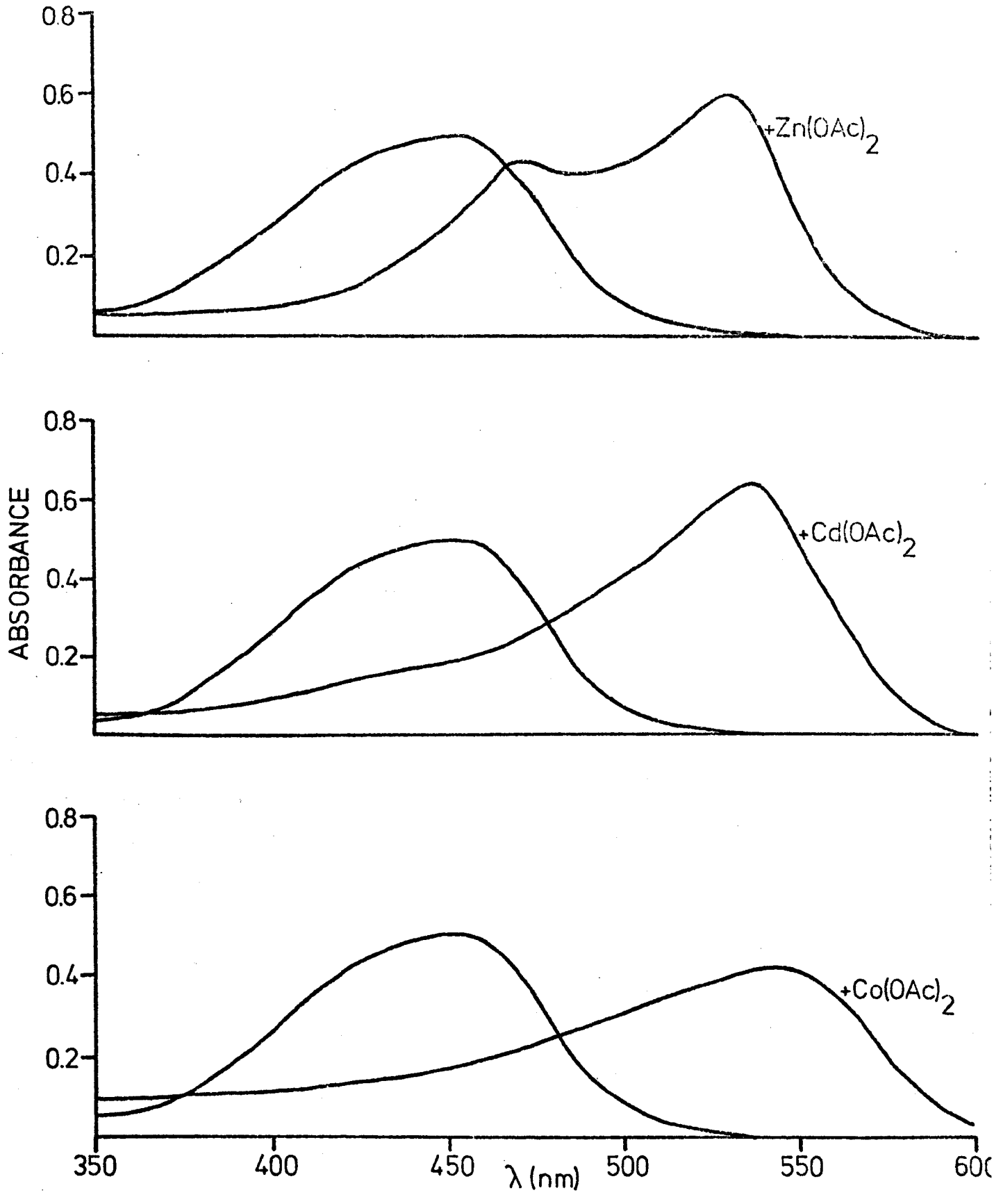


FIGURE 4.8. THE ADDITION OF 30µl OF 0.1M SOLUTIONS OF THE STATED METAL ACETATES IN DMF TO 10^{-5}M SOLUTIONS OF BILIRUBIN IN DMF

Methods.

Isolation of the Zinc - Bilirubin Complex from DMF Solution.- Bilirubin (100mg, 0.17mmole) was dissolved in purified DMF (150ml) and zinc acetate dihydrate (150mg , 0.68 mmole) dissolved in DMF (5ml) added. The solution was allowed to stand for 10 minutes, in the dark and then most of the solvent, (130ml) was removed in vacuo, below 40°C. The resulting dark red - brown solution was set aside for one hour in the dark, the precipitate isolated by vacuum filtration and washed with a little DMF. The last traces of solvent were removed at 0.15mm Hg overnight.

i.r. (Nujol mull) ν_{max} : 3,300(m), 1660(s), 1630(s). 1575(br,s)$cm.^{-1}$

Analysis (B) : Found (i) C, 49.64; H, 5.24; N, 7.16; Zn, 16.89%.

(ii) C, 49.72; H, 5.07; N, 7.10; Zn, 16.77%.

If the complex has the structure proposed then the molecular formula is $C_{43}H_{52}O_{12}Zn_3$, which requires C, 49.51; H, 5.23; N, 8.06; Zn, 18.80%.

Mass Spectrum (PCMU) : Accurate mass of the ion corresponding to DMF(C_3H_7NO) = 73.0538. C_3H_7NO requires 73.0528.

Accurate mass of the ion corresponding to acetic acid ($C_2H_4O_2$) = 60.0211. $C_2H_4O_2$ requires 60.0211.

The complex prepared using zinc deuteroacetate (167 mg, 0.68mmole) was isolated in a similar manner.

i.r. (Nujol mull) ν_{max} : 3,310(m), 1655(s), 1630(s), 1560(s) $cm.^{-1}$

Mass Spectrum (FCMU) : Accurate mass of the ion corresponding to DMF(C_3H_7NO) at m/e 73 = 73.0527. C_3H_7NO requires 73.0528. Accurate mass of the ion corresponding to CD_3COOH = 63.0400. $C_2HD_3O_2$ requires 63.0400.

Determination of the Quantitative Absorption Spectra.- The quantitative absorption spectra of the complexes were determined by their preparation in situ, and all determinations were carried out using a Cary 14 Spectrophotometer. A solution of bilirubin or mesobilirubin in the appropriate solvent, having an optical density at the absorption maximum, between 0.1 and 1.0, was prepared. Zinc acetate dihydrate, dissolved in the same solvent, was added until the extinction at the absorption maximum of the complex reached a maximum value. This generally required 6 - 12 equivalents of the zinc acetate dihydrate. The molarity of the bilirubin solution was determined spectrophotometrically, using the extinction coefficients in Table 4.1, allowing the extinction coefficient of the complex to be determined, assuming the complex to contain one tetrapyrrole residue. The average of several determinations in each case is shown in Table 4.1.

TABLE 4.1.

Tetrapyrrole.	Solvent.	Free Ligand. λ_{max}	ε	Complex. λ_{max}	ε
Bilirubin.	DMF.	448nm.	58,700	526nm.	58,000
	DMA.	448nm.	54,200	526nm.	74,000
	DMSO.	454nm.	59,200	528nm.	63,000
Mesobilirubin.	DMF.	420nm.	50,300	432nm.	64,900
	DMSO.	427nm.	53,200	485nm.	55,800

Determination of the ^1H n.m.r. Spectra of the Zinc Complexes of Bilirubin and Mesobilirubin in $^2H_6$ - DMSO.- Bilirubin (30mg, 51μmole) was dissolved in $^2H_6$ - DMSO (0.5ml) in a sample tube and a 2.2M solution of zinc deuteroacetate in deuterium oxide (100μl, 4 equivalents of Zn(II)) added and the spectrum recorded. A further quantity of the zinc deuteroacetate solution was added (50 μl) and the spectrum re - recorded. The ^1H n.m.r. spectrum of the mesobilirubin complex was obtained in a similar manner.

Zinc Deuteroacetate Dideuterohydrate.- Perdeuteroacetic acid (5ml, 5g, 0.078mole) was diluted with deuterium oxide (15ml), zinc oxide (4.0g, 0.049mole) added and the suspension heated gently to boiling point. The supernatant was decanted and any cloudiness cleared by the addition of a few drops of CD_3COOD. The solution was allowed to cool, the crystals collected by vacuum filtration and dried in vacuo over phosphorus pentoxide. Yield 4.54g, (51%).

1 - ^{14}C - Zinc Acetate.- 1 - ^{14}C - Acetic acid was obtained as the sodium salt (95% isotopic abundance in the carbon atom). Two methods were used to convert this to zinc acetate: a) 1 - ^{14}C - Sodium acetate (50 μCi) was diluted with the unlabelled salt (2g) dissolved in water (2ml). This solution was allowed to percolate through a column of ion exchange resin (Dowex x 50, H^+ form, 70ml wet vol. of resin). The acid fraction was collected, stirred overnight with an excess of basic zinc carbonate, filtered and the filtrate reduced in volume to about 10ml and allowed to crystallise. The crystals were collected by vacuum filtration, dried in vacuo over phosphorus pentoxide, ground, and sieved through a 70 mesh sieve. b) Two samples of 1 - ^{14}C - labelled zinc acetate were obtained by diluting a sample of 1 - ^{14}C- sodium acetate (250μCi) with different amounts of cold zinc acetate (about 2g and 3g) dissolved in water. Crystals were obtained and processed as described in a).

Determination of the Specific Activities of the Preparations of 1 - ^{14}C- Zinc Acetate.- A sample of radioactive zinc acetate (20 - 50 mg) was dissolved in water (1ml) and the activities of 10, 20, - 60 μl aliquots determined using the dioxan based scintillation medium described in Chapter 2, section 2.3. The specific activities of the three preparations were found to be i) 6.28 x 10^6 cpm/mmole (prepared by method a)), ii) 3.29 x 10^7 cpm/mmole and iii) 4.61 x 10^7cpm/mmole. (both prepared by method b)).

Isolation of the Zinc - Bilirubin Complex, Labelled with 1 - ^{14}C - Acetate, from DMF Solution.- Samples of the labelled complex were prepared exactly as described above for the "cold" complex. The i.r. spectrum and the X-ray powder photograph of each preparation were identical to those obtained from the "cold" complex.

Determination of the Specific Activities of the Samples of Labelled Complex from DMF solution.- A convenient approach was to utilise the limited solubility of the DMF complex in DMSO. Quenching curves were constructed for the chemical quenching by the DMSO and the colour quenching by the complex, using DMSO and a DMSO solution of the DMF complex respectively, in similar ways to that already described in Chapter 2, section 2.3. The quenching curves are shown in Figure 4.9 and Figure4.10 respectively. The quenching by DMSO is low (about 3% per 0.1mg in 10 ml) whereas that by the complex is greater (about 30% per 0.05mg in 10ml)

A sample of the 1 - ^{14}C - acetate labelled complex (10mg) was suspended in DMSO (10ml) and the suspension stirred, in the dark at room temperature until a solution was obtained (about 2 hours). Aliquots (0.1ml - 0.6ml) were counted and the specific activity of the complex determined, after correction for quenching. The specific activities of the three samples prepared from the three preparations of 1 - ^{14}C - zinc acetate were found to be i) 5.94×10^6 cpm/mmole, ii) 2.97×10^7 cpm/mmole, and iii) 4.85×10^7 cpm/mmole respectively, which indicates the presence of i) 1.88, ii) 1.80, and iii) 2.10 acetate ions respectively per molecule of complex.

Attempts were made to determine the specific activities of these samples employing the technique of suspension counting using the thixatropic agent Cab - O - Sil (500mg/10ml). [150] The specific activities of the 1 - ^{14}C - zinc acetate preparations were readily determined in this way. However when applied to the DMF complex, the method failed because of quenching within the particles themselves and attempts to construct a quenching curve led to a series of random results even when the latter had been passed through a fine - mesh sieve.

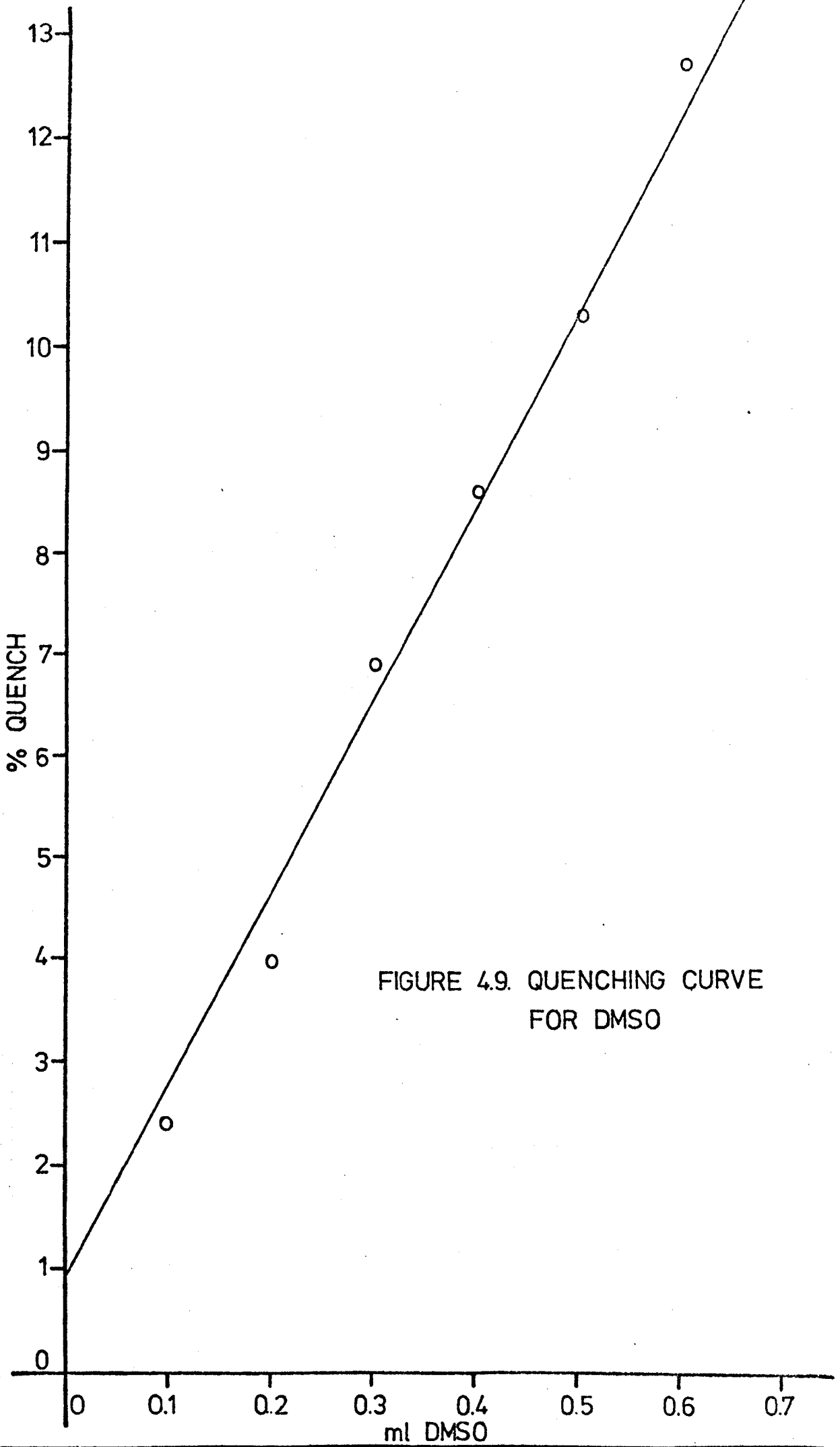


FIGURE 4.9. QUENCHING CURVE FOR DMSO

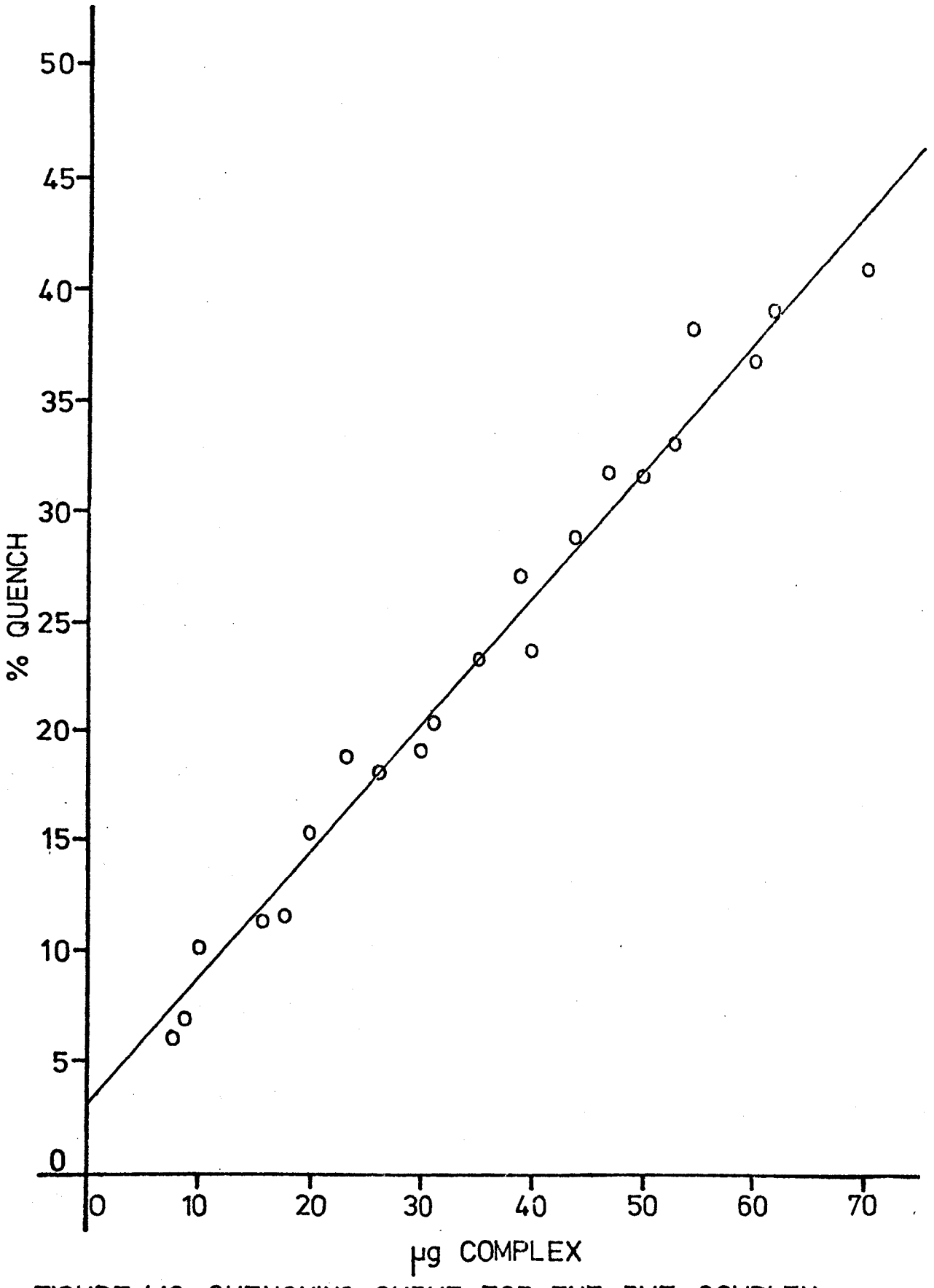


FIGURE 4.10. QUENCHING CURVE FOR THE DMF COMPLEX

Determination of the Zinc : Tetrapyrrole Ratios in the Complexes Examined.- The method used was similar to that already described,[144] all solutions being made up to a volume of 10ml. The molarities of the pigment solutions used (and hence also of the zinc acetate solutions) ranged from 0.5×10^{-5}M to 2.0×10^{-5}M, the exact concentration being determined spectrophotometrically. The zinc acetate solutions were prepared by dilution of an M/10 solution in the appropriate solvent.

Recovery of Bilirubin and Bilirubin Dimethyl Ester from the Complexes Formed in DMF Solution and of Bilirubin from the Complexes Formed in DMSO Solution.- Bilirubin (100mg, 0.17mmole) was dissolved in DMF or DMSO (100ml) and zinc acetate (150mg, 0.68mmole) added. The solutions were allowed to stand at room temperature for 30 minutes in the dark and were then poured into water (1l) containing a few drops of glacial acetic acid. This solution was extracted with chloroform (3 x 250ml) and the organic layer washed with water (1 x 100ml) containing a little sodium bicarbonate, then with water alone (1 x 100ml). The chloroform solution was dried over anhydrous Na_2SO_4 and the solvent removed in vacuo to leave an orange residue which was maintained at 1mm Hg overnight. Recovery of bilirubin was 71mg (71%) (DMF) and 62mg (62%) (DMSO).

u.v. (Chloroform) : From DMF, λ_{max} 454nm (ε 57,900),
Lit.,[88] λ_{max} 543nm (ε58,800).
From DMSO, λ_{max} 454nm (ε 59,700),
Lit.,[88] λ_{max} 453nm (ε 62,200)

The i.r., ^{1}H n.m.r. and the mass spectra were identical to those described in Chapter 2, the Appendix and Chapter 5 respectively.

In a similar manner, bilirubin dimethyl ester was recovered from DMF solutions containing two and four equivalents of zinc acetate. Recovery was 61% (2 equivalents Zn(II)) and 63% (4 equivalents of Zn(II))

Qualitative absorption, i.r., ^{1}H n.m.r. and mass spectra of both samples were identical to those observed for an authentic sample of bilirubin dimethyl ester described in Chapter 5.

Control experiments for the recovery of bilirubin and its dimethyl ester from the same processes indicated that the recovery never exceeded 70% of the theoretical amount.

Bilirubin Dimethyl Ester.- This was prepared as described in Chapter 5, section 5.3.

α,α' - Dimethoxybilirubin Dimethyl Ester.- This was prepared as already described in Chapter 2, section 2.3.

CHAPTER 5.

ESTERS OF BILIRUBIN.

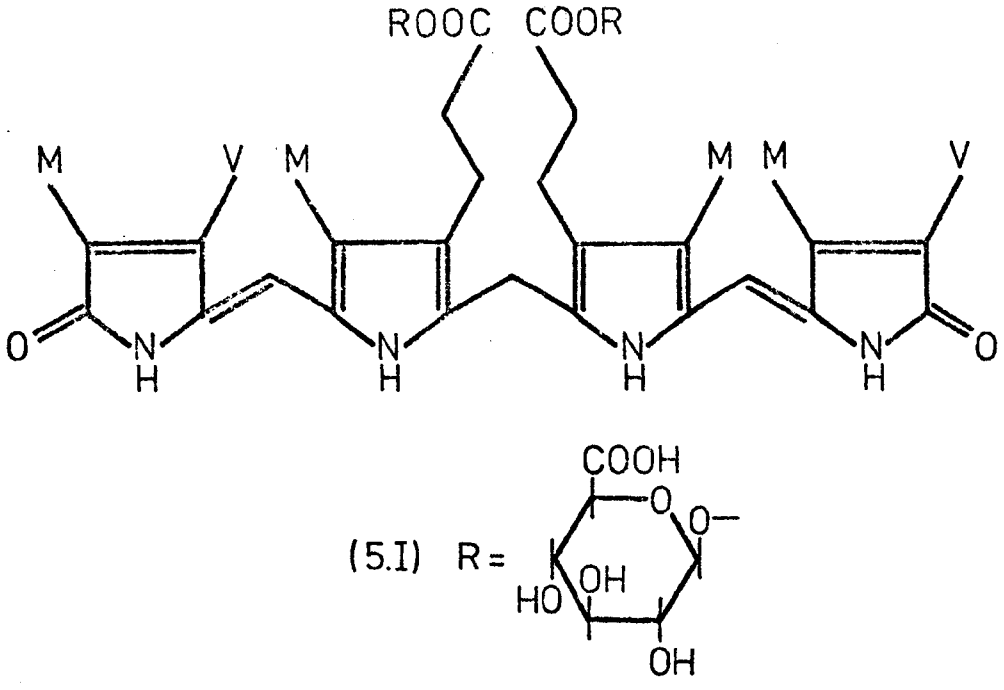
5. ESTERS OF BILIRUBIN.

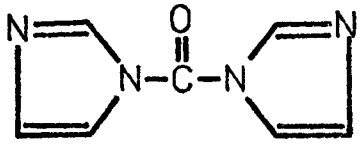
5.1. Introduction.

The methods available for the isolation of bilirubin conjugates from bile are lengthy and give products of uncertain purity, due either to the incomplete separation of the compounds in bile or to the formation of artefacts during the isolation procedures.[77, 78] The first bilirubin conjugate to be identified was the diglucuronide (5.I.) in which the propionic acid side chains of bilirubin are esterified by the C(I) hydroxyl groups of β - D - glucuronic acid.[61] Recently, other sugar derivatives have been identified in bilirubin conjugates isolated from human bile, and it is likely that bilirubin is excreted in part as acyl glycosides of complex oligosaccharides[70] (see Chapter 1, section 1.5.2.). In dog bile, bilirubin can be excreted as the acyl glycosides of β - D - glucose and β - D - xylose.[68, 69, 151]

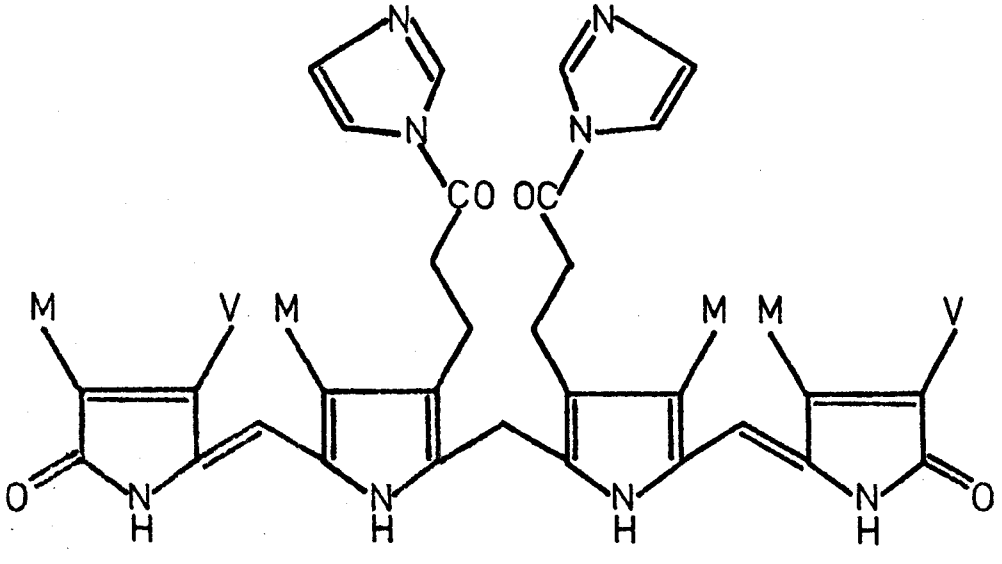
Thus there is a need for synthetic bilirubin conjugates of defined chemical structure to confirm these various naturally occurring conjugates. Any chemical method for the synthesis of bilirubin conjugates must allow the stereospecific esterification of a particular hydroxyl group of a polyhydroxylated saccharide residue. Moreover, in view of the known instability of bilirubin under acid[86] or alkaline[87, 136, 137, 152] conditions, the esterification must take place under mild conditions and preferably also with the minimum use of protecting groups. The methods currently available for the synthesis of the dimethyl ester of bilirubin such as diazomethane,[102,153] methanol - boron trifluoride,[154] or methanol - hydrogen chloride[111] are unsuitable as these procedures cannot be easily adapted for the esterification of specific hydroxyl groups in mono - or oligo - saccharides.

The reaction between bilirubin diimidazolide (5.III.)


ROOC COOR
M V M M M V
O N H N H N H N H O
(5.I) R =
COOH
O
O–
HO
OH
OH


N
O
N
N–C–N

(5.II)


N N
N N
CO OC
M V M M M V
O N H N H N H N H O

(5.III)

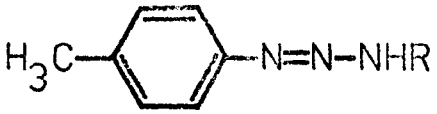
(prepared from bilirubin and N,N' - carbonylbisimidazole (5.II.) in DMF) and glucuronic acid [155, 156] has yielded products with mobilities on TLC identical to the bilirubin mono - and diglucuronides isolated from bile. [78] The synthetic diglucuronide gave a direct van den Bergh reaction, was hydrolysed by alkali, indicating an ester, but was not cleaved by β - glucuronidase. Since TLC of the ethyl anthranilate azopigment showed the presence of several components, [156] the most likely explanation is that random esterification of bilirubin by any of the hydroxyl groups of the glucuronic acid has occurred. N - Protected amino acids have been esterified specifically by the free C(I) hydroxyl group of otherwise fully protected glucuronic acid in a related manner. [157] The disadvantage of this method if applied to bilirubin would be the need for the removal of protecting groups with as little degradation of the bilirubin molecules as possible.

The reaction between diazotised aromatic amines and a primary alkyl amine in neutral solution yields 1 - alkyl-3-aryl triazenes [158,159] These compounds, eg. 1 -alkyl-3-p-tolyltriazenes (5.IV.), will esterify carboxylic acids to give the corresponding alkyl esters in high yield under mild conditions. An extension of the method for the preparation of 1 - amino - β - D - glucose [160] to other sugars could allow the preparation of the corresponding carbohydrate triazenes. Thus, in theory, a method exists for the synthesis, under mild conditions, of bilirubin conjugates with specific sugar residues.

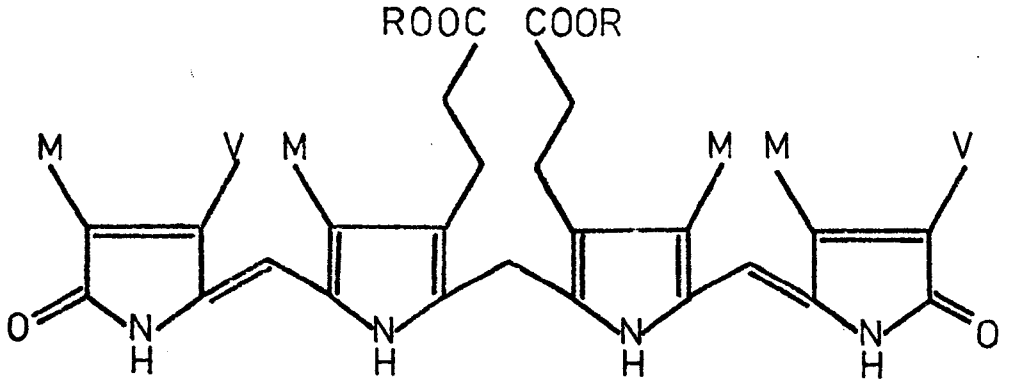
5.2. Results and Discussion.

5.2.1. Preparation and Identification of the Esters.

The reaction between bilirubin and the triazenes (5.IVa, b,d,e,) in chloroform solution at room temperature yields the dimethyl (5.Va.) and the hitherto unknown diethyl (5.V.b.), diisopropyl (5.Vc.)



(5.IV) a)R=CH_3, b)R=CH_2CH_3, c)R=$CH_2CH_2CH_3$, d)R=$CH(CH_3)_2$, e)R=$CH_2C_6H_5$



(5.V) a)R=CH_3, b)R=CH_2CH_3, c)R=$CH(CH_3)_2$, d)R=$CH_2C_6H_5$

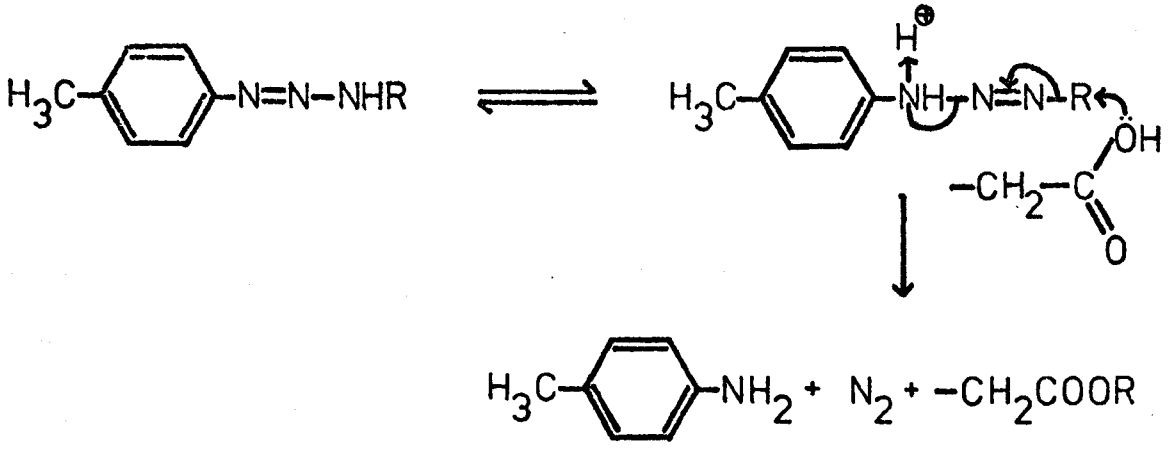


FIGURE 5.1. A MECHANISM FOR THE ESTERIFICATION OF CARBOXYLIC ACIDS USING TRIAZENES

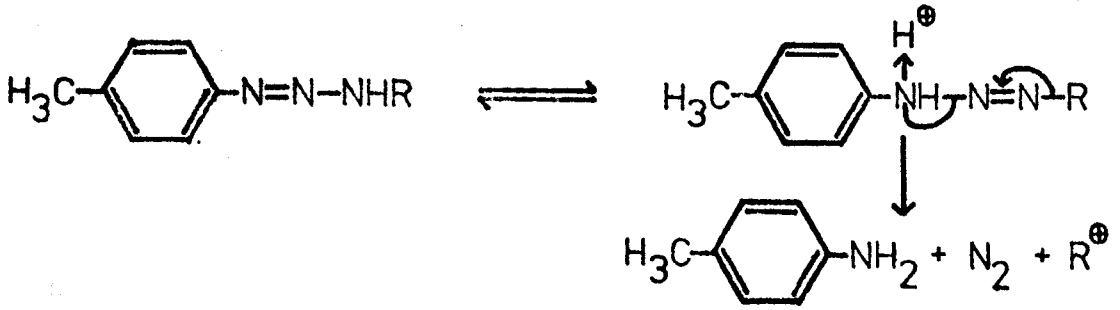


FIGURE 5.2. THE DISSOCIATION OF TRIAZENES

and dibenzyl (5.Vd.) esters of bilirubin which have been identified by the usual physical techniques.

The mechanism of the reaction is uncertain, but it is likely that, in chloroform solution, attack by the carboxylic acid residues of bilirubin on the alkyl groups of one tautomer of the triazene produces the ester, nitrogen and p - toluidine, as shown in Figure 5.1. The stereospecificity of the reaction is unknown, but it appears that, in chloroform solution, the decomposition of the triazenes does not involve carbonium ions, formed as shown in Figure 5.2., as intermediates. An examination, by ^{1}H n.m.r. spectroscopy, of the product from the reaction between phenylacetic acid and 1 - n - propyl-3-p-tolyltriazene(5.IVc.) in chloroform solution, failed to reveal any signals due to iso - propyl groups, indicating that the reaction had proceeded without the rearrangement of an intermediate n - propyl carbonium ion to an iso - propyl carbonium ion. However, it has been observed that substantial rearrangement of the n - propyl group occurs in polar solvents.[161] These two observations suggest that the stereospecificity of the reaction may be solvent dependent.

The properties of bilirubin dimethyl ester prepared in this manner are identical to those of this ester obtained, for example, using diazomethane,[88] while the other esters prepared have been identified by i.r., absorption, mass and ^{1}H n.m.r. spectroscopy, as well as by elemental analysis whenever possible.

The i.r. spectra of the esters, when recorded as Nujol mulls, KBr discs or chloroform solutions, show bands at 1735cm.$^{-1}$ characteristic of non - hydrogen bonded ester carbonyl groups.[115e] A band at 3340cm.$^{-1}$ is also observed, which is assigned to the N - H vibrations of the lactam and pyrrole rings[115b,c] since the frequency is too low for O -H vibrations. Thus it appears that the

esters retain the bislactam tautomeric form of bilirubin. In these and other respects, the spectra, as Nujol mulls, agree with that published for the dimethyl ester.[111]

The esters all show absorption maxima at 400nm in chloroform solution and at 450nm in methanol or DMSO solution, as does a sample of authentic dimethyl ester.[88] The reasons for these differences are discussed more fully below.

Mass spectrometric analysis of the esters gave accurate mass values for the molecular ions consistent with the expected molecular formulae as shown in Table 5.1. The cracking patterns of the esters are

TABLE 5.1.

Ester	Molecular Formula	Required	Found
Dimethyl	$C_{35}H_{40}N_4O_6$	612.2948	612.2943
Diethyl	$C_{37}H_{44}N_4O_6$	640.3261	640.3271
Diisopropyl	$C_{39}H_{48}N_4O_6$	668.3574	668.3582
Dibenzyl	$C_{47}H_{48}N_4O_6$	764.3574	764.3596

shown in Figures 5.3 to 5.6 with those of bilirubin, mesobilirubin and α,α' - dimethoxybilirubin dimethyl ester (Figures 5.7 to 5.9) included for comparison and completeness. In each case the fragmentation pattern is essentially the same as that already observed for bilirubin.[162,163] For all the molecules, except α,α' - dimethoxybilirubin dimethyl ester, the parent ion is quite strong, but the two principal ions (5.VI.) and (5.VII.), one of which is the base peak in each spectrum

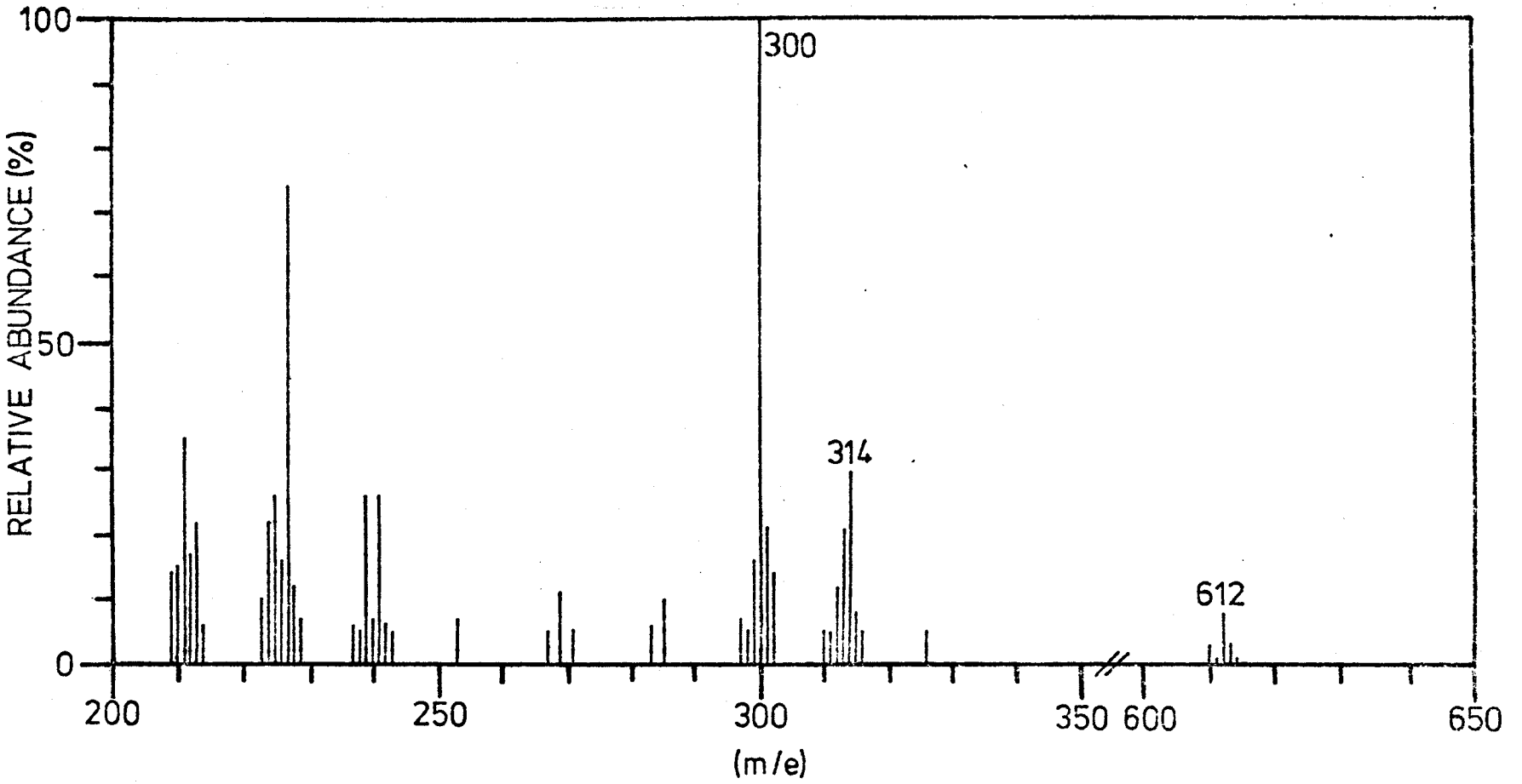


FIGURE 5.3. THE PARTIAL MASS SPECTRUM OF BILIRUBIN DIMETHYL ESTER

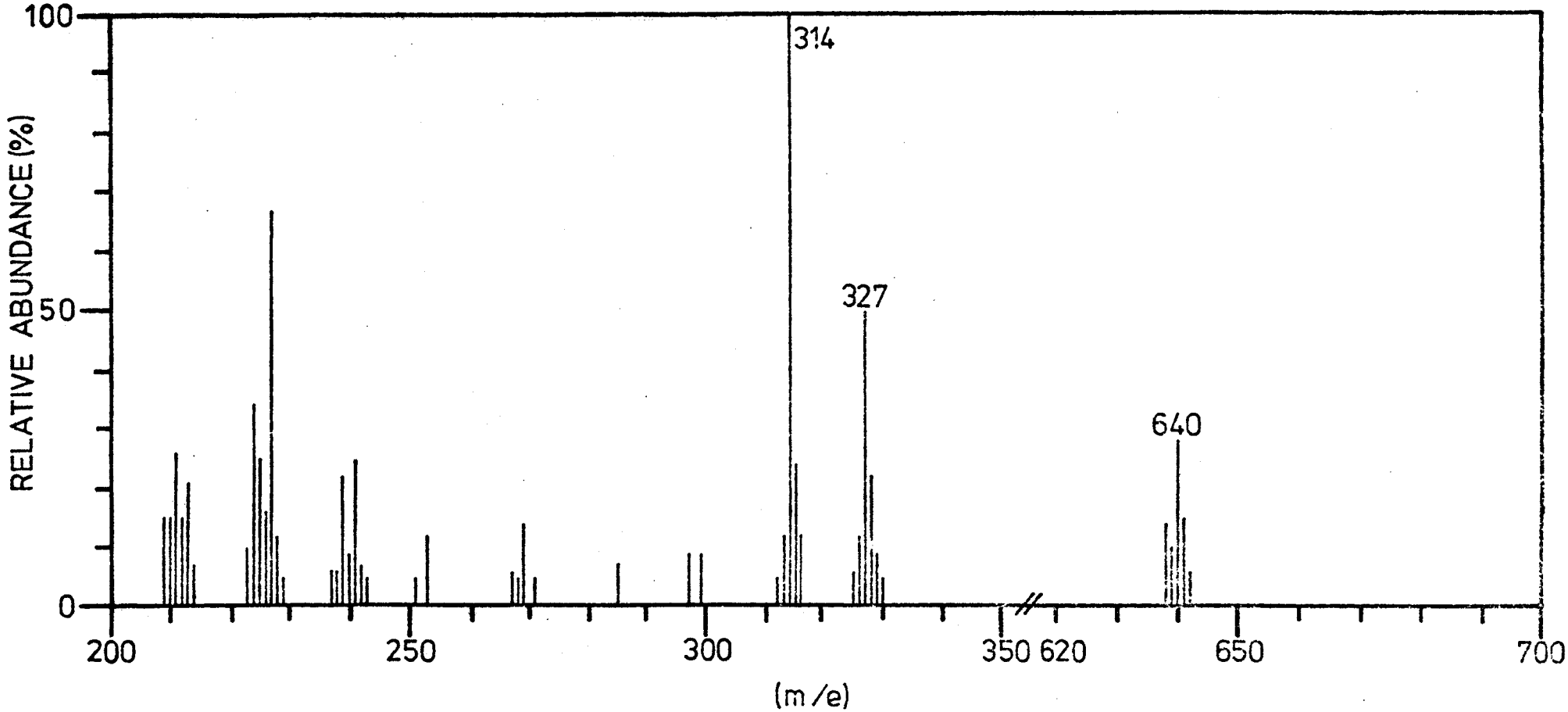


FIGURE 5.4. THE PARTIAL MASS SPECTRUM OF BILIRUBIN DIETHYL ESTER

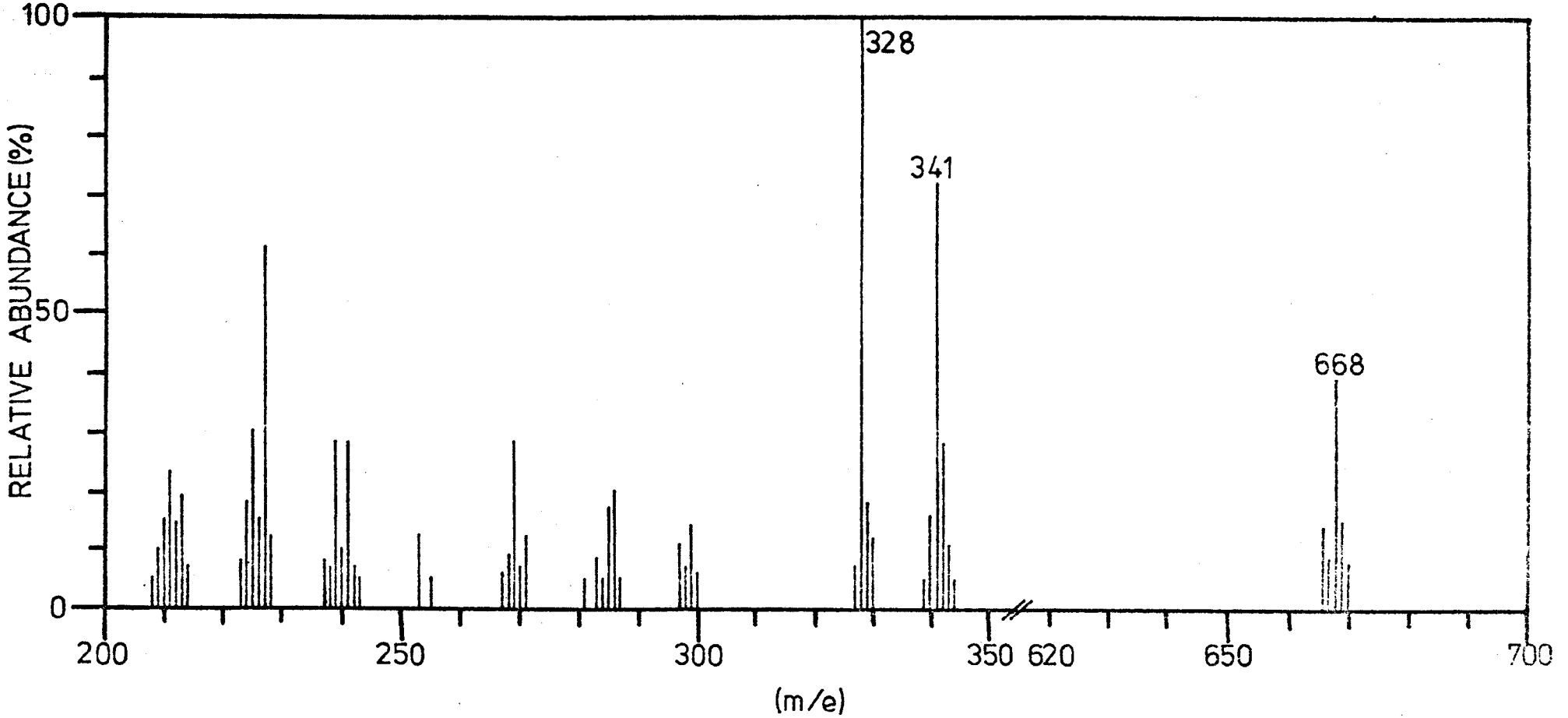


FIGURE 5.5. THE PARTIAL MASS SPECTRUM OF BILIRUBIN DIISOPROPYL ESTER

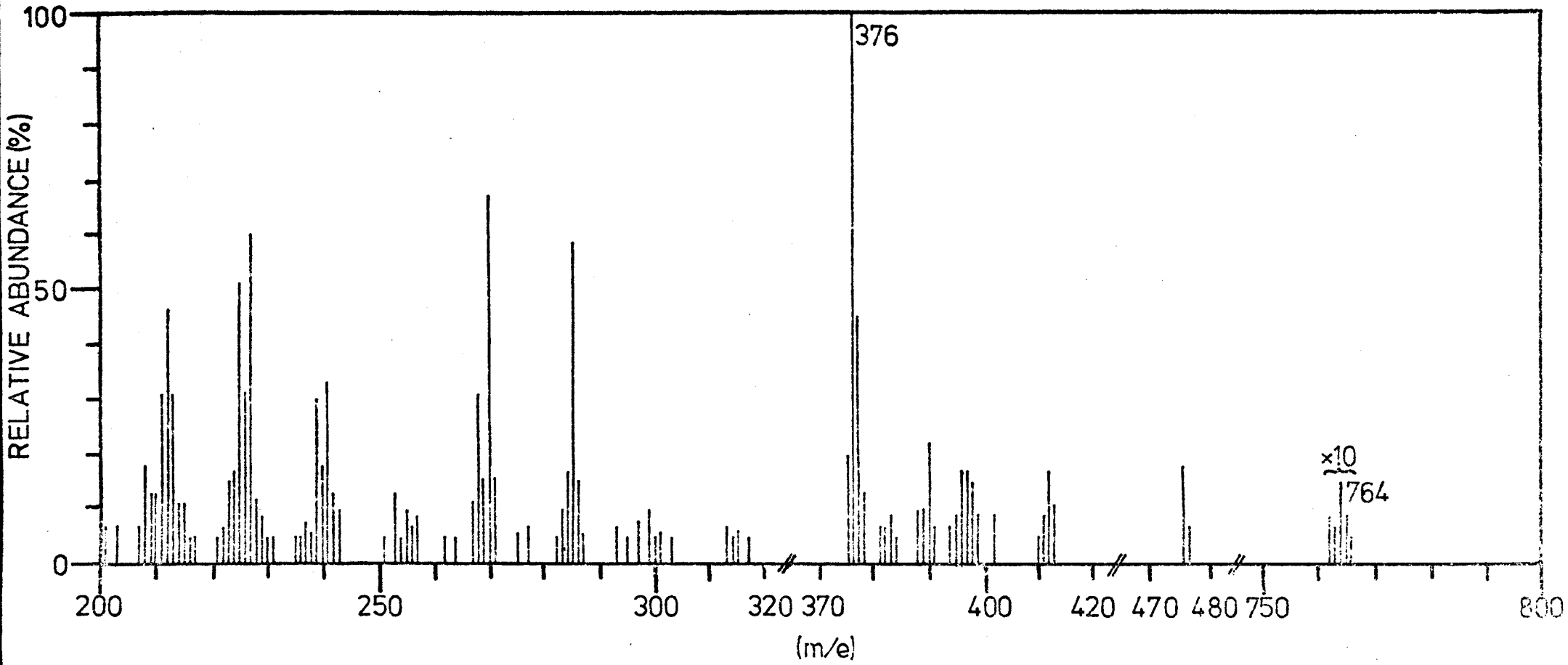


FIGURE 5.6. THE PARTIAL MASS SPECTRUM OF BILIRUBIN DIBENZYL ESTER

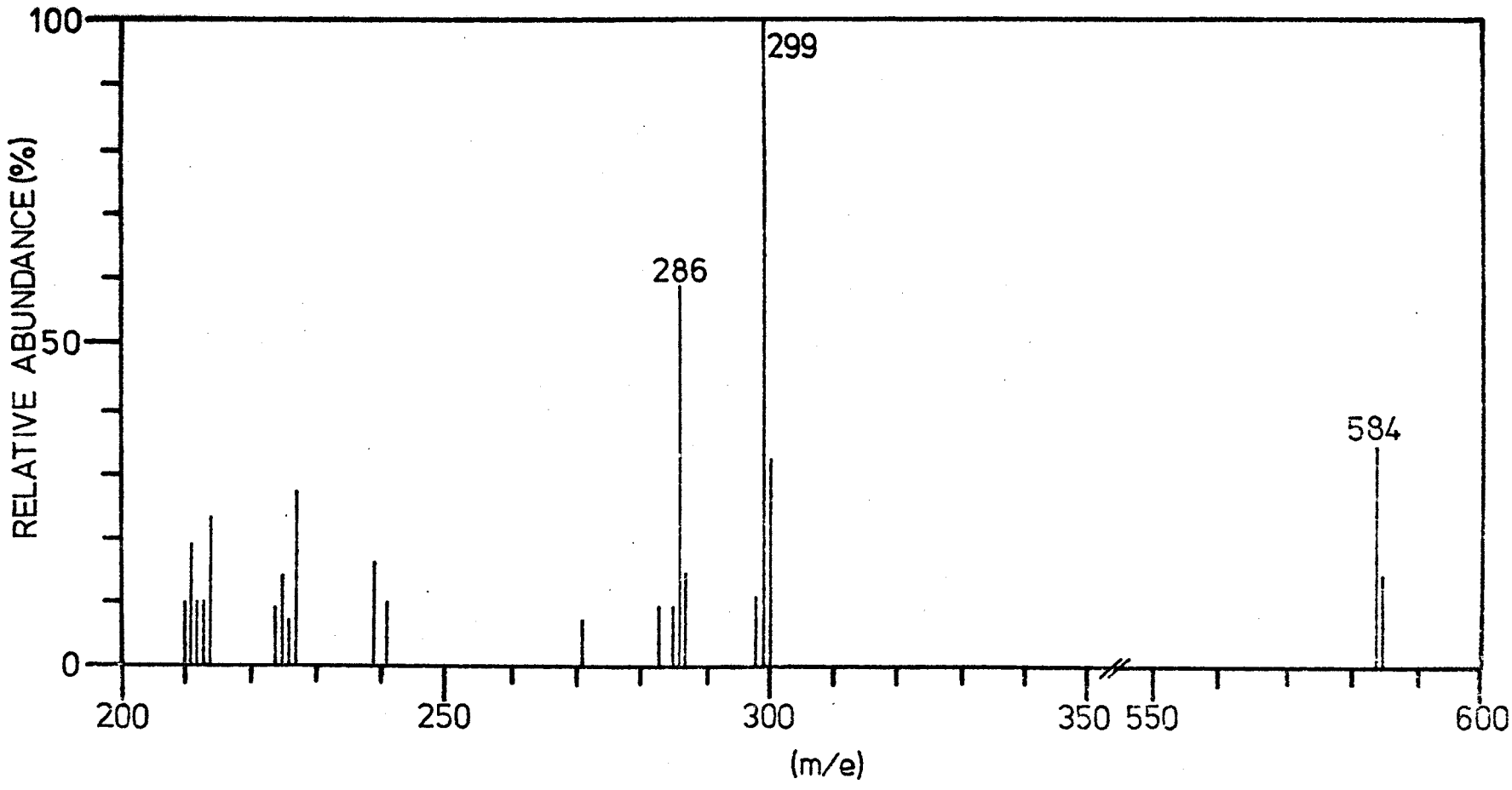


FIGURE 5.7. THE PARTIAL MASS SPECTRUM OF BILIRUBIN

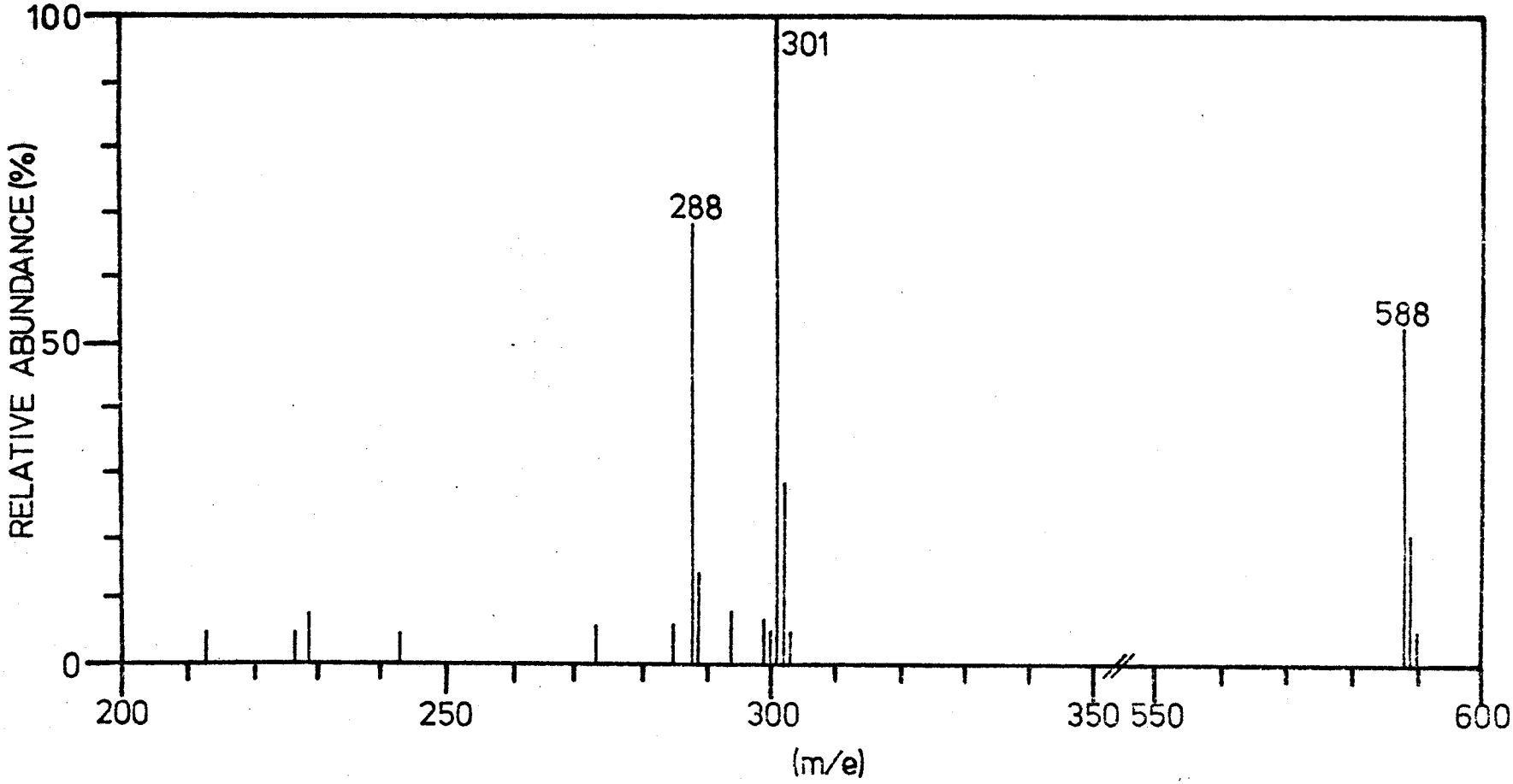


FIGURE 5.8. THE PARTIAL MASS SPECTRUM OF MESOBILIRUBIN

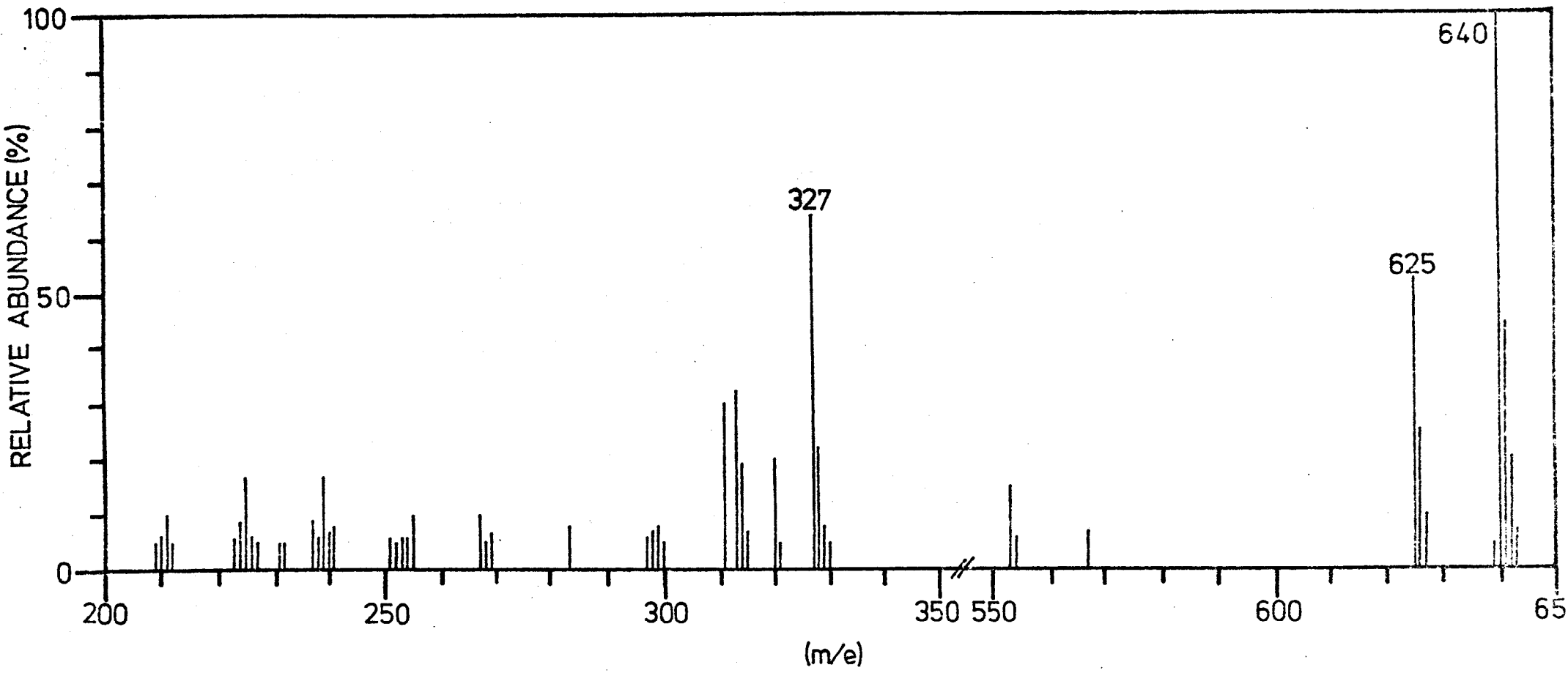
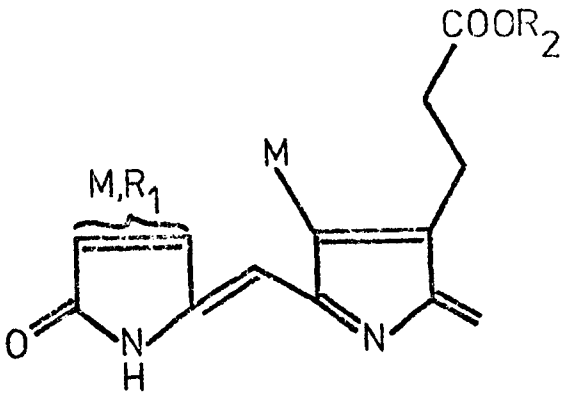
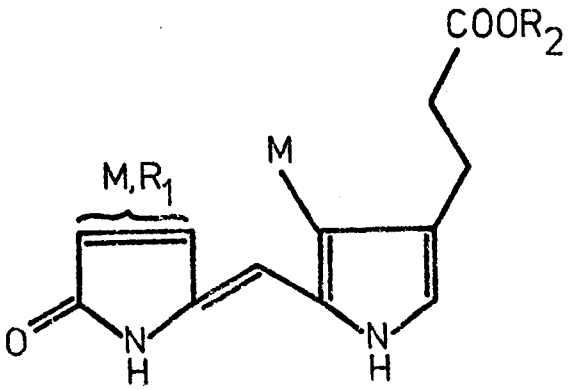


FIGURE 5.9. THE PARTIAL MASS SPECTRUM OF α,α'-DIMETHOXYBILIRUBIN DIMETHYL ESTER



(5.VI)



(5.VII)

IN EACH CASE: R_1=V, EXCEPT WHEN R_1=E FOR MESOBILIRUBIN

R_2=ALKYL, EXCEPT WHEN R_2=H FOR BILIRUBIN AND MESOBILIRUBIN

(except for the dibenzyl ester) arise from cleavage occurring at the central methylene bridge. For both of these ions, two alternative structures may be written depending on which way the cleavage occurs and there is no reason to suppose that one may be favoured rather than the other. In the case of the dibenzyl ester the molecule fragments to give one principal ion only, corresponding to (5.VII.). The remaining minor fragments in every case may be derived by side chain cleavages of these two main fragments and the cracking patterns below m/e 200 are very similar, as one might expect, to that obtained for bilirubin.[162] The cracking pattern of α,α^{1} dimethoxybilirubin dimethyl ester is very similar to those already discussed except that the parent ion is also the base peak and the two half - molecule fragments are much lower in intensity. This behaviour may be the result of this compound being a bislactim.

The ^{1}H n.m.r. spectra of the diethyl, diisopropyl and dibenzyl esters recorded in deuterochloroform (Figures 5.10 to 5.12) resemble that of the dimethyl ester already published,[88] except for the resonances of the esterifying alkyl groups. Thus, the diethyl ester shows a quartet at 5.86τ and a triplet at 8.74τ (6H) due to the ethyl groups, the diisopropyl ester shows a septet at 4.98τ (2H) and a doublet at 8.77τ (12H) due to the isopropyl groups, while the dibenzyl ester shows singlets at 2.70τ (10H) and 4.89τ (4H) due to the benzyl groups. The spectrum of the dimethyl ester in $^{2}H_{6}$ -DMSO differs from that in deuterochloroform,[88] the major differences being observed in the resonances of the C- methyl groups (v.infra). These changes are also observed for the previously unknown esters prepared in this work.

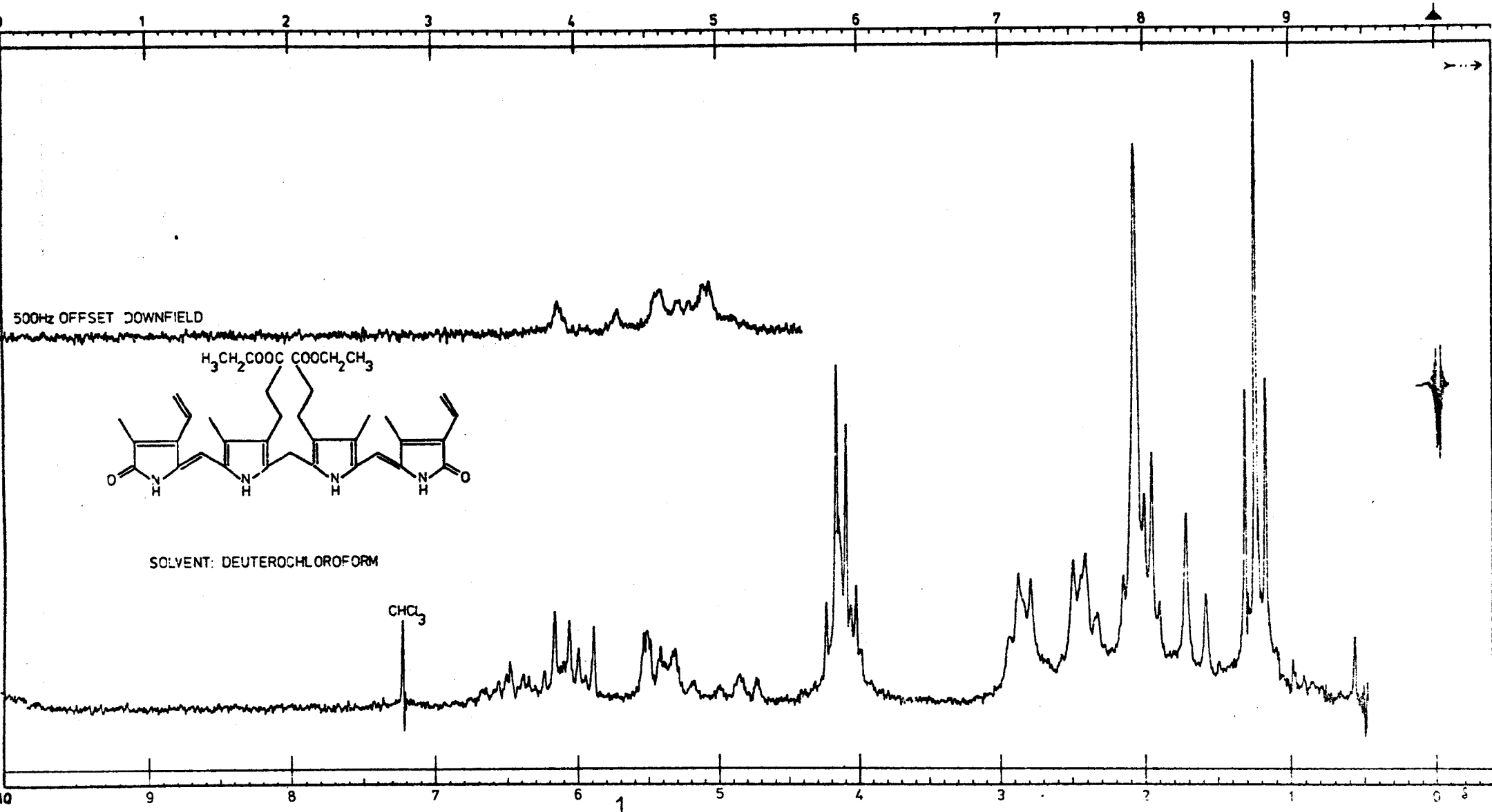


FIGURE 5.10. THE 100MHz ^{1}H N.M.R. SPECTRUM OF BILIRUBIN DIETHYL ESTER

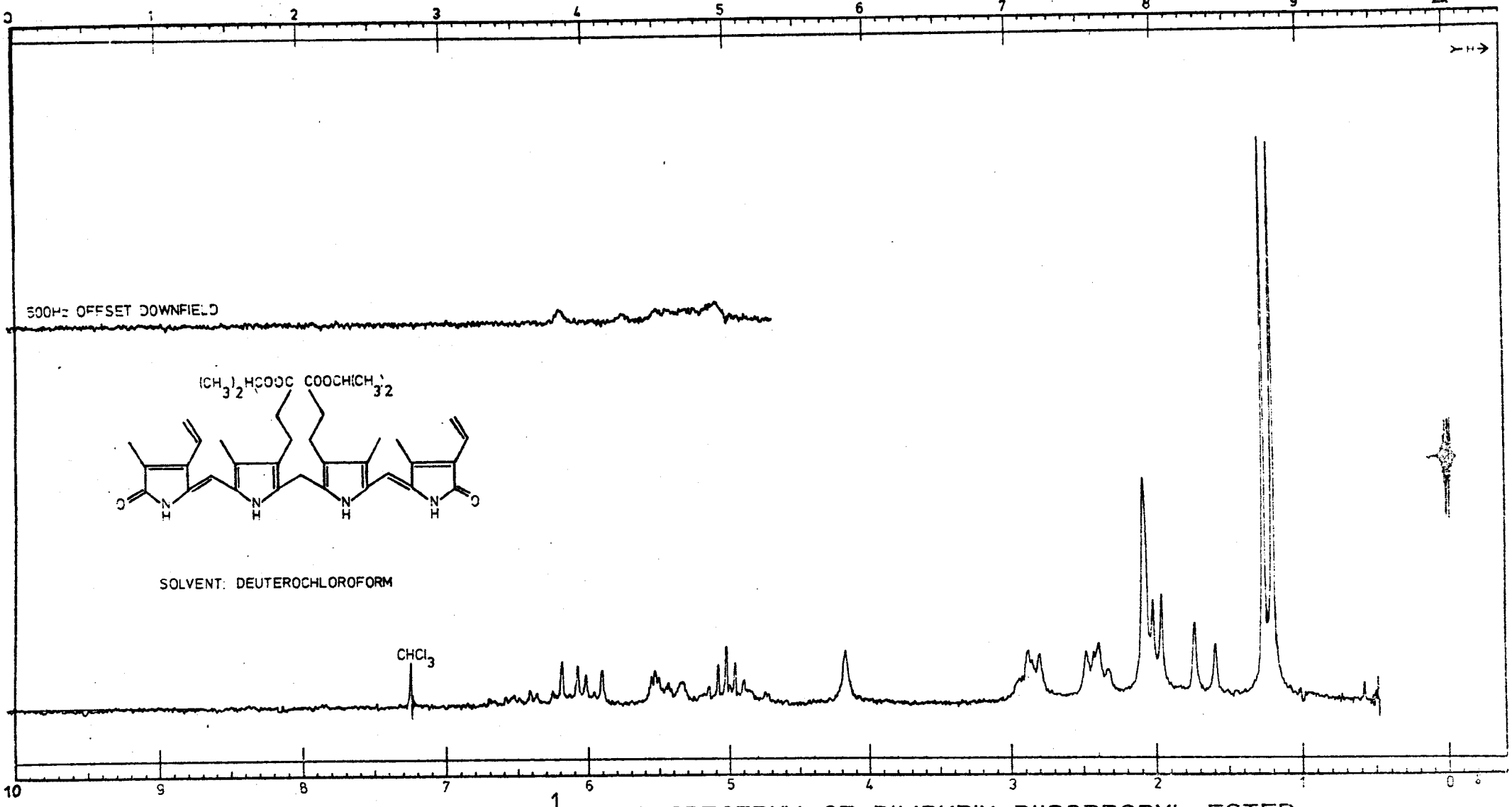


FIGURE 5.11. THE 100MHz ^{1}H N.M.R. SPECTRUM OF BILIRUBIN DIISOPROPYL ESTER

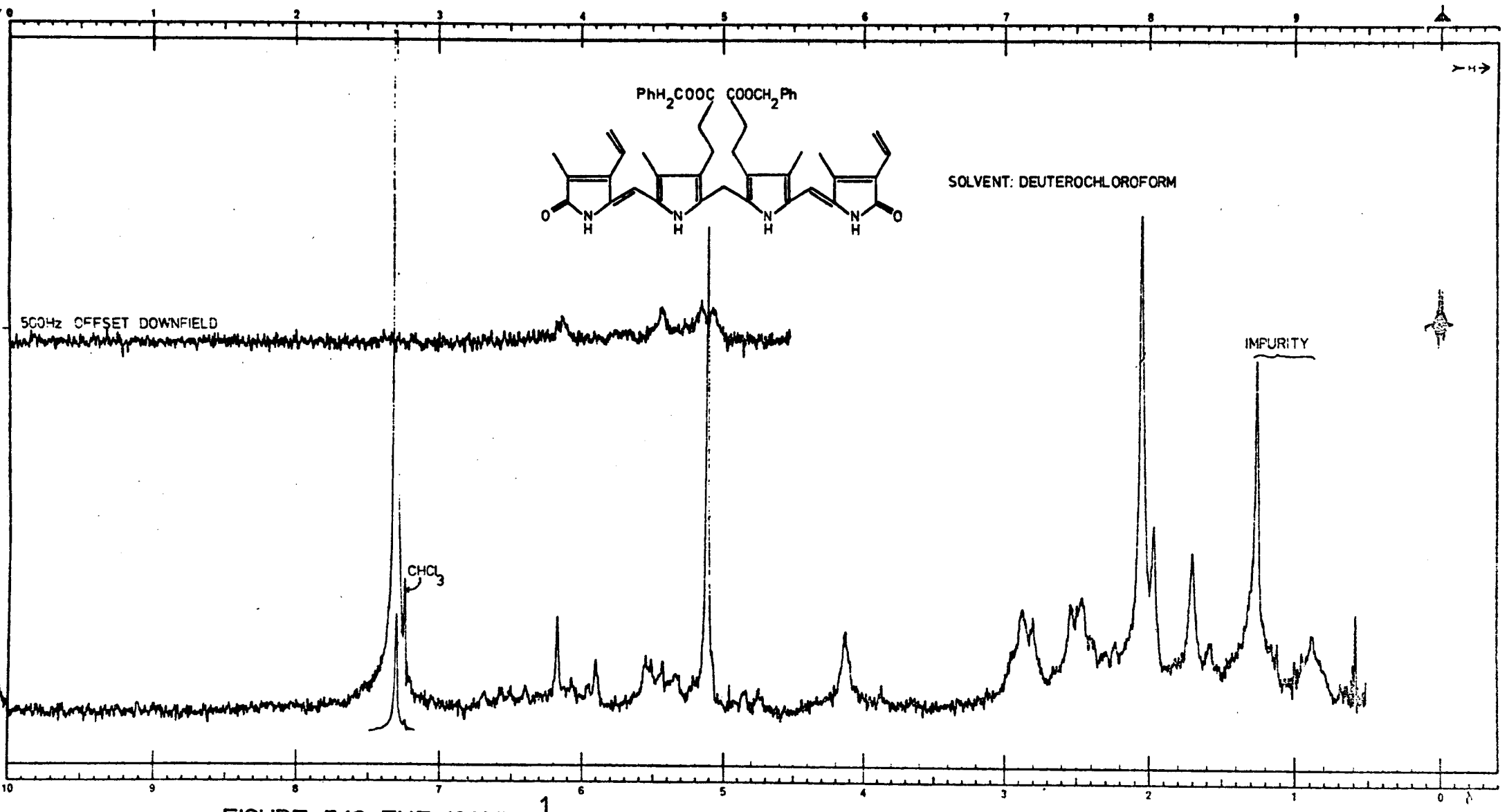


FIGURE 5.12. THE 100MHz ^{1}H N.M.R. SPECTRUM OF BILIRUBIN DIBENZYL ESTER

5.2.2. Tautomerism and Solvent Effects.

It has been shown [88] and confirmed in this work that bilirubin exists as the bislactam tautomer and, a priori, a change to the bislactim tautomer is not expected on methylation of bilirubin to the dimethyl ester, since the carboxyl groups are not conjugated with the dipyrrylmethene chromophores. Since the positions of the absorption maxima of chloroform solutions of bilirubin dimethyl ester (405nm) and bilirubin (450nm) differ, it has been suggested that tautomerism - from the bislactim in bilirubin to the bislactam in the dimethyl ester - does occur. This change was thought to arise from loss of hydrogen bonding between the propionic acid side chains and the two terminal hydroxyl groups of the bislactim tautomer of bilirubin. [111]

The absorption maximum of the dimethyl ester in chloroform solution is nearer to that of α,α' - dimethoxybilirubin dimethyl ester (fixed in the bislactim form) than to that of bilirubin (Table5.2.) suggesting that the dimethyl ester might exist as the bislactim tautomer in this solvent. This possibility has already been considered and

TABLE 5.2.

Compound	Solvent	λ_{max}
Bilirubin	$CHCl_3$	454nm
	DMSO	454nm
Bilirubin Dimethyl Ester	$CHCl_3$	400nm
	MeOH	448nm
	DMSO	453nm
α,α'-Dimethoxy-bilirubin Dimethyl Ester	$CHCl_3$	418nm
	DMSO	424nm

rejected because the i.r. spectrum of the dimethyl ester in carbon tetrachloride (in which the absorption maximum occurs at 400nm) shows no hydroxyl groups to be present.[88] In polar solvents, like DMSO, strong aggregation between solvent and solute molecules is thought to account for the bathochromic shift to about 450nm.[88]

The published ^{1}H n.m.r. spectrum of bilirubin dimethyl ester in deuterochloroform[88] shows five resonances, at 8.25τ, 8.04τ, 7.97τ, 7.94τ, and 7.92τ, said to arise from the C-methyl groups of various molecular species present, although the nature of these is not described. If the monomer only were present, then three resonances only should be observed in the ratio 2 : 1 : 1, assuming, for reasons given in the Appendix, that the C-methyl groups of the two pyrrole rings will resonate with the same chemical shifts. In $^{2}H_{6}$ -DMSO however, the spectrum differs from that observed in deuterochloroform. The C-methyl protons now resonate as four singlets in the ratio 1 : 1 : 1 : 1 with chemical shifts similar to those observed for bilirubin (Figure 2.3.) and is interpreted as indicating the presence of a single molecular species.[88]

In this work, the deuterochloroform spectrum of a sample of the dimethyl ester prepared by the triazene method shows five similar high field resonances, but at 8.42τ, 8.28τ, 8.04τ, 7.98τ, and 7.92τ (Figure 5.13.). The spectrum of the same sample recorded in $^{2}H_{6}$ -DMSO shows the expected changes in the chemical shifts of the C-methyl groups (Figure 5.14.). If the signal due to the central methylene bridge protons corresponds to two protons, then the resonances due to the C-methyl groups correspond to rather more than the expected twelve protons. Samples of the dimethyl ester, prepared using different commercial samples of bilirubin, give ^{1}H n.m.r. spectra in which the ratio of the intensities of the

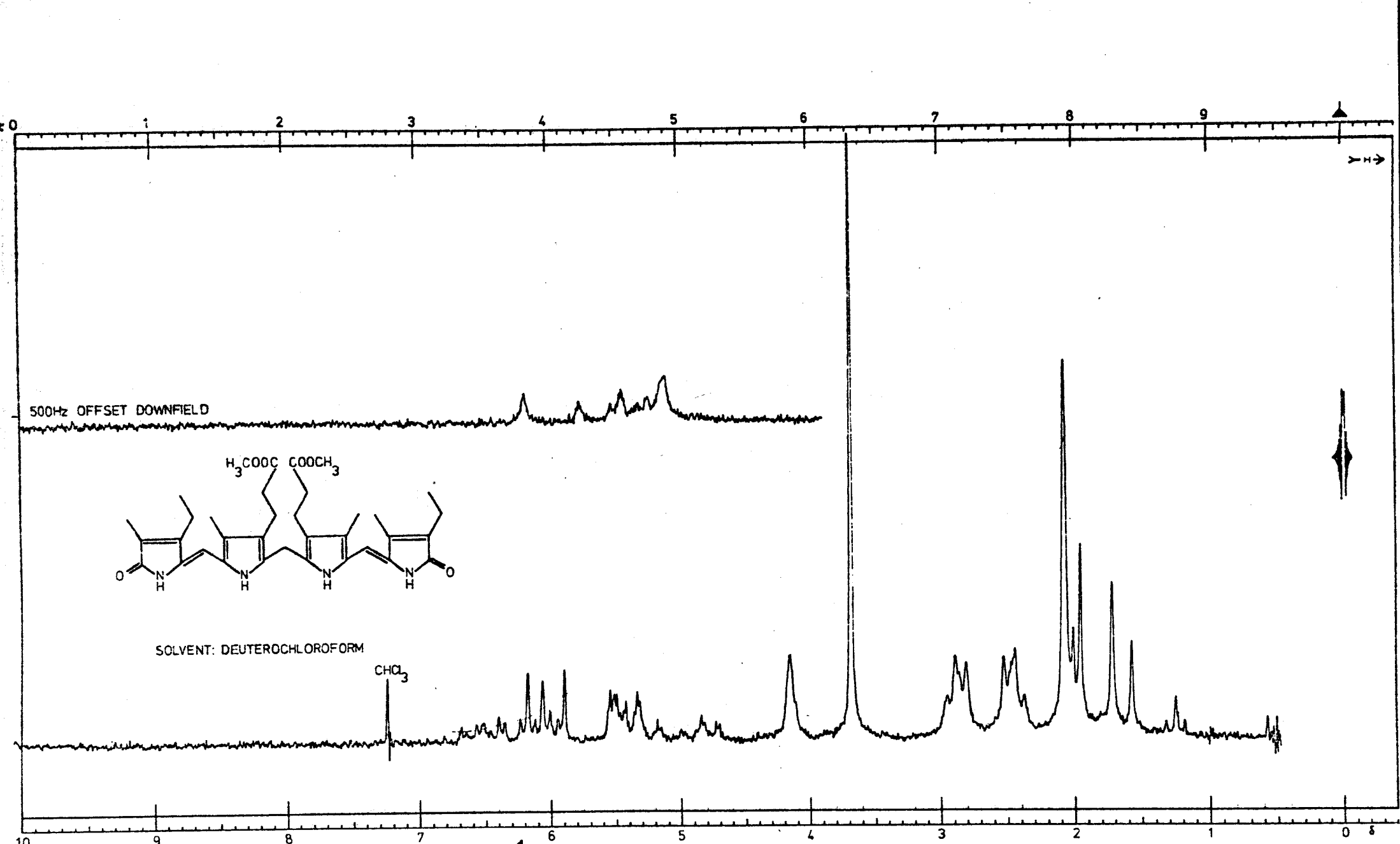


FIGURE 5.13. THE 100MHz ^{1}H N.M.R. SPECTRUM OF BILIRUBIN DIMETHYL ESTER

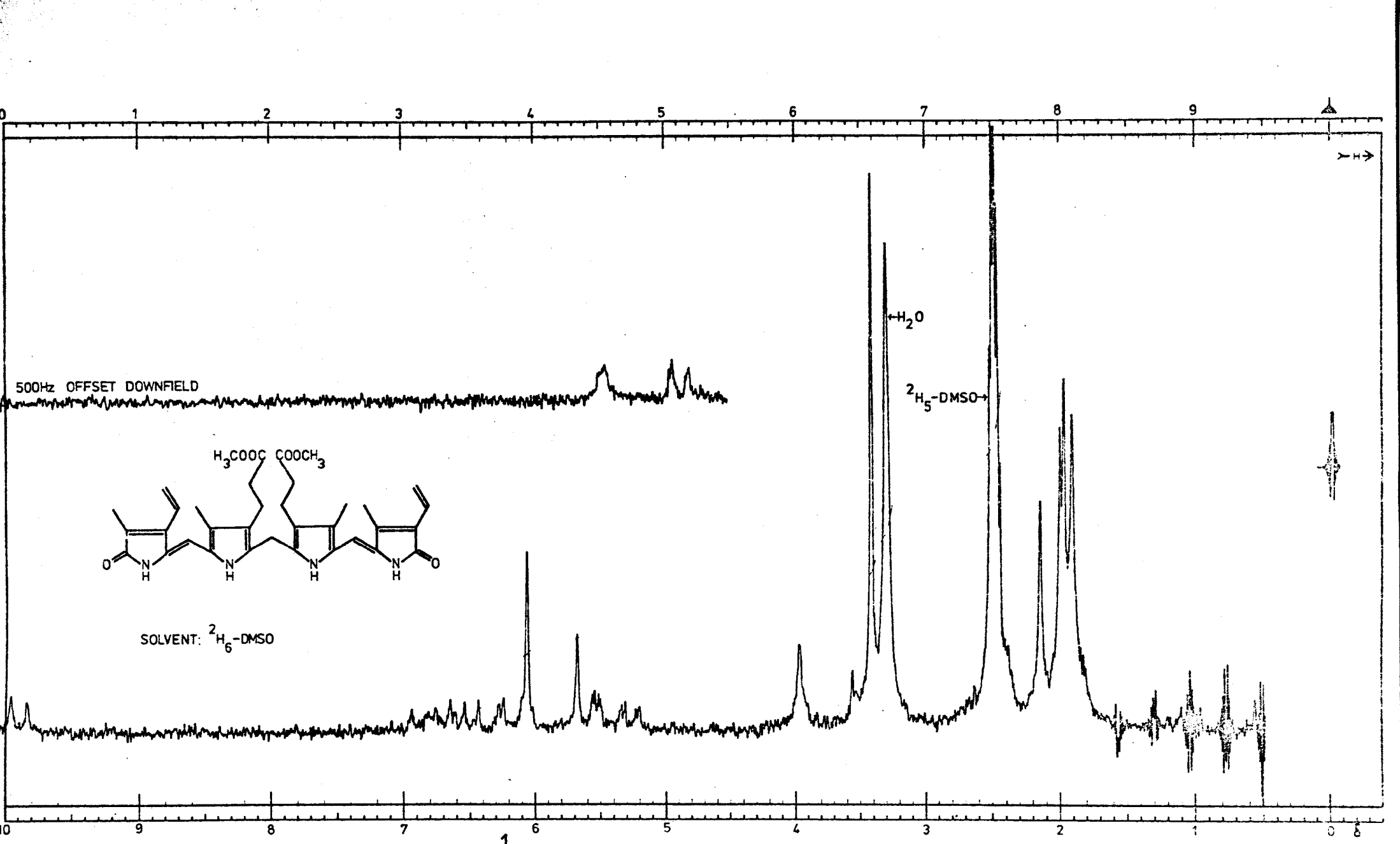
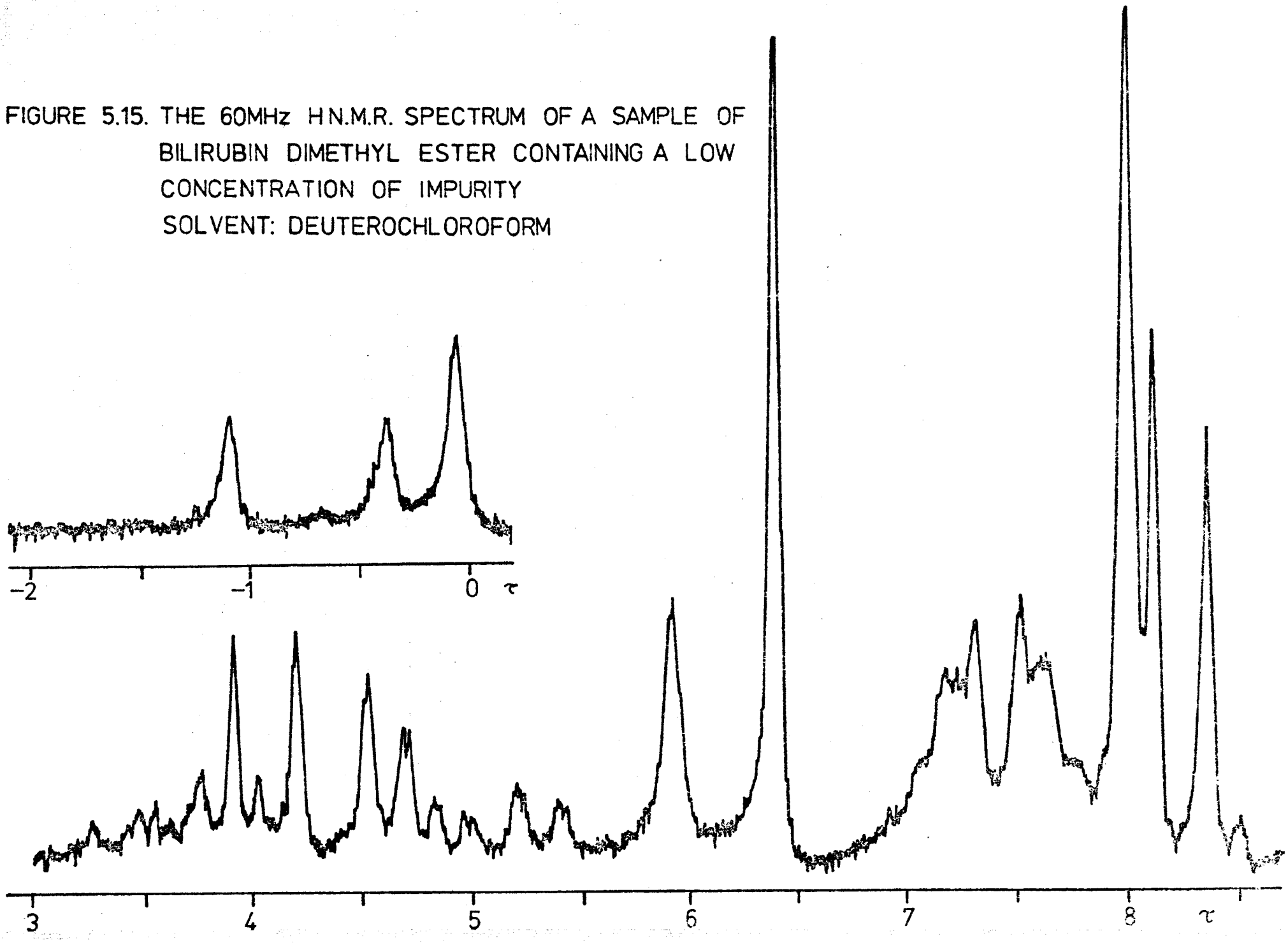


FIGURE 5.14. THE 100MHz ^{1}H N.M.R. SPECTRUM OF BILIRUBIN DIMETHYL ESTER

resonances at 8.42τ and 7.98τ are the same for all the samples of bilirubin used but in which the ratio of these resonances to the remaining high field C-methyl resonances varies considerably. Furthermore, for a sample of the dimethyl ester in which the intensities of these two resonances are negligible, the ^{1}H n.m.r. spectrum in deuterochloroform is invariant over the concentration range 0.025M to 0.15M, indicating that only one molecular species is present. Even though the concentration range examined was varied by less than ten - fold, if several molecular species were formed their presence should have been detected by the presence of extra resonances, especially at the higher concentrations, since the published spectrum was recorded with an ester concentration of 0.05M.[88] The resonances observed at 8.42τ and 7.98τ are therefore ascribed to bilirubin - like impurities although their exact nature must remain obscure. The presence of the isomers bilirubin IIIα and XIIIα should not give rise to any new C-methyl resonances as already described.[88] The presence of bilirubin IXβ , IXγ and IXδ is considered unlikely, for while these isomers could give rise to extra resonances, their natural abundance[81 - 83] is too low to account for the peak intensities observed. Concomitant with the decrease in the amount of impurity, the low field region of the spectrum, showing N - H resonances, simplifies, until, with a low concentration of impurity, peaks at −0.15τ (2H), −0.48τ (1H) and −1.20τ (1H) only are observed (Figure 5.15.) as expected for the monomeric dimethyl ester.

These results suggest that one species only is present in deuterochloroform (and therefore chloroform) so the existence of several molecular species of the ester in non - polar solvents seems doubtful. This leaves two possibilities to account for the

FIGURE 5.15. THE 60MHz H N.M.R. SPECTRUM OF A SAMPLE OF BILIRUBIN DIMETHYL ESTER CONTAINING A LOW CONCENTRATION OF IMPURITY
SOLVENT: DEUTEROCHLOROFORM



difference in the absorption spectra and these are a) keto - enol tautomerism despite the i.r. evidence described above [88] and b) solvent effects.

In a manner similar to that described to distinguish between N-H and O-H resonances in the ^{1}H n.m.r. spectrum of bilirubin (Chapter 2.), the bislactam tautomer was found to be the only tautomer present in both $^{2}H_{6}$ -DMSO and deuterochloroform. Thus in each case, all the low field signals collapse on the addition of deuterium oxide while the addition of water has no effect.

If a keto - enol tautomerism, monitored by n.m.r. spectroscopy, is disturbed by any physical means, then concomitant with the decrease in intensity of resonances due to one tautomer, there should be an increase in the intensity of resonances due to the other tautomer, unless the tautomerism is so rapid that only the time averaged signals are observed. One such method is to vary the temperature, and the high field regions of the spectra obtained from a deuterochloroform solution of the dimethyl ester at various temperatures are shown in Figure 5.16. The main effects observed are, that on reducing the temperature, the resonance at 8.28 τ moves downfield rapidly until at -60°C it coalesces with the resonance at 8.04 τ . The singlet, due to the pyrrole C-methyl groups, observed at 7.92 τ at ambient temperature is just resolved at -60°C into two overlapping singlets at 7.86 τ and 7.92 τ . On raising the temperature from -60°C to ambient, the chemical shifts observed initially are regained. Therefore, these observations are not of a keto - enol tautomerism and may be explained on the basis that at ambient temperature there is free rotation about the bonds between the central methylene bridge carbon atom and the dipyrrylmethene subunits (5.VIII.), with, on average, the two halves of the molecule

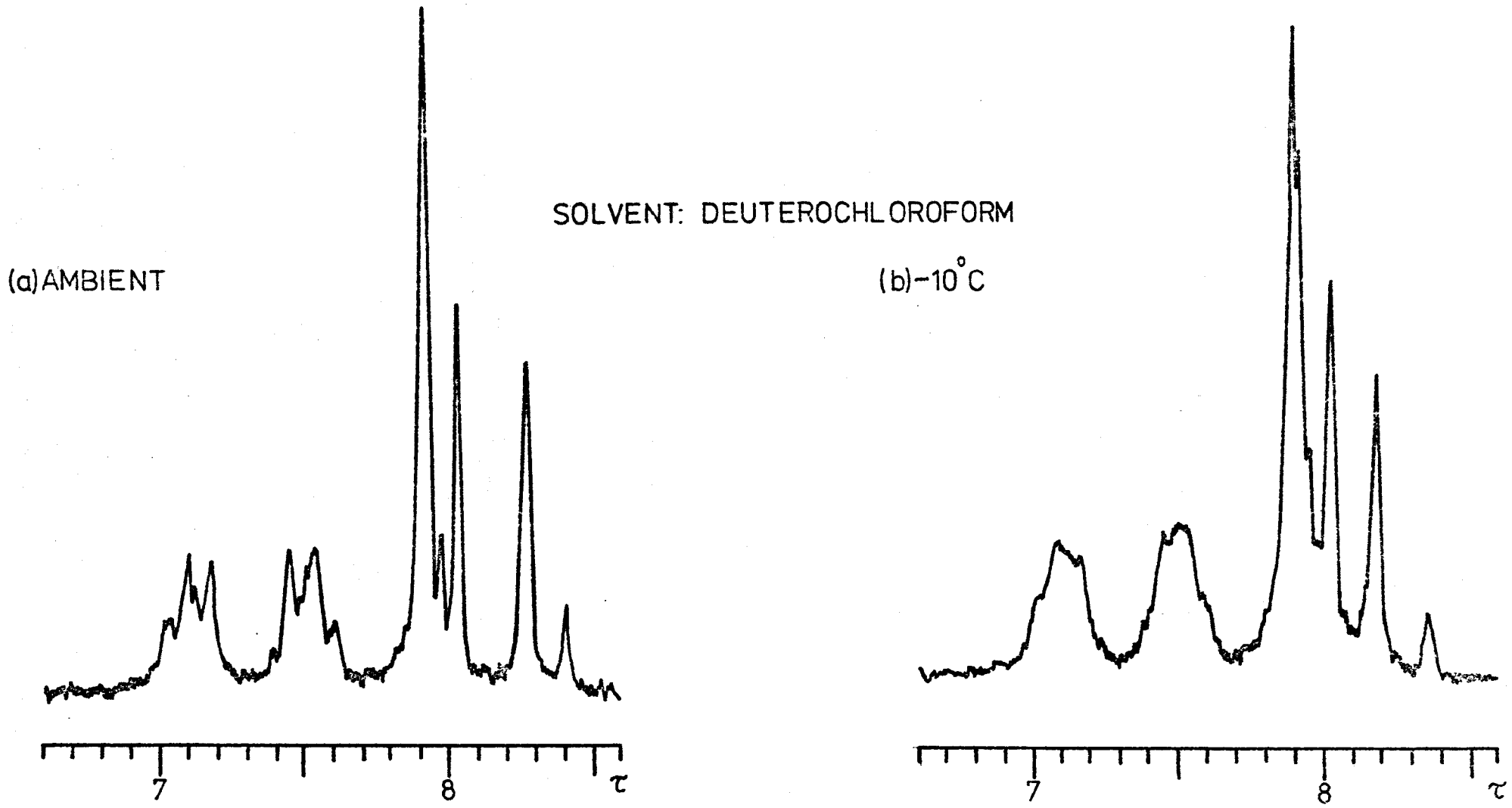


FIGURE 5.16. THE HIGH FIELD REGIONS OF THE 100MHz ^{1}H N.M.R. SPECTRA OF BILIRUBIN DIMETHYL ESTER RECORDED AT VARIOUS TEMPERATURES

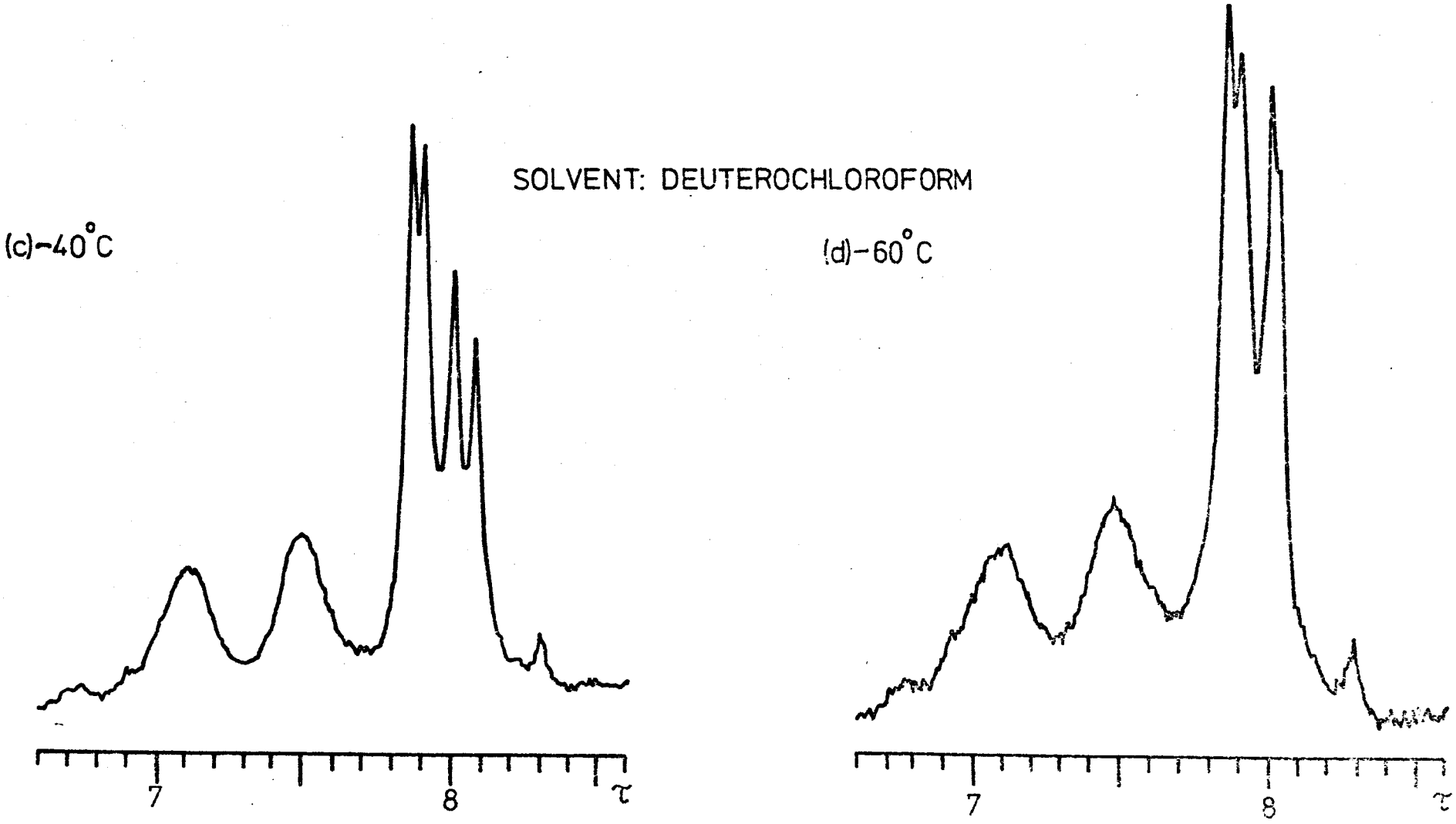
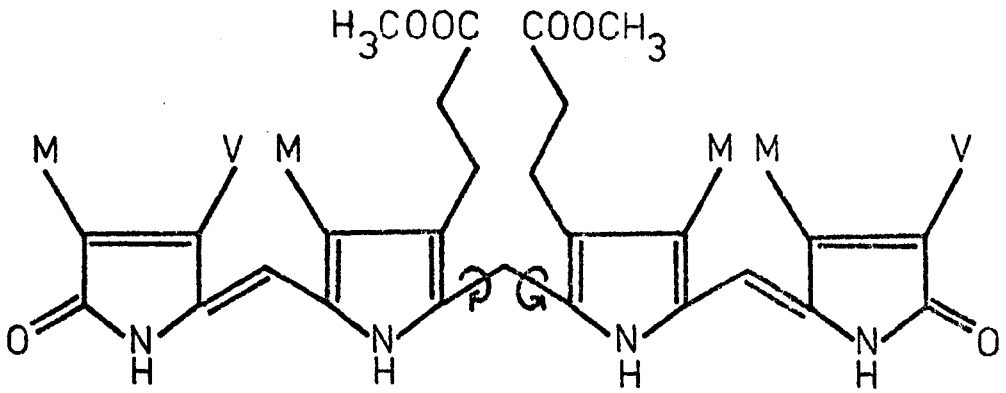


FIGURE 5.16 CONTINUED

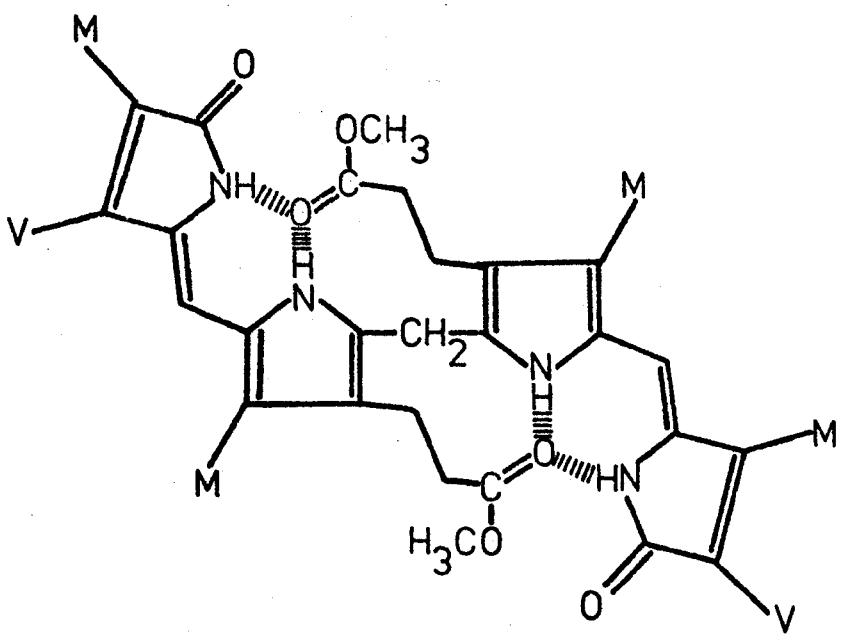
experiencing the same environment. On lowering the temperature, these rotations become progressively more restricted with the molecules being "frozen out" probably in form (5. IX.) rather than in form (5.VIII.). The form (5.IX.) is preferred because of the possibility of hydrogen bonding in a manner similar to that already proposed for bilirubin (Chapter 2.).

A keto - enol equilibrium can be disturbed by altering the dielectric constant of the medium by the addition of another solvent. Thus, the progressive addition of DMSO to a solution of bilirubin dimethyl ester in chloroform causes a shift in the position of the absorption maximum from 400nm to 450nm, similar to that observed on the addition of methanol.[111] The examination by ^{1}H n.m.r. spectroscopy of this process (using deuterated solvents) causes changes in the C-methyl resonances as shown in Figure 5.17. These changes are similar to those observed using variable temperature techniques described above and also are not due to a keto - enol tautomerism.

Therefore, the observed differences in the absorption spectra of bilirubin dimethyl ester are most likely due to solvent effects which cause the molecule to adopt different conformations in the two solvents.[64] Solutions of bilirubin in chloroform and DMSO have the same absorption maxima (Table 5.2.). These two solvents differ widely in polarity, so the observed absorption maxima must be a reflection of the strong intramolecularly hydrogen bonded structure adopted by bilirubin (Chapter 2.) in both solvents rather than of any solvation effects of the two solvents. In theory, bilirubin in chloroform should absorb at 420nm [109] so the observation of the absorption maximum at higher wavelength may be due to interactions between the dipyrrylmethene chromophores. In the


H3COOC
COOCH3
M
V
M
M
M
V
P
G
O
N
H
N
H
N
H
N
H
O

(5.VIII)


M
O
OCH3
NH
O
C
V
M
H
N
CH2
N
H
M
M
O
HN
C
H3CO
O
V

(5.IX)

FIGURE 5.17. THE HIGH FIELD REGIONS OF THE 60MHz ^{1}H N.M.R. SPECTRA OF BILIRUBIN DIMETHYL ESTER IN DEUTEROCHLOROFORM AFTER THE ADDITION OF THE STATED VOLUMES OF $^{2}H_{6}$-DMSO

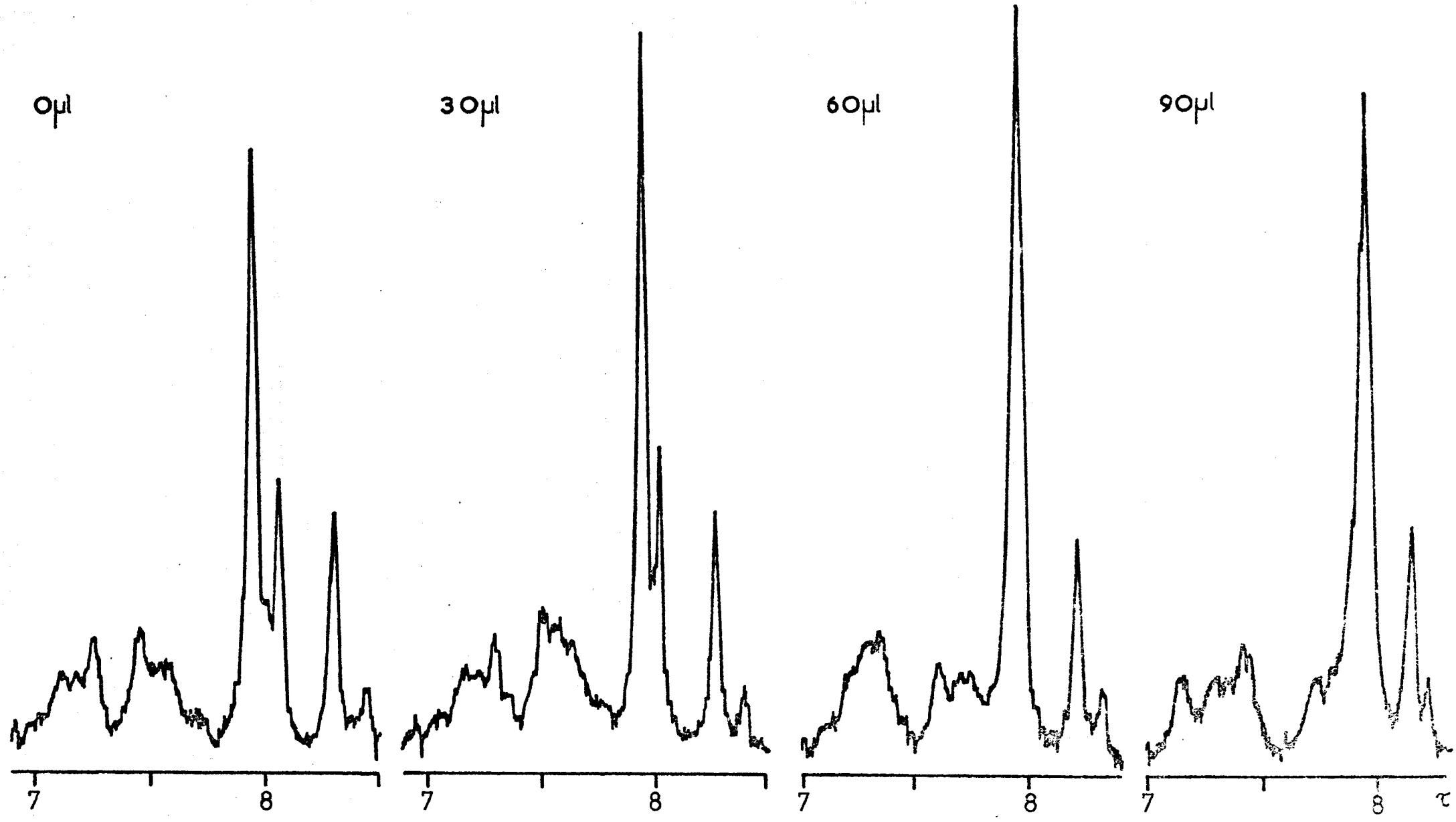


FIGURE 5.17.

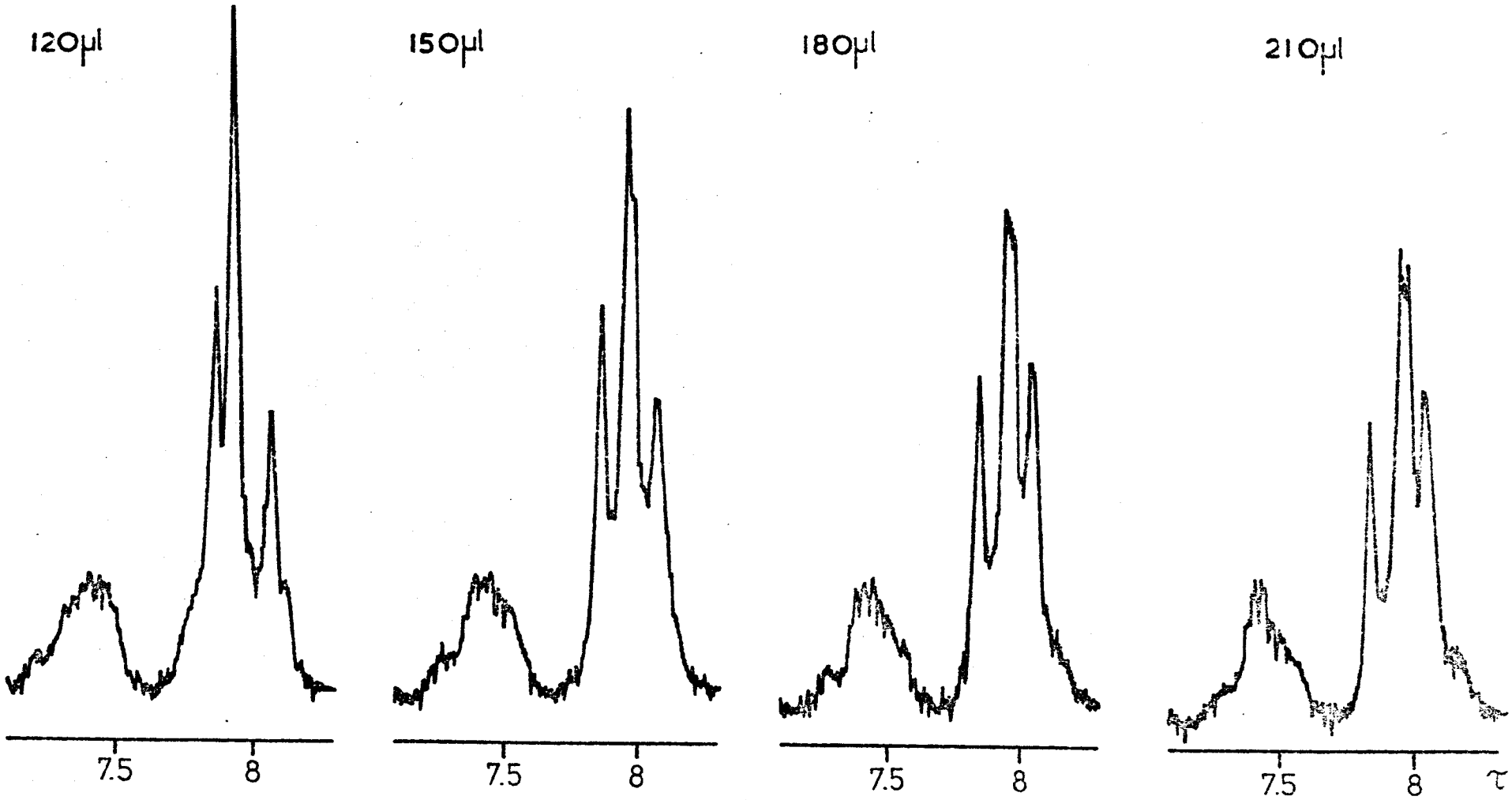


FIGURE 5.17. CONTINUED

case of the dimethyl ester however, the differences in solvation properties of the two solvents become significant in the absence of any intramolecular hydrogen bonding at ambient temperatures. Hydrogen bonding between the ester and DMSO solvent molecules, particularly at the lactam residues, could force the molecule to adopt a conformation similar to that of bilirubin, thus allowing the same interactions between the dipyrrylmethene subunits leading to the same absorption maximum. The interaction between solute and solvent in chloroform solution will be much weaker than in DMSO, therefore the dimethyl ester may adopt a random configuration with less interactions between the chromophores leading to an absorption maximum at shorter wavelength.

α, α' - Dimethoxybilirubin dimethyl ester has solvent independent absorption maxima (Table 5.2.) and the ^1H n.m.r. spectra recorded in deuterochloroform and $^2H_6$ - DMSO are very similar (Figures 5.18. and 5.19. respectively). The differences between the C-methyl resonances are minimal, with a singlet being observed at 8.06τ (3H) in each solvent. The singlet at 7.86τ (9H) in deuterochloroform is resolved into three singlets (two overlapping) at 7.84τ , 7.89τ and 7.91τ in $^2H_6$ - DMSO. The absence of any solvent dependency here must result from the non - polar nature of this compound compared with bilirubin and its dimethyl ester. There is no hydrogen bonding and the chloroform and the DMSO molecules interact with the α, α' - dimethoxybilirubin dimethyl ester to an equal degree, again leading to the adoption, by the solute, of a random conformation leading to similar absorption maxima in each solvent.

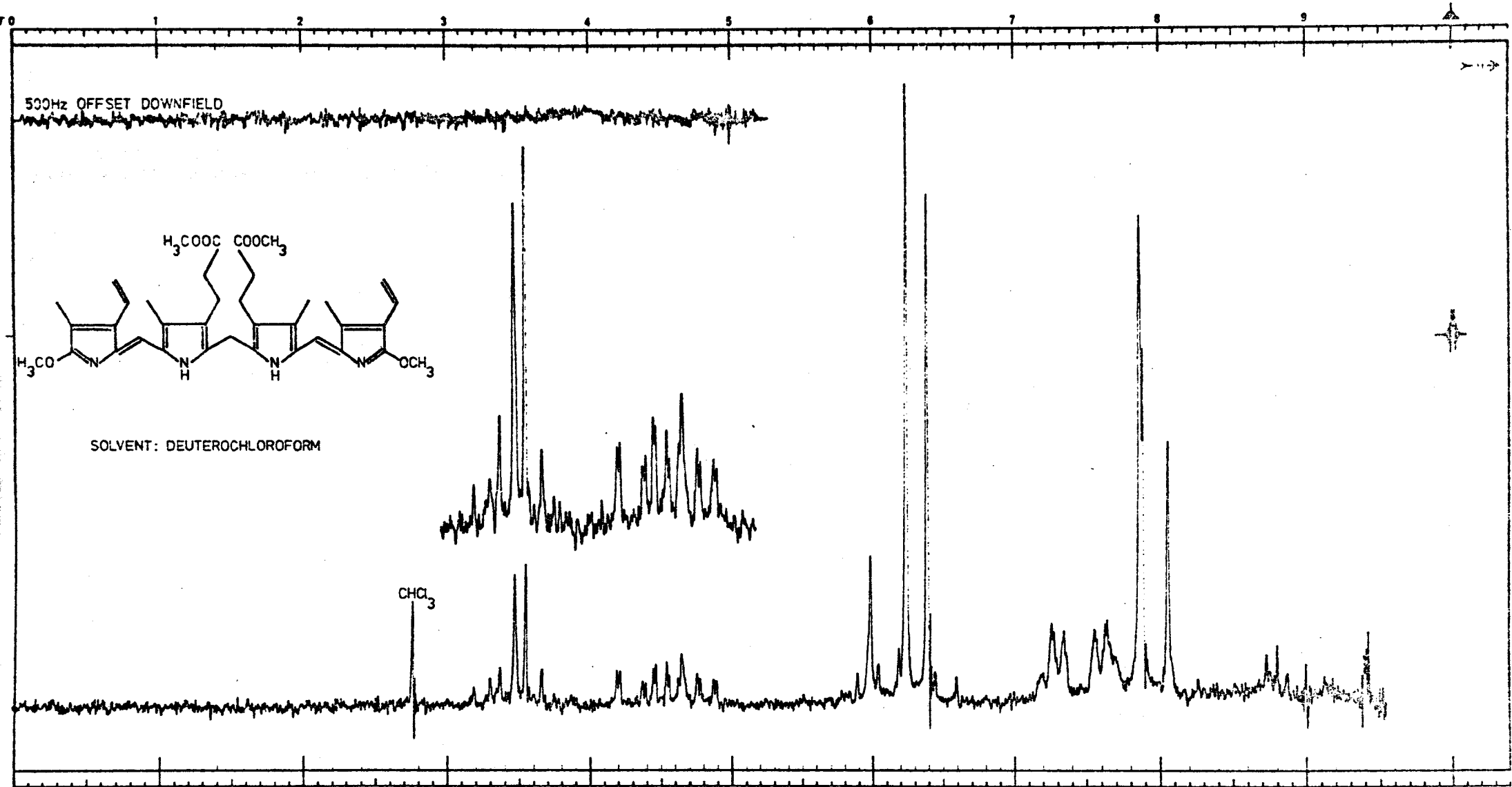


FIGURE 5.18. THE 100MHz ^{1}H N.M.R. SPECTRUM OF α,α'-DIMETHOXYBILIRUBIN DIMETHYL ESTER

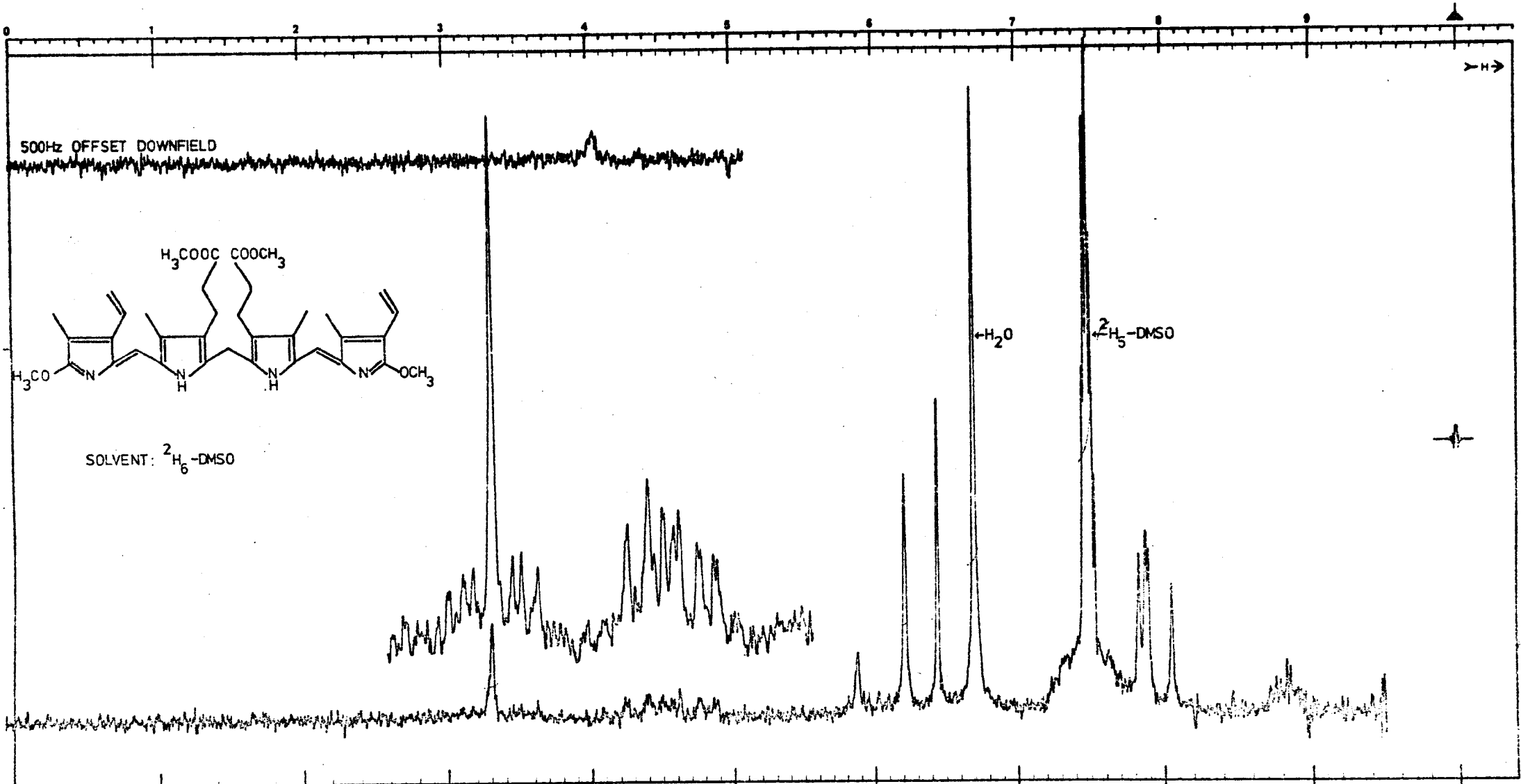


FIGURE 5.19. THE 100MHz H.N.M.R. SPECTRUM OF α,α'-DIMETHOXYBILIRUBIN DIMETHYL ESTER

5.3. Experimental

Materials.

Bilirubin was obtained from BDH Chemicals Ltd., Fluka A.G. and Sigma Chemical Company and was used without further purification.

1 - Alkyl -3-p- Tolyltriazenes were obtained from Willow Brook Laboratories, Inc. and were used without further purification.

Petroleum Ether (B.p. 60 - 80°C), Chloroform and Methanol were distilled before use.

Methods.

Bilirubin Dimethyl Ester.- Bilirubin (120mg, 0.2mmole) was suspended in chloroform (120ml) and 1 - methyl -3-p- tolyltriazene (150mg, 1mmole) added. The mixture was stirred, in the dark, at room temperature for 12 hours. During this time, the reaction mixture darkened and the bilirubin dissolved. Excess triazene was decomposed by shaking the chloroform solution with dilute HCl (1N, 100ml). The organic layer was then shaken with saturated aqueous sodium bicarbonate (100ml), with water (2 x 100ml) and finally dried over anhydrous Na_2SO_4. After filtration and removal of the solvent *in vacuo* at 40°C, the residue was applied to a column (45cm x 1.5cm o.d.) of neutral alumina. Elution with chloroform - petroleum ether (b.p. 60 - 80°C) (1 : 1, v/v) followed by pure chloroform removed most of the impurities. The bilirubin dimethyl ester was eluted with chloroform - methanol (20 : 1, v/v), and was finally purified by PLC on silica gel, developing the chromatogram with benzene - ethanol (25 : 2, v/v).[164] The orange band, R_F = 0.7, was removed from the plate and the ester eluted from the adsorbent with chloroform. Evaporation of the chloroform *in vacuo* followed by crystallisation, from methanol, gave bilirubin dimethyl ester (56.5mg, 46%), m.p. 198 - 200°C. Lit.,[111] 200 - 205°C.

i.r. (Nujol mull) ν_{max} : 3340 (br, m), 1736 (m) $cm.^{-1}$

u.v. (Chloroform) : λ_{max} 400nm (ε 55,600). Lit.,[88] λ_{max} 405nm (ε 55,800).

^1H n.m.r. (100MHz, $CDCl_3$) : See Table 5.3.

Analysis (B) : Found C,68.61; H, 7.09; N, 8.94 %. $C_{35}H_{40}N_4O_6$ requires C,68.65; H, 6.60; N, 9.15 %.

Mass Spectrum (PCMU) : The cracking pattern is shown in Figure 5.3, and the accurate mass of the molecular ion in Table 5.1.

Bilirubin Diethyl Ester.- This was synthesised from bilirubin (120mg, 0.2mmole) and 1 - ethyl -3-p- tolyltriazene (325mg, 2.0mmole) as described above. Crystallisation, from hexane, of the product obtained from PLC gave bilirubin diethyl ester (13mg, 10%), m.p. 197-198°C.

i.r. (Nujol mull) ν_{max} : 3340 (br, m), 1753 (s) $cm.^{-1}$

u.v. (Chloroform) : λ_{max} 400nm (ε 55,100).

^1H n.m.r. (100MHz, $CDCl_3$) : See Table 5.3.

Analysis (S) : Found C, 69.37; H, 6.88; N, 8.29%. $C_{37}H_{44}N_4O_6$ requires C, 69.34; H, 6.93; N, 8.74%.

Mass Spectrum (PCMU) : The cracking pattern is shown in Figure 5.4, and the accurate mass of the molecular ion in Table 5.1.

Bilirubin Diisopropyl Ester.- This was synthesised from bilirubin (120mg, 0.2mmole) and 1 -isopropyl -3-p- tolyltriazene (335mg, 2.0mmole) as described above. Crystallisation, from hexane, of the product

obtained from PLC gave bilirubin diisopropyl ester (20mg, 15%) m.p. 179 - 181°C.

i.r. (Nujol mull) ν_{max} : 3350 (br,m), 1730(s) $cm.^{-1}$

u.v. (Chloroform) : λ_{max} 400nm (ε54,200).

^1H n.m.r. (100MHz, $CDCl_3$) : See Table 5.3.

Analysis (B) : Found C, 69.63; H, 7.27; N, 8.46%.
$C_{39}H_{48}N_4O_6$ requires C, 70.02;
H, 7.24; N, 8.37%.

Mass Spectrum (PCMU) : The cracking pattern is shown in Figure 5.5, and the accurate mass of the molecular ion in Table 5.1.

Bilirubin Dibenzyl Ester.- This was synthesised from bilirubin (120mg, 0.2mmole) and 1 - benzyl -3-p- tolyltriazene (450mg, 2.0mmole) as described above. Despite repeated attempts, material obtained from PLC (23mg, 14%) could not be crystallised and an analytically pure sample of bilirubin dibenzyl ester was not obtained.

u.v. (Qualitative, Chloroform) : λ_{max} 400nm.

^1H n.m.r. (100MHz, $CDCl_3$) : See Table 5.3.

Mass Spectrum (PCMU) : The cracking pattern is shown in Figure 5.6, and the accurate mass of the molecular ion in Table 5.1.

The Reaction Between Phenylacetic Acid and 1-n-Propyl-3-p-Tolyltriazene.- To phenylacetic acid (272mg, 2.0mmole) in chloroform (50ml) was added 1-n-propyl-3-p-tolyltriazene (390mg, 2.2mmole) with stirring. Immediate evolution of nitrogen was observed and the solution was set aside, at room temperature, for 30 minutes.

Excess triazene was removed with HCl in the usual manner, the chloroform solution was dried (anhydrous Na_2SO_4) and evaporated *in vacuo*, at 40°C, to leave a yellow oil which was examined by ^1H n.m.r. spectroscopy (60MHz) without further purification. No signals corresponding to isopropyl groups could be detected in the ^1H n.m.r. spectrum of a carbon tetrachloride solution of the oil.

TABLE 5.3.

Dimethyl Ester	Diethyl Ester	Diisopropyl Ester	Dibenzyl Ester	Assignment.
		8.73(d,12H,J=7Hz)		Methyl protons of isopropyl ester groups.
	8.74(t,6 H,J=7Hz)			Methyl protons of ethyl ester groups.
8.27(s, 8.04(s, } 12H) 7.92(s,	8.27(s, 8.03(s, } 12H) 7.92(s,	8.26(s, 8.04(s, } 12H) 7.91(s,	8.30(s, 8.04(s, } 12H) 7.95(s,	Methyl group on C(2) Methyl group on C(17) Methyl groups on C(7) and C(13)
7.32 (A_2B_2system,8H)	7.34 (A_2B_2system,8H)	7.36 (A_2B_2system,8H)	7.32 (A_2B_2system,8H)	Methylene groups of propionic ester side chains on C(8) and C(12)
6.31(s,6H)				Methyl protons of methyl ester groups
	5.85*(q,J=7Hz)			Methylene protons of ethyl ester groups
5.84(s,br,2H)	*	5.82(s,br,2H)	5.86(s,br,2H)	Methylene protons on C(10)
5.28 - 3.32 (m,8H)	5.28 - 3.30 (m,8H)	5.28 - 3.30 (m,8H)	5.26 - 3.32 (m)**	Vinyl groups on C(3) and C(18) and methine protons on C(5) and C(15)
		4.98(sp,2H,J=7Hz)		Methine protons of isopropyl ester groups
			4.89 (s)**	Methylene protons of benzyl ester groups.
			2.70(s,10H)	Phenyl ring protons of benzyl ester groups.
-0.10 - -1.18 (m,br,4H)	-0.10 - -1.16 (m,br,4H)	-0.08 - -1.18 (m,br,4H)	-0.07 - -1.14 (m,br,4H)	Protons on ring nitrogen atoms.

* The quartet and methylene singlet overlap; the total integral corresponds to 6H.

** Total integral corresponds to 12H.

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APPENDIX.

INTERPRETATION OF THE ^{1}H N.M.R. SPECTRUM

OF BILIRUBIN.

INTERPRETATION OF THE ^{1}H N.M.R. SPECTRUM OF BILIRUBIN.

The spectrum of bilirubin at 100MHz in $^{2}H_{6}$ -DMSO (Figure A.1.) is discussed in conjunction with that of mesobilirubin under the same conditions (Figure A.2.).

Examination of the structural formula of bilirubin suggests that the C(7) and C(13) methyl groups will resonate at approximately the same frequency, while the chemical shifts of the C(2) and C(17) methyl groups will differ because of the relative positions of these groups with respect to the lactam carbonyl groups and the C(3) and C(18) vinyl groups.[113b] Accordingly, the resonances at 7.96τ and 8.00τ are assigned, though in no special order, to the C(7) and C(17) methyl groups.

In methacrolein (A.I.) and crotonaldehyde (A.II.), model compounds for the A and D lactam rings respectively, the methyl resonances appear at 8.22τ and 8.09τ respectively.[165] The case of bilirubin is complicated by the presence of the vinyl groups, but it is assumed that any inductive and spatial effects of the vinyl groups on the methyl groups are the same in each case, so that by direct comparison the resonances at 8.07τ and 7.82τ are assigned to the C(2) and C(17) methyl groups respectively.

If, in mesobilirubin, it is assumed that the methyl groups resonate in the same relative positions as in bilirubin, the C(2) and C(17) methyl group resonances apparently move upfield, by roughly equal amounts, to 8.22τ and 7.94τ respectively, while the C(7) and C(13) methyl resonances remain essentially unchanged at 7.99τ and 8.01τ . Thus, the assumptions made above concerning the effects of the vinyl groups seem valid. The two model compounds resemble more closely the lactam rings of mesobilirubin than those of bilirubin, so the methyl resonance at 8.22τ in methacrolein (≡ ring A)

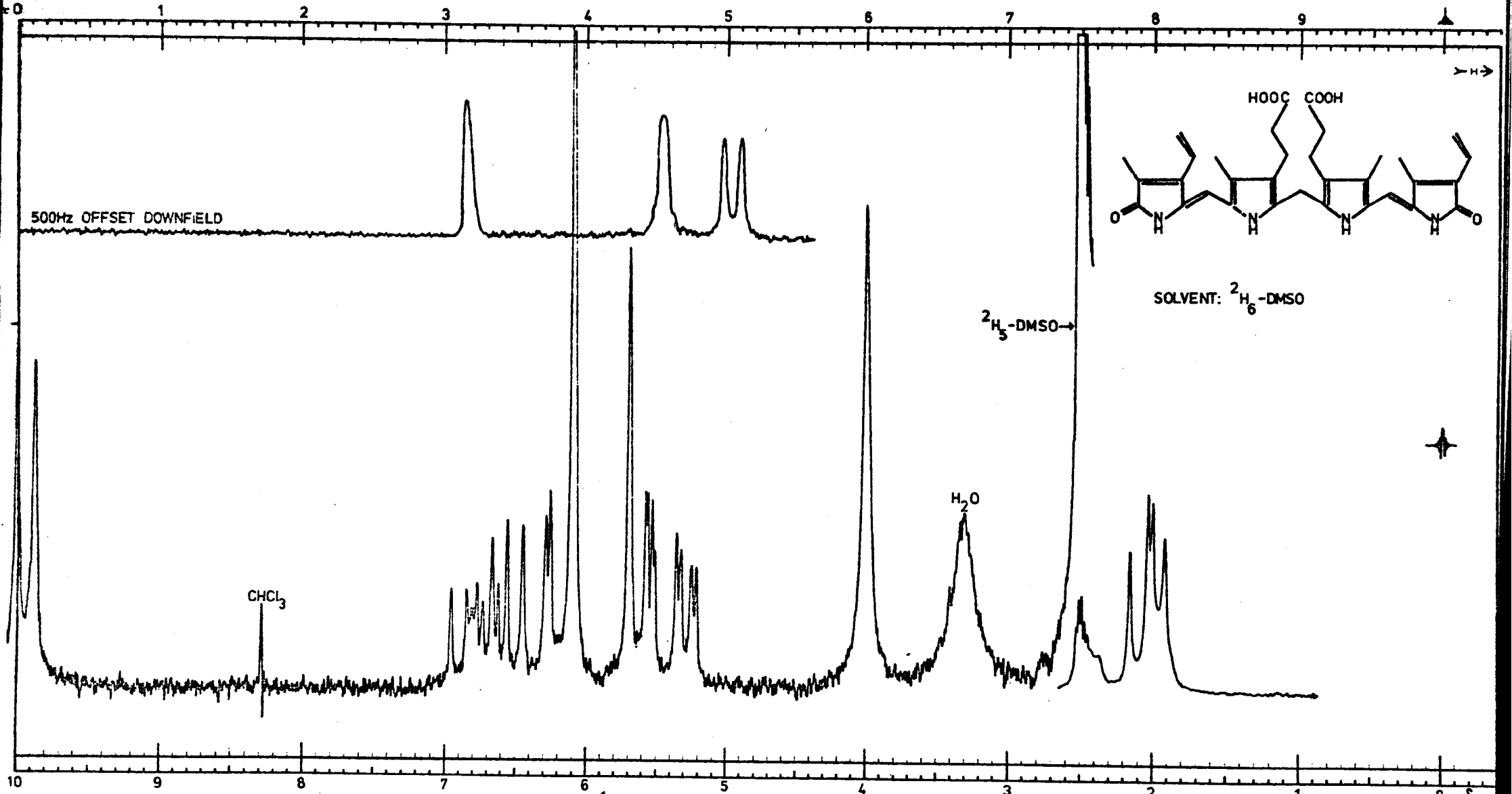


FIGURE A.1. THE 100MHz ^{1}HN.M.R. SPECTRUM OF BILIRUBIN

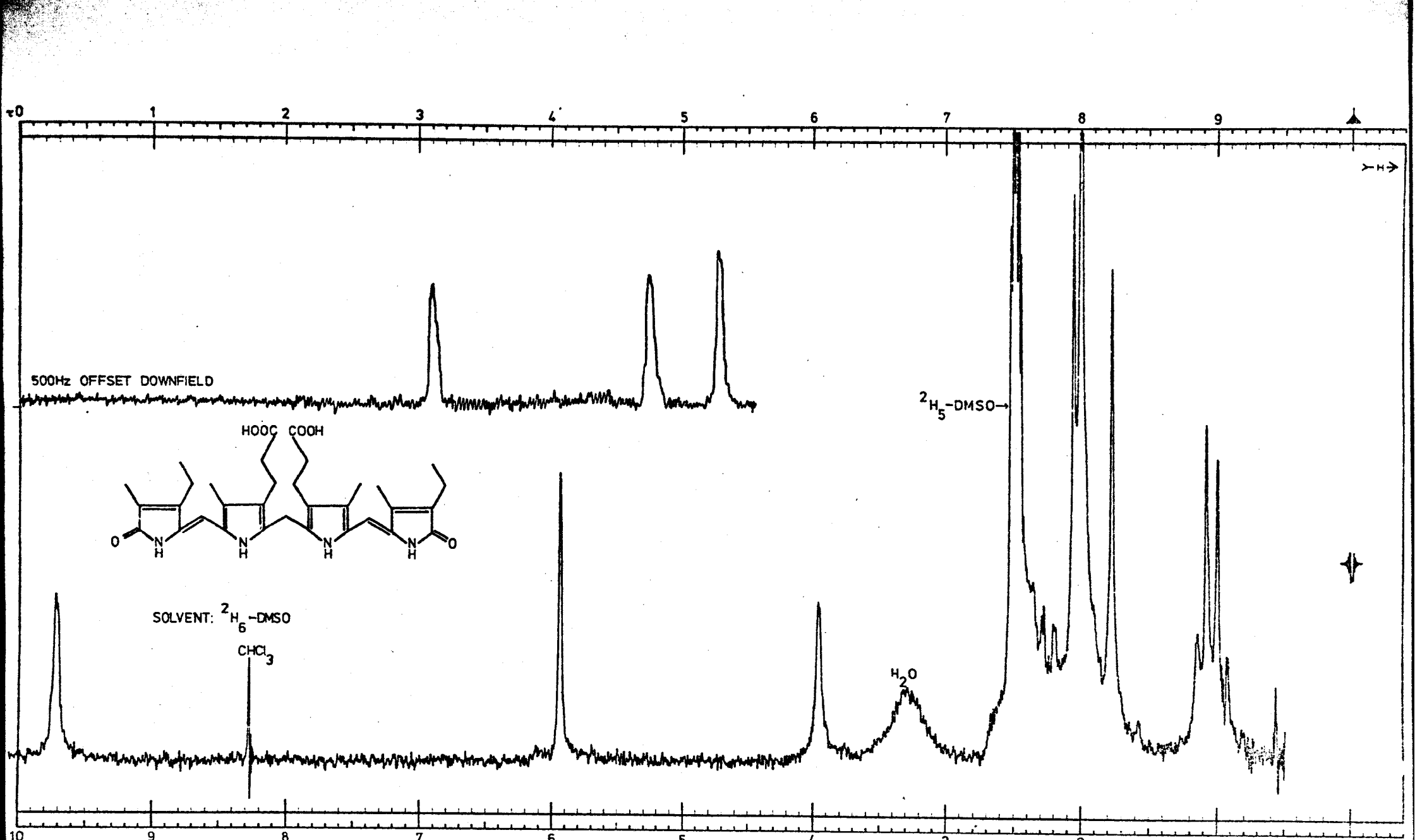


FIGURE A.2. THE 100MHz ^{1}H N.M.R. SPECTRUM OF MESOBILIRUBIN

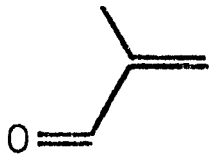
confirms the assignment of the C(2) methyl group in mesobilirubin (also at 8.22 τ) and hence in bilirubin also.

The methylene groups of the propionic acid side chains on C(3) and C(12) in both compounds are partly buried beneath the methyl and the $^2H_6$ -DMSO resonances. They would appear as an A_2B_2 system.

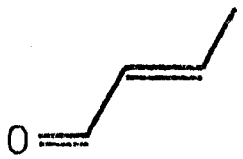
The singlets at 6.00 τ in bilirubin and 6.04 τ in mesobilirubin are assigned to the central methylene bridge protons in each case. The two protons of the C(5) and C(15) methine bridges resonate as a singlet at 4.05 τ in mesobilirubin, while in bilirubin, analysis of the signals between 3.04 τ and 4.78 τ (v.infra), shows that the resonances of these protons comprise part of the singlet at 3.91 τ . The singlet at 3.91 τ and part of the singlet at 4.30 τ have been assigned previously, one each , to these protons.[111] The signal at 3.91 τ has also been assigned to both these protons alone.[88]

The remainder of the resonances between 3.04 τ and 4.78 τ in bilirubin arise from the C(3) and C(18) vinyl groups, which appear to resonate as two first order ABX systems, $A_1B_1X_1$ and $A_2B_2X_2$. The theoretical first order analysis of the ABX system is shown in Figure A.3 [113c] and the vinyl spectrum of bilirubin will be analysed with reference to this.

The signals between 3.04 τ and 3.55 τ comprise the X protons and since the S_{BX} values are identical for both X_1 and X_2 these protons are only distinguishable by the S_{AX} values, which are different. The two groups are the signals at 3.04, 3.14, 3.22, and 3.32 τ (X_1) with $S_{A_1X_1}$ = 19Hz and $S_{B_1X_1}$ = 10Hz and those at 3.26, 3.36, 3.44 and 3.54 τ (X_2) with $S_{A_2X_2}$ = 16 Hz and $S_{B_2X_2}$ = 10Hz. Theoretically, from the first order approach, the singlets within a group should have equal intensities. This is only true in the present



(A.I)



(A.II)

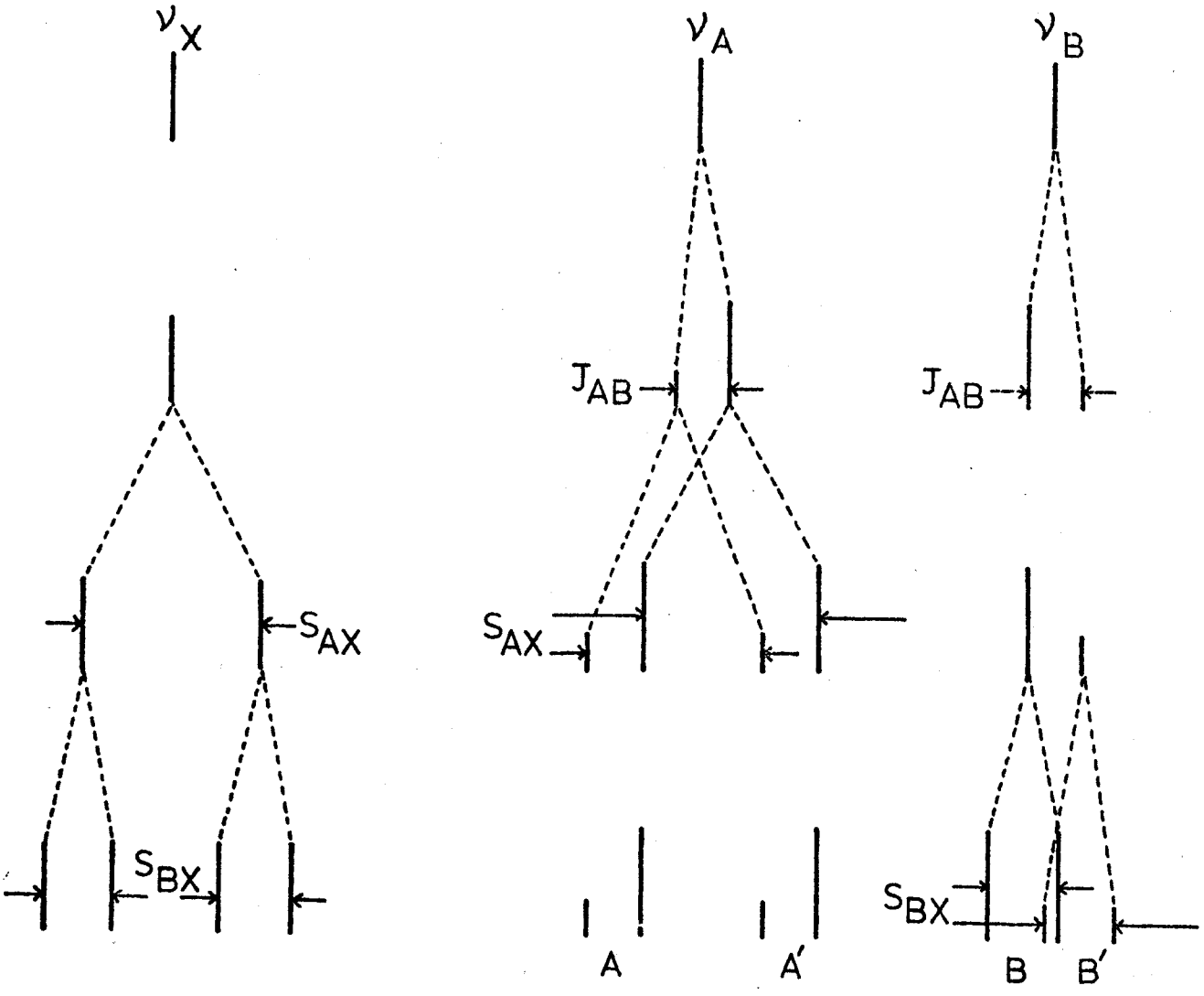


FIGURE A.3. THE THEORETICAL FIRST ORDER ANALYSIS OF AN ABX SPECTRUM (From ref. 113c)

case if the shoulder to low field on the singlet at 3.32τ and half of the singlets at3.44τ and 3.54τ are due to the presence of impurities or the presence of bilirubin IXβ, IXγ or IXδ - the presence of bilirubin IIIα or XIIIα should serve merely to increase the intensities of all the singlets within a group. If this is not the case then the increased intensities must be due to extra lines from the vinyl groups, so that the first order approach used here is not strictly valid.[113c]

The theoretical model requires that the AB protons should give rise to four pairs of doublets* in this case; only five doublets are observed in the spectrum. The pair centred at 4.70τ are complete and represent one proton. The highfield part of the doublet at 3.72τ must be obscured by the singlet at 3.91τ while the lowfield counterparts of the doublets at 4.43τ and 4.47τ must themselves form the singlet at 4.31τ.

Inspection of the spectrum readily shows two values for J_{AB}, thus the doublet at 3.72τ and the two doublets centred at 4.70τ represent part of one pair of AB protons (J_{AB} = 4Hz) while the doublets at 4.43τ and 4.47τ represent part of the other AB protons J_{AB} = 2Hz). Consider the case where J_{AB} = 4Hz. By direct comparison with the theoretical model, the doublets centred at 4.70τ arise from the B proton, while the counterpart of the doublet at 3.72τ is only symmetrically located under the singlet at 3.91τ if S_{AX} = 19Hz = $S_{A_1X_1}$. Thus the $A_1B_1X_1$ system is identified and the complete analysis is shown in Figure A.4.

By a similar argument, the doublets at 4.47τ and 4.43τ

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* The term "doublet" is used here to describe one half of an AB - type quartet rather than the 1 : 1 doublet produced when one proton is split by another on an adjacent carbon atom, each being in a different chemical environment.

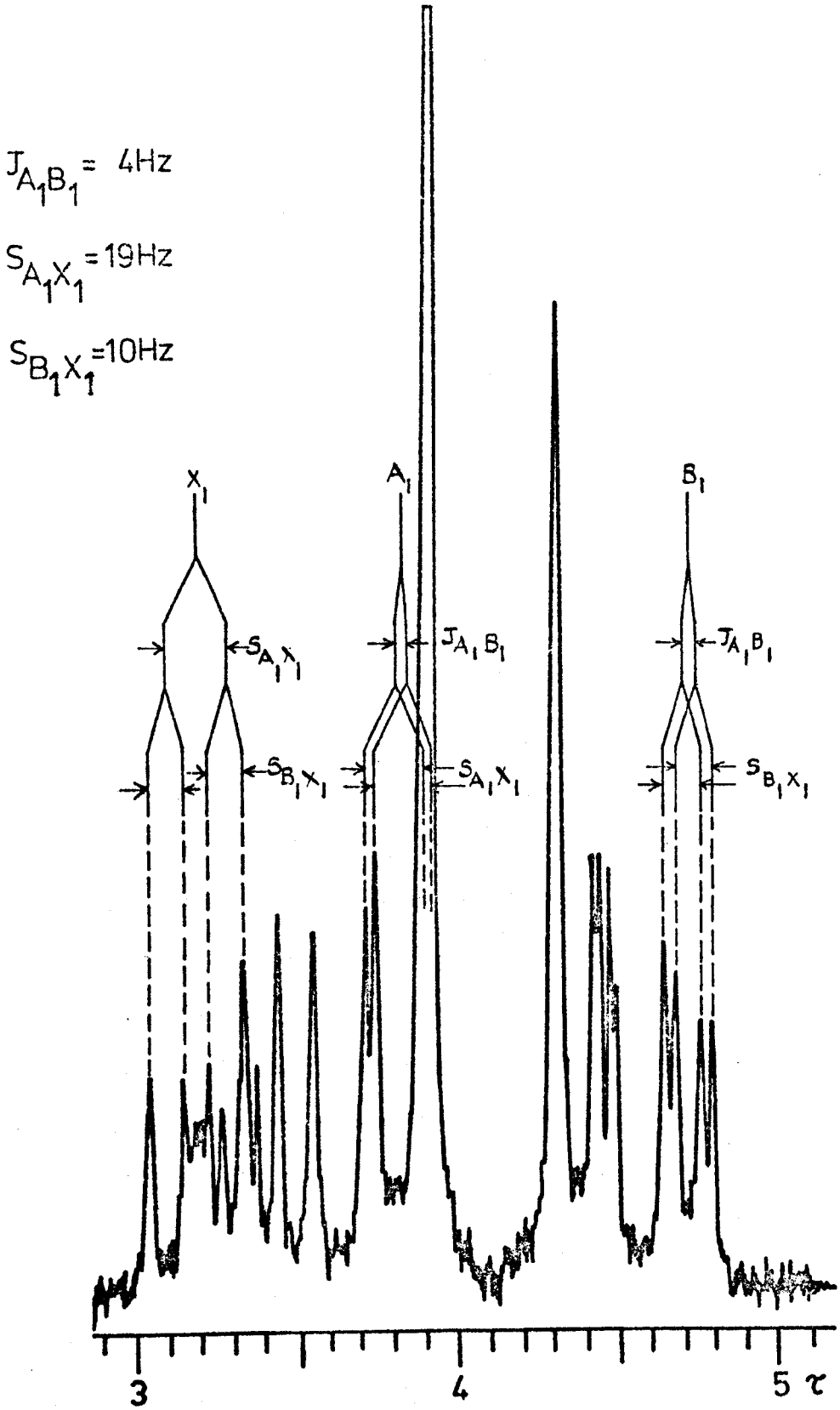

FIGURE A.4. THE ANALYSIS OF THE $A_1B_1X_1$ VINYL GROUP

represent parts of the signals due to the A_2 and B_2 protons respectively. The analysis of the $A_2B_2X_2$ system is shown in Figure A.5., where it is seen that the singlet at 4.30 τ is, in fact, compounded from two overlapping doublets.

The two ABX systems are not specifically assigned to the C(3) and C(18) vinyl groups. Although X_1 resonates at lower field than X_2, the resonance positions of A_1B_1 and A_2B_2 bear no relationship to these nor to each other. However if X_1 and X_2 are regarded as protons of a methyl group, then the argument used to distinguish the C(2) and C(17) methyl groups suggests that $A_1B_1X_1$ and $A_2B_2X_2$ are the C(3) and C(18) vinyl groups respectively.

The resonance at lowest field, -1.90 τ , in bilirubin (and mesobilirubin) is assigned to the carboxylic acid protons for reasons already given in section 2.2.1 of this thesis. The remaining low field signals must then be due to the pyrrole and lactam N -H groups. Pyrroles and lactams can usually be distinguished, since pyrrole N-H protons are exchangeable in D_2O/DCl, whereas lactam N-H protons are exchangeable only in D_2O/NaOD. [113d] In the case of bilirubin and mesobilirubin all the N-H protons are exchanged with D_2O alone (Figure 2.4.) . Mesobilirubin shows two singlets at +0.20 τ (2H) and - 0.35 τ (2H), whereas bilirubin shows singlets at +0.10 τ (1H), -0.02 τ (1H) and -0.45 τ (2H), the latter being resolved on scale expansion into two overlapping singlets 0.03 τ apart. The resonances at -0.45 τ in bilirubin and -0.35 τ in mesobilirubin are assigned to the N-H protons of the pyrrole rings. These are essentially magnetically equivalent in both compounds and those in bilirubin would be expected to change in position but little on reduction of the vinyl groups. The remaining singlets at +0.10 τ and -0.02 τ in bilirubin and the signal at +0.20 τ in mesobilirubin are assigned to the lactam N-H protons of rings A and D, although no specific assignment is made in

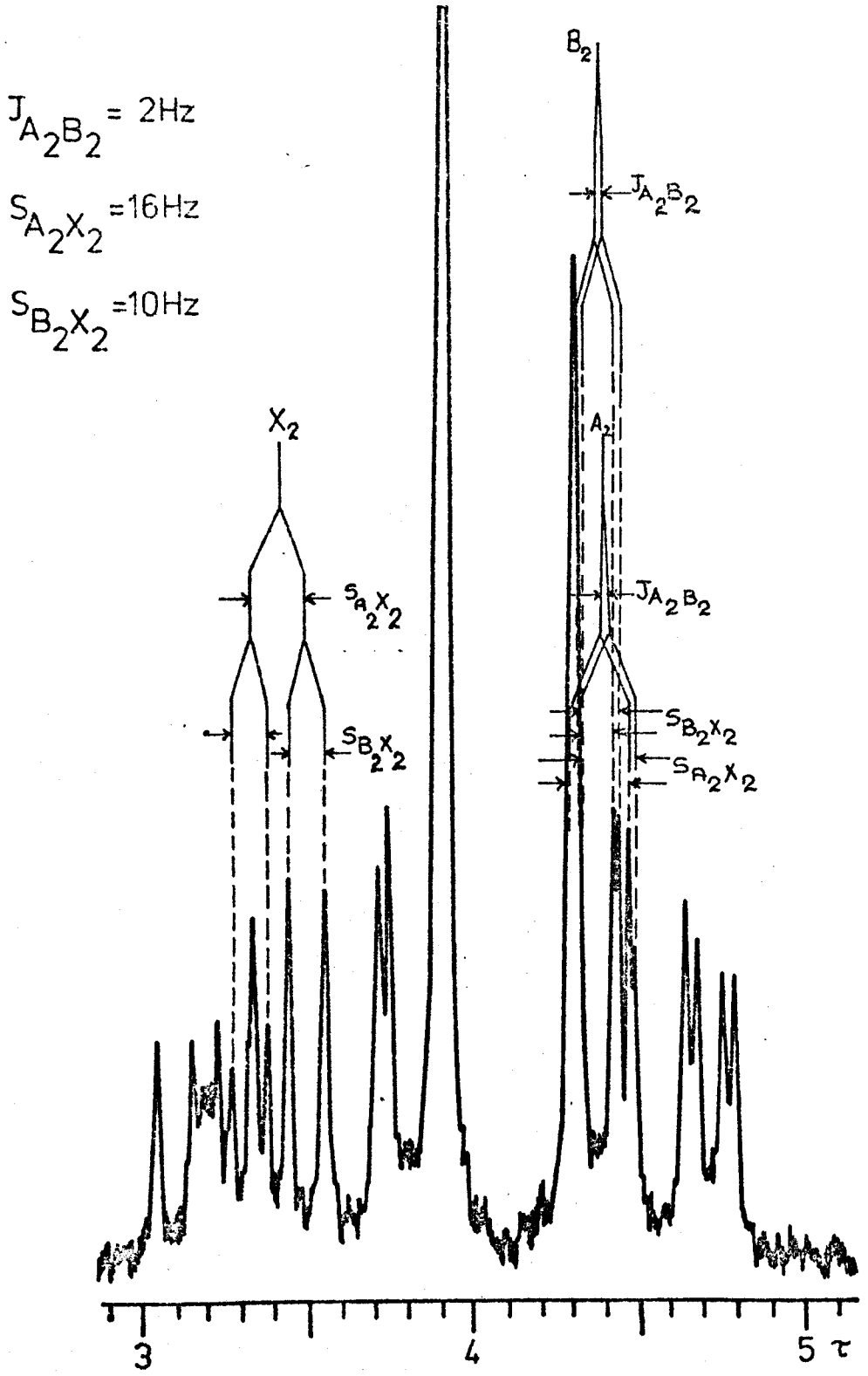

FIGURE A.5. THE ANALYSIS OF THE $A_2B_2X_2$ VINYL GROUP

the case of bilirubin. In the published assignment,[88] the resonances at +0.10τ and -0.04τ are assigned to the pyrrole N-H protons while that at -0.45τ is assigned to the lactam N-H protons.

Publications arising from this work

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Short Communication

Tautomerism and Hydrogen Bonding in Bilirubin

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(*Received* 13 *April* 1971)

The tautomerism of bilirubin has been the subject of recent investigations (Kuenzle, 1970; Nichol & Morell, 1969) and we now present additional evidence that the lactam form is the predominant tautomer in solution and in the solid state.

The ^1H n.m.r. spectrum of bilirubin (40mg, 70μmol) in carefully dried [$^2H_6$]dimethyl sulphoxide (0.5ml) exhibits broad singlets at $\delta = 11.90$ (2H), 10.50 (2H), 10.10 (1H) and 9.95 (1H) p.p.m. (Kuenzle, 1970), but no signal at 5.25p.p.m., which has been attributed (Nichol & Morell, 1969) to the enolic protons of the lactim tautomer. We have found that, although all low-field signals collapse on addition of deuterium oxide (20μl, 1mmol), when water (10μl, 0.5mmol) is added to a similar solution of bilirubin in [$^2H_6$]dimethyl sulphoxide only the signal at 11.90p.p.m. disappears. The n.m.r. spectra of solutions of *N*-benzoylglycine or 2,4-dimethylpyrrole-3-propionic acid (Morsingh & MacDonald, 1960) in dry [$^2H_6$]dimethyl sulphoxide are also affected by the addition of water. In these cases the signals due to hydroxylic protons disappear, whereas signals due to protons attached to nitrogen atoms persist. These results suggest that in bilirubin two protons are attached to oxygen atoms and that the lactam tautomer is the predominant form in solution in dry [$^2H_6$]dimethyl sulphoxide.

Tritiated bilirubin was prepared by direct exchange (Brodersen, Flodgaard & Krogh-Hansen, 1967) in an atmosphere of dry nitrogen. The specific radioactivity of two preparations, carefully dried to constant radioactivity, indicated that 5.95 (average of seven determinations) and 6.03 (average of six determinations) atoms of tritium had been incorporated into each molecule of bilirubin. An earlier report that only four atoms of tritium are incorporated per bilirubin molecule (Brodersen *et al.* 1967) may be explained by the extreme rapidity of exchange of the hydroxylic protons with adventitious water.

Additional evidence that all six protons in bilirubin can undergo exchange is provided by i.r. spectroscopy. The i.r. spectrum (Nujol mull or potassium chloride disc) of fully deuterated bilirubin, prepared as described above by direct exchange in an atmosphere of dry nitrogen, has no absorption maxima in the region 3000–3600cm^{-1} due to O–H or N–H vibrations. If a potassium bromide disc of deuterated bilirubin is prepared, deuteron–proton exchange with the slightly hygroscopic matrix produces absorption maxima in the region of 3400 and 3225cm^{-1}, as found for undeuterated bilirubin (Newbold & LeBlanc, 1964). The

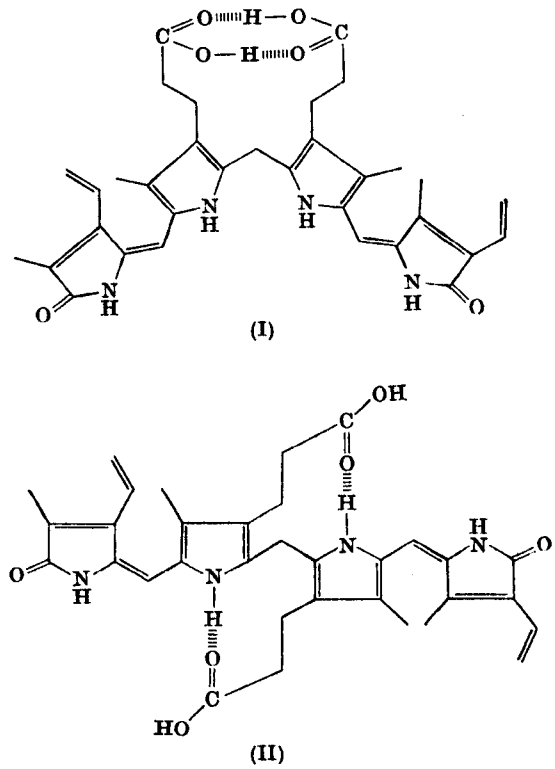

Fig. 1. Tautomeric forms of bilirubin.

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carbonyl stretching vibration of the propionic acid side chains of undeuterated bilirubin appears in the i.r. spectrum at 1690cm^{-1} (Brodersen *et al.* 1967; Fog & Jellum, 1963; Newbold & LeBlanc, 1964). We have found that the position of this band does not alter whether the sample is prepared as a Nujol mull, potassium bromide disc or in solution (chloroform or dioxan). In contrast, the carbonyl stretching vibration of 2,4-dimethylpyrrole-3-propionic acid is strongly dependent on the medium, ranging from 1690cm^{-1} (Nujol mull) to 1735cm^{-1} (dioxan solution).

These results suggest that the carbonyl groups of the propionic acid side chains in bilirubin participate in strong intramolecular hydrogen bonds and that the hydrogen-bonding does not alter with medium. Two structures (I and II, Fig. 1) are possible on steric grounds (Brodersen *et al.* 1967) and we believe that the conformation of the molecule is the same in solution and in the solid state. Hydrogen-bonding between the hydroxyl groups of the propionic side chains and the π-electron system of the pyrrole rings (Kuenzle, 1970) should not lower the carbonyl stretching frequency to 1690cm^{-1} and we consider that this form of bonding is unlikely in bilirubin.

Materials. Bilirubin was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and recrystallized from chloroform–methanol (Found: C, 67.9; H, 6.0; N, 9.2; $C_{33}H_{36}N_4O_6$ requires C, 67.8; H, 6.2; N, 9.6%). It had a light-absorption maximum in chloroform at 454nm (ϵ 59400). Deuterated bilirubin had an absorption maximum in chloroform at 450nm (ϵ 58300).

Infrared spectra were recorded with a Perkin–Elmer 621 spectrometer and ^{1}H n.m.r. spectra were recorded with a Perkin–Elmer R12 spectrometer. The tritium content of samples was measured with a Packard 4000 scintillation counter by using a dioxan-based scintillation medium containing naphthalene (60g/l), 2,5-diphenyloxazole (4g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g/l).

We thank the Medical Research Council for financial support. Requests for reprints should be sent to D. W. H.

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Short Communications

The Reaction between Bilirubin and Aromatic Diazo Compounds

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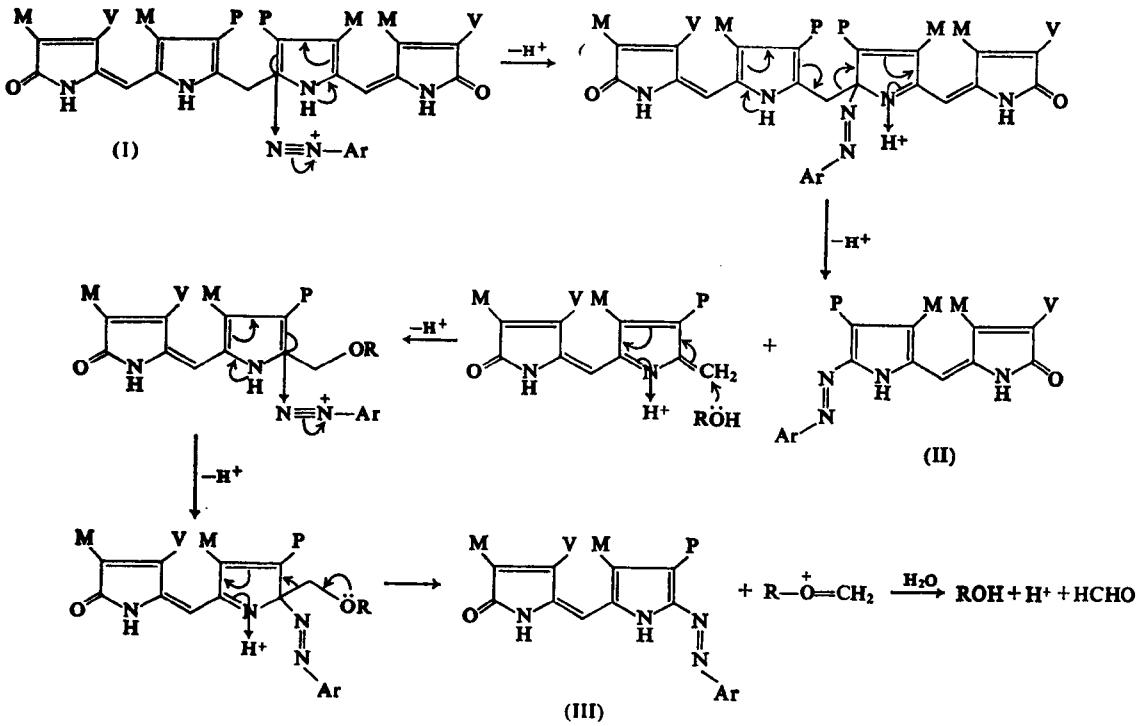
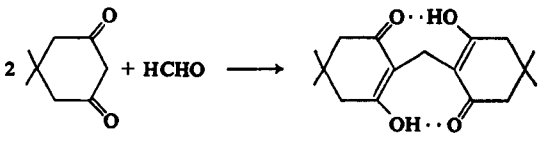
(*Received* 18 *February* 1972)

Bilirubin (I) reacts with diazotized aromatic amines and is cleaved at the central methylene bridge to form two isomeric azopigments (II and III). The reaction between bilirubin (I) and diazotized sulphanilic acid is used to determine the concentrations of bilirubin and its conjugates in sera [the van den Bergh reaction (Hijmans van den Bergh & Snapper, 1913)]. The structures of the azopigments derived from diazotized ethyl anthranilate and aniline have been established (Jansen & Stoll, 1971), and the reaction with diazotized ethyl anthranilate has been used in the determination of the structures of bilirubin conjugates (Compernolle *et al.*, 1970). The van den Bergh reaction is thought to occur in two steps, and it has been suggested that the central methylene bridge carbon atom of bilirubin (I) is released as formaldehyde during the second step of the reaction (Overbeek *et al.*, 1955). However, formaldehyde could not be detected in the distillate from the van den Bergh reaction by using 3-methyl-2-benzothiazolone hydrazone, which reacts with formaldehyde to form a blue colour (Fog & Jellum, 1962).



Scheme 1. *Suggested mechanism for the formation of formaldehyde from bilirubin (I)*



(IV) (V)

Scheme 2. *Reaction between dimedone and formaldehyde*

A mechanism for the formation of formaldehyde from bilirubin (I) is suggested in Scheme 1; it should be noted that initial attack by the diazonium ion could occur at either of the pyrrole rings flanking the central methylene bridge.

We now report that formaldehyde is formed during the van den Bergh reaction and can be detected as formaldehyde dimethone (V), the product of the reaction between formaldehyde and 5,5-dimethylcyclohexane-1,3-dione (dimedone) (IV) (Scheme 2).

Experimental

With free bilirubin, the van den Bergh reaction can be carried out in a chloroform–methanol medium. Since both these reagents contain C_1 units that could give rise to formaldehyde, this solvent system was abandoned in favour of a 1,2-dichloroethane–2-methylpropan-2-ol medium, although the reaction was much slower in this medium.

Bilirubin (195mg, 0.33mmol) was suspended in a mixture of 1,2-dichloroethane (50ml) and 2-methylpropan-2-ol (50ml). A solution of benzenediazonium chloride (2mmol) was added and the mixture stirred in a closed flask, in the dark, at room temperature for 30min. A further quantity of benzenediazonium chloride (2mmol) was added and the solution stirred for 30min more. The reaction mixture was then evaporated to dryness, the solvents being distilled into a solution of dimedone (200mg) in 2-methylpropan-2-ol (50ml), with the receiver connected to a second vessel containing a similar solution of dimedone in 2-methylpropan-2-ol. The dimedone solutions were combined and heated under gentle reflux for 30min. After cooling and removal of the solvents *in vacuo*, t.l.c. of the residue (on silica gel, with chloroform as solvent) showed the presence of formaldehyde dimethone (V), R_F 0.44. No formaldehyde dimethone (V) was obtained from two control reactions, one in which the benzenediazonium chloride was omitted and the other in which the bilirubin was omitted. The formaldehyde dimethone (V) was isolated by elution from a silicic acid column with light petroleum (b.p. 60–80°C)–diethyl ether (3:1, v/v). The isolated material was identical in all respects with an authentic sample of formaldehyde dimethone (V). The recovery of the compound (V) was up to 20% (20mg) of the theoretical amount. When 1,2-dichloroethane (50ml) and 2-methylpropan-2-ol (50ml) containing formaldehyde [10mg, equivalent to 195mg of bilirubin (I)] were distilled as above, the formaldehyde dimethone (V) recovered never exceeded 20% of the theoretical amount (100mg).

Materials

Bilirubin was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and was purified by the method of Fog (1964) before use (Found: C, 67.9; H, 6.0; N, 9.2; $C_{33}H_{36}N_4O_6$ requires C, 67.8; H, 6.2; N, 9.6%). It had a light-absorption maximum in chloroform at 454nm (ϵ 59400). 1,2-Dichloroethane and 2-methylpropan-2-ol were distilled before use, and the dimedone was used without further purification.

We thank the Medical Research Council for financial support. Requests for reprints should be sent to D. W. H.

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83 **Intramolecular Hydrogen Bonds in Bilirubin**

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A specific system of intramolecular hydrogen bonds is probably present in bilirubin, as attested by the following observations: (1) Isotope exchange experiments demonstrate six exchangeable protons in bilirubin, of which two are attached to oxygen. The lactam tautomer therefore predominates. (2) The carboxyl stretching vibration at 1,690 cm^{-1} in the infra-red spectrum of bilirubin is at an unusually low wave number, and does not vary with change of matrix or solvent. (3) The titration curve of bilirubin has a two-equivalent step at about pH 7.5, which we believe is the dissociation of the pyrrole amide groups. This pKa contrasts with the expected for such an amide (about 10). The pKa of the propionic carboxyl groups is probably less than 4, whereas the expected value is 4.8. Bilirubin probably is a *bis*-anion between pH values of about 5 and 7, and a *tetra*-anion above pH 8. (4) Molecular models show that the proposed hydrogen bonds are strain-free, and the planarity of each pair of pyrrole rings is preserved.

This system of hydrogen bonds is probably broken on conjugation. Some promoters of the diazo reaction may act by disrupting these hydrogen bonds, e.g. urea, caffeine, guanidine and acetate. Intramolecular hydrogen bonding may explain the extreme insolubility of free bilirubin.

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