BBA 26141

STUDIES ON THE ISOMERIC COMPOSITION OF BILIVERDIN AND BILIRUBIN BY MASS SPECTROMETRY

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(Received January 13th, 1969)

SUMMARY

The isomeric composition of bile pigments produced both *in vivo* and *in vitro* has been investigated using both mass and NMR spectrometry. Bile pigments from mammalian, avian and algal sources were all found to be of the IXα type although traces of other isomers were present in the pigments derived from the two former sources. Biliverdin produced by coupled oxidation of haemoglobin and ascorbic acid in the presence of oxygen is also of the IXα type. Biliverdin produced by coupled oxidation of pyridine protohaemochrome and either hydrazine or ascorbic acid by oxygen is of the type IXβ or IXδ. Spectroscopic data are given for this compound which resembles closely the bile pigment formed by guinea pig liver homogenate and pyridine protohaemochrome.

INTRODUCTION

Determination of the isomeric composition of bile pigments has, to this date, been carried out by oxidation of the bile pigments with potassium permanganate to the pyrrole carboxylic acids (I) and (II) (refs. 1-3). This method has been used extensively by GRAY and coworkers but suffers from the disadvantage that yields of the acids are low. By this means it has been determined that whereas bilirubin from natural sources is the IXα isomer, biliverdin produced by coupled oxidation of pyridine protohaemochrome in the presence of hydrazine and oxygen appeared to be a mixture of isomers. This work has been used as one of the major pieces of evidence for the enzymic formation of biliverdin during the oxidative degradation of the haem group of haemoglobin *in vivo*. The discovery of an activity in liver extract[4,5] was taken by NAKAJIMA and coll.[5-7] to support this theory; recently, however, three independent groups (refs. 8,9 and A. W. NICHOL, unpublished results) have found that the activity present in liver is due to a dialysable heat-stable factor. This finding suggested a re-examination of the isomeric nature of biliverdin produced by non-enzymic coupled oxidation of porphyrin-Fe (II) substrates. A suitable approach to this problem was suggested by the fact that the principal fragmentation process in the mass spectrum of bilirubin is cleavage at the central methylene group yielding two dipyrrole fragments[10]. Thus the cleavage of bilirubins IXα and IXγ should yield ions of m/e 300, *e.g.* (III)

while, the IXβ and IXδ isomers should yield the ions of *m/e* 360 and 239, *e.g.* (IV) and (V) for the β isomer.

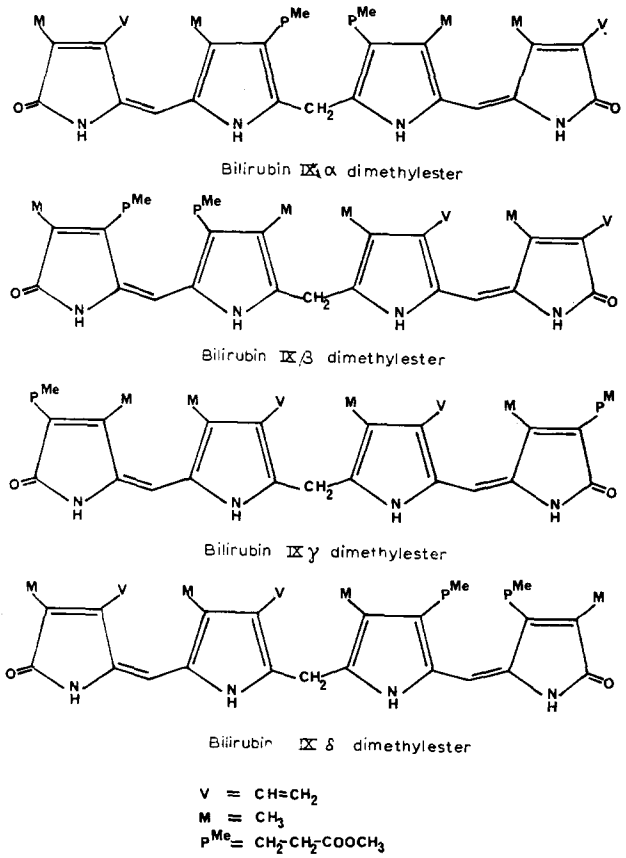
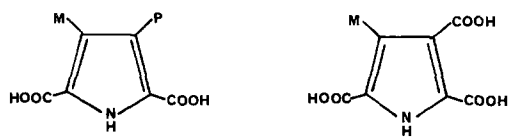


Fig. 1. Isomeric bilirubin dimethyl esters which may be formed from protoporphyrin IX.



I II

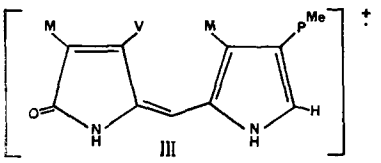
MATERIALS AND METHODS

Bilirubin from ox gall stones was from Sigma Chemical Co. and was crystallised from chloroform–methanol before use. Haemin from horse blood was from the same supplier. Crystalline haemoglobin was prepared from horse blood by the method of SUZUKI, KAJITA AND HANAOKA[11]. Biliverdin dimethyl ester was prepared from ox

bilirubin by the method of LEMBERG[12] as modified by NICHOL AND MORELL[13]. Hydrolysis to the free acid was accomplished by allowing the ester to stand overnight in 10 M HCl.

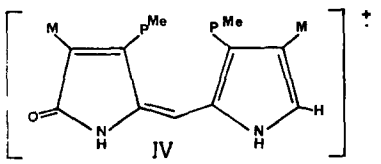
Alumina for chromatography was Schuchardt neutral, activity I. Chloroform for chromatography was destabilised before use by washing 4 times with deionised water and drying over anhydrous potassium carbonate.

Melting points are uncorrected and were determined on a Reichert hot stage microscope. Nuclear magnetic resonance spectra were run on a Varian A-60 spectrometer while mass spectra were obtained on an A.E.I. MS-9 machine at a temperature of 250°. All visible spectra were determined on a Perkin–Elmer 137 ultraviolet spectrophotometer.



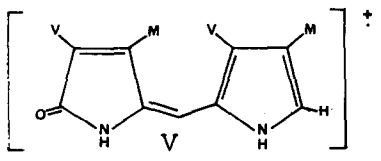
III

m/e 300



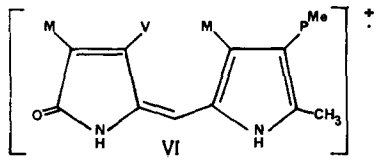
IV

m/e 360



V

m/e 239



VI

m/e 314

Preparation of biliverdin dimethyl ester by coupled oxidation

From haemin and hydrazine. Haemin (2.0 g) was dissolved in a mixture of pyridine (400 ml) and water (1 l) and treated with a solution of hydrazine sulphate (5.2 g) in 0.5 M NaOH (132 ml). Oxygen was rapidly passed into the solution which was maintained at 60° in a water bath. Samples were withdrawn at frequent intervals and examined with the hand spectroscope after treatment with a few crystals of sodium dithionite. Oxygenation was stopped when the band at 557 mμ due to *meso*-oxyprotohaemochrome disappeared to be replaced by the three-banded spectrum of verdohaemochrome (λ_{max} 500, 531, 656 mμ; I>II>III). The reaction required about

30 min. The solution was extracted with chloroform (3×) and the chloroform extracts washed with water (4×) and evaporated to dryness. The residue was dissolved in methanol (500 ml) containing concentrated H_2SO_4 (20 ml) and allowed to stand overnight. After pouring into saturated sodium acetate solution, the mixture was extracted with ether (3×) followed by 10% HCl (3×). The HCl extracts were immediately treated with saturated sodium acetate solution and the biliverdin methyl ester re-extracted into ether. The ether extracts were washed with water followed by 5% $NaHCO_3$, dried and evaporated to dryness. The residue was chromatographed on alumina (40 g) in chloroform–0.2% methanol. The blue band was collected and, after evaporation, the residue was crystallised from methanol (10 ml) by standing at 4° for 3 days. Biliverdin dimethyl ester was obtained as a mixture of needles and rosettes of prisms (0.1 g), m.p. 208–212°.

From haemin and ascorbic acid. Haemin (2 g) was treated exactly as above, except that ascorbic acid (80 g) adjusted to pH 7.5 with aqueous NaOH (100 ml) replaced hydrazine. The temperature was maintained at 40°, the reaction requiring about 3 h. At this time, although the band at 557 mμ had not completely disappeared, yields were found to be optimal since continued oxygenation resulted in the decomposition of verdohaemochrome to brown products with diffuse spectra. Biliverdin dimethyl ester (0.15 g) was obtained after esterification with methanolic H_2SO_4 followed by chromatography as above. The products crystallised as needles and rosettes, m.p. 207–210°.

From haemoglobin and ascorbic acid. Horse haemoglobin (40 g dry weight) was dissolved in 0.25 M Na_2HPO_4 (1 l) and treated with a solution of ascorbic acid (40 g) in water (100 ml) which had been adjusted to pH 7.5. The mixture was incubated in a shallow tray at 37° for 20 h with a few drops of chloroform to inhibit bacterial growth. The incubated mixture, which contained a heavy precipitate of choleglobin, was poured into acetic acid (500 ml), stirred for 1 h and then poured into acetone (2 l) containing 10 M HCl (20 ml). The precipitate was filtered off and the filtrate extracted with ether in small batches after pouring into saturated sodium acetate. The ether extract was washed with water (6×), dried, evaporated to dryness and the residue esterified with methanolic sulphuric acid as above. Biliverdin dimethyl ester was recovered by a procedure identical with that given above as elongated plates (0.05 g), m.p. 203–205°.

From the eggs of the emu (Dromaius novae-hollandiae). The shells of three freshly laid emu eggs (310 g) were broken by hammering and dissolved in 10 M HCl (1 l) overlayered with ether. The residue was filtered off with muslin gauze and extracted with methanol (500 ml). The methanolic extract was evaporated to dryness and the residue esterified with methanolic sulphuric acid. Biliverdin (oocyan) dimethyl ester was recovered as above as blue elongated plates (0.062 g), m.p. 208–212°.

From guinea pig liver homogenate and pyridine protohaemochrome. Three male guinea pigs were each injected intraperitoneally with a solution of ascorbic acid (0.15 g) and prednisolone 21-phosphate (3 mg) in 0.9% NaCl (10 ml). After 4 h the animals were anaesthetised with ether and their livers removed. Subsequent steps were carried out after the method of NAKAJIMA AND GRAY[7]. About 1 mg of biliverdin was obtained.

Mesobilirubin dimethyl ester

Attempted preparation of this compound from the phycoerythrobilin of the red seaweed *Corallina sp.* by the method of LEMBERG AND BADER[14] gave only mesobili-

verdin. Mesobilirubin obtained from the R-phycoerythrin of *Ceramium rubrum* was a gift from Dr. Lemberg and was converted to the ester by solution in chloroform, treatment with diazomethane for 1 min and precipitation with light petroleum. Since phycoerythrobilin is an isomer of mesobiliverdin it is probable that this mesobilirubin is derived from the urobilinoid component of R-phycoerythrin.

Reduction of biliverdin dimethyl ester

Several chemical methods have been used for the reduction of biliverdin to bilirubin. The most generally useful methods have been zinc dust in aqueous ammonia[15], alkaline sodium dithionite[16], HI (ref. 12) and zinc dust in acetic acid[17]. Of these the first two methods were found to be suitable only for biliverdin free acid, while HI tended to yield impure products. Zinc in acetic acid gave rapid reduction of the ester although some precautions were necessary to prevent autoxidation of the product. The following general method was used.

Biliverdin dimethyl ester (0.05 g) was dissolved in acetic acid (10 ml) and shaken under nitrogen with zinc dust (0.5 g) until the supernatant solution became a clear golden yellow. The solution was immediately filtered under nitrogen and the filtrate poured into ether. The ether solution was washed free of acetic acid with dilute $NaHCO_3$. After drying over K_2CO_3 in the cold under nitrogen the solution was evaporated to small volume (5 ml) and poured into light petroleum (30 ml). The flocculent precipitate was centrifuged, washed with light petroleum and dried *in vacuo*.

Mass spectrometric investigation

As found by COLE, CHAPMAN AND SIEGELMAN[3] the major peaks in the biliverdin dimethyl ester preparations arise from fragmentations of the side chains. All the preparations are isomeric with M^+ at 610. Biliverdin dimethyl ester from ox gall stone bilirubin ($FeCl_3$ oxidation), from emu eggs and from the coupled oxidation of haemoglobin showed mass spectra close to that obtained for biliverdin dimethyl ester by CULE, CHAPMAN AND SIEGELMAN[3] except that the peak of *m/e* 313 was replaced by one at 314. The mass spectrum of biliverdin dimethyl ester prepared by coupled oxidation of pyridine protohaemochrome with either ascorbate or hydrazine was similar but showed an additional intense peak of *m/e* 368. The spectrum is given in Table I. Biliverdin methyl ester prepared by oxidation of ox gall stone bilirubin with hydrogen peroxide also showed an intense peak at *m/e* 642. The same peak appeared in biliverdin dimethyl ester prepared by oxidation of bilirubin with $FeCl_3$ without intermediate isolation of biliverdin–$FeCl_3$ or if refluxing with $FeCl_3$ was too prolonged. Since the visible spectra of these products showed a shift towards the blue (Table IV) it appears likely that the contaminant is formed by oxidation of the vinyl chains possibly with formation of a di-epoxide.

In contrast to the biliverdin preparations, the bilirubin dimethyl esters showed weak molecular ions at *m/e* 612 with intense peaks resulting from fragmentation at the central methylene group (Table I). The dimethyl esters of bilirubin derived from ox gall stones, emu eggs and coupled oxidation of haemoglobin all showed base peaks of *m/e* 300 (*e.g.* III). The appearance of an ion of mass 14 units higher than the base peak is unusual when compared with the free acid in which the corresponding peak is only 13 units removed from the base peak[10]. It probably corresponds to the ion (VI). In the 70-eV spectrum fragmentation of the propionic ester side chains gives rise to a com-

TABLE I

MASS SPECTRA OF BILE PIGMENT DIMETHYL ESTERS

Origin of product	*70 eV, m/e* (%)	*11 eV, m/e* (%)
Biliverdin from coupled oxidations of protohaemochrome	610(100), 595(20), 579(15), 551(10), 537(32), 523(48), 368(35), 355(21), 312(23), 300(21), 285(11), 281(15), 269(13), 257(9), 241(18), 239(15), 236(10), 227(23), 225(21)	
Bilirubin from ox gall stones	612(5), 374(1), 360(2), 314(28), 300(100), 285(22), 269(30), 253(10), 241(32), 239(30), 227(98), 213(55)	612(4), 314(39), 300(100)
Bilirubin from emu eggs	612(4), 374(1), 360(3), 314(35), 300(100), 285(16), 269(21), 253(11), 241(35), 239(33), 227(100), 213(38)	
Haemoglobin and ascorbate	612(6), 374(2), 360(3), 314(35), 300(100), 269(15), 253(23), 241(35), 239(32), 227(95), 213(41)	
Pyridine protohaemochrome and ascorbate	612(9), 374(86), 360(100), 342(19), 329(36), 314(61), 300(95), 287(92), 285(75), 269(45), 255(92), 253(52), 241(96), 239(86), 227(95), 213(95)	
Pyridine protohaemochrome and hydrazine	612(5), 374(92), 360(100), 342(15), 329(40), 314(45), 300(80), 287(92), 285(73), 269(30), 255(95), 253(62), 241(95), 239(91), 227(82), 213(75).	374(25), 360(100), 253(15), 239(33)

plex pattern of peaks in the region *m/e* 300–200. Peaks at *m/e* 253 and 239 are probably formed by loss of $2H+COOCH_3$ from the ions (III) and (VI). A much simpler spectrum was obtained at 11 eV. At this energy fragmentation occurred only at the central methylene group to yield ions of *m/e* 314 and 300. The lower energy spectrum is thus more diagnostic of isomeric type. Mesobilirubin from Ceramium phycoerythrin gave a mass spectrum of the above type with a molecular ion of *m/e* 616 and dipyrrolic fragments of 2 mass units higher than the bilirubin derived from animal sources. Peaks of mass 374 and 360 were absent.

The low energy spectrum of bilirubin dimethyl ester derived from the coupled oxidation of pyridine protohaemochrome in the presence of either hydrazine or ascorbic acid once again showed a weak molecular ion of *m/e* 612. However, the dipyrrolic fragments had *m/e* 374, 360 (IV) 253 and 239 (V and isomers). The compound is thus distinct from the natural isomer and must be either bilirubin IXβ or IXδ. The 70-eV spectrum also showed peaks corresponding to fragmentation of the propionic ester side chains.

Nuclear magnetic resonance investigation

Nuclear magnetic resonance spectra of biliverdin dimethyl ester from ox gall stones and biliverdin dimethyl ester from coupled oxidation of pyridine protohaemochrome were determined in both deuterochloroform and deuterotrifluoracetic acid. The spectra for the latter compound are given in Fig. 2. The peaks and assignments for both compounds are given in Table II. The two compounds show quite different spectra and the absence of coinciding peaks confirms the mass spectroscopic results which show that biliverdin formed by coupled oxidation is a pure compound not contami-

nated with either the IXα or IXγ isomers. Three of the propionic ester methylene groups appear at much higher field in the *in vitro* produced isomer as compared with natural biliverdin dimethyl ester (for the spectrum of this compound see ref. 3). This shift is probably associated with the position of one of the propionic ester groups on the less deshielded terminal pyrrole rings. Protonation of both compounds causes a general downfield shift except in the case of one of the methyl signals and one of the

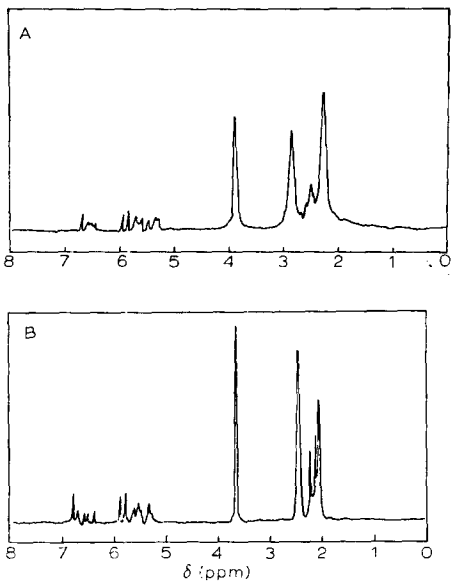


Fig. 2. Nuclear magnetic resonance spectra of biliverdin dimethyl ester formed by coupled oxidation of pyridine protohaemochrome and hydrazine. (A) in deuterotrifluoracetic acid and (B) in deuterochloroform. Chemical shifts are in parts per million (δ) from internal tetramethylsilane.

TABLE II

NMR SPECTRA OF BILIVERDIN DIMETHYL ESTER ISOMERS

From ox gall stone bilirubin			*From pyridine protohaemochrome and hydrazine*		
Peak (δ, ppm)		*Assignment*	*Peak (δ, ppm)*		*Assignment*
C^2HCl_3	CF_3COO^2H		C^2HCl_3	CF_3COO^2H	
1.80(3H)	1.67(3H)		2.07	2.28	2 ring CH_3, 1′ CH_2 of
2.05(6H)	2.25(3H)	ring CH_3	2.12(12H)	2.50(12H)	terminal propionyl
2.16(3H)	2.49(6H)		2.22		and 2′CH_2 of both propionyls
2.53(4H)m	3.94(4H)m	2′CH_2 of propionyls	2.47(8H)	2.87(8H)	2 ring CH_3 and 1′CH_2 of propionyl
2.86(4H)m	3.41(4H)m	1′CH_2 of propionyls	3.65(6H)	3.88(6H)	OCH_3
3.67(6H)	3.88(6H)	OCH_3	5.33(2H)m	5.42(2H)m	vinyl CH_2
			5.56(2H)m	5.67(2H)m	vinyl CH_2
5.75(2H)m	5.52(2H)m	methines	5.78(1H)	5.85(1H)	methine
5.93(2H)	5.83(2H)	and vinyls	5.90(1H)	5.90(1H)	methine
6.77(3H)m	6.03(1H)		6.55(2H)m	6.52(2H)m	vinyl CH
7.12(2H)m	6.62(3H)m		6.79(1H)	6.67(1H)	methine

vinyl signals of natural biliverdin which move slightly upfield. This shift may be caused by protonation of the terminal carbonyl groups resulting in loss of deshielding of the group in the adjacent β position. The absence of this shift in the IXβ (δ) isomer may indicate a different tautomeric form.

Visible spectra of biliverdin and bilirubin

The visible spectra of the biliverdin and bilirubin preparations are shown in Table IV. No change occurred in the spectra of the biliverdin esters on hydrolysis to the free acid. On the other hand all the bilirubin preparations absorbed at longer wavelengths than their esters. This difference has been shown by NICHOL AND MORELL[13] to be due to a difference in tautomeric form. The spectra of the bilirubin free acids were obtained by reduction of the biliverdin free acids using zinc and acetic acid. The product was extracted into chloroform, washed free of acetic acid with water and the chloroform solution used for the spectral determination.

Paper chromatography of biliverdin isomers

Attempts to separate biliverdin isomers by standard paper chromatography techniques[19] were unsuccessful. All the biliverdin dimethyl esters had R_F 0.20 using the kerosene–chloroform (4:2.6, v/v) system and R_F 0.62 in the *n*-propanol–kerosene (1:5, v/v) system. On Whatman No. 1 impregnated with 5% citric acid and using ethyl acetate as ascending solvent all the pure preparations had R_F 0.01 but those containing oxidised material (*m/e* 642) showed a spot of R_F 0.50. This material was not present in the biliverdin formed by guinea pig liver homogenate. No separation of the free acids resulted using the descending 2,6-lutidine–water (5:3.5 v/v) solvent system. The R_F value was 0.71.

Permanganate oxidation of biliverdin isomers

The oxidation to pyrrole carboxylic acids was carried out on the biliverdin free acids. On oxidation with potassium permanganate under acidic conditions only pyrrole carboxylic acid (II) was obtained from all preparations and from haemin. This presumably was due to over-oxidation. More consistent results were obtained by suspending the bile pigment or protohaemin (about 20 mg) in 1 M K_2CO_3 (10 ml) and oxidising by the slow addition of saturated potassium permanganate until the super-

TABLE III

R_F VALUES OF PYRROLE CARBOXYLIC ACIDS STAINED PINK WITH DIAZOTISED SULPHANILIC ACID AND NaOH (ref. 7).

Compound oxidized	*Butan 1-ol–acetic acid–water (4:1:5, by vol.)*	*Ethanol–aqueous ammonia–water (20:1:4, by vol.)*
Biliverdin from ox gall stones	0.82	0.04
Biliverdin by coupled oxidation of haemochrome	0.82, 0.51	0.04, 0.37
Protohaemin (acids I and II respectively)	0.82, 0.51	0.04, 0.37

TABLE IV

VISIBLE ABSORPTION SPECTRA OF BILIVERDIN AND BILIRUBIN PREPARATIONS IN CHLOROFORM

Method of preparation	*Biliverdin*		*Bilirubin* λ_{max} (mμ)	
	λ_{max} (mμ)	*RII/I*	*Free acid*	*Dimethyl ester*
Oxidation of ox gall stones bilirubin with $FeCl_3$	379, 668	3.66	450	410
Oxidation of ox gall stones bilirubin with H_2O_2	380, 662	3.62		
Oxidized contaminant of above	382, 660	3.51		
From emu eggs	380, 668	3.76	450	410
Coupled oxidation of haemoglobin and ascorbate	379, 666	3.56	440	409
Coupled oxidation of haemochrome and ascorbate	376, 647	3.20	403	385
Coupled oxidation of haemochrome and hydrazine	376, 645	3.19	402	385
Guinea pig liver homogenate and haemochrome	381, 650	3.28	408, 435*	385
Mesobilirubin from phycoerythrin			434	400

* Shoulder.

natant solution was colourless. After heating the solutions for 10 min in the water bath they were filtered and acidified with HCl. The subsequent procedures were identical with those used by NAKAJIMA AND GRAY[7]. The acids were detected chromatographically and the results are summarised in Table III. The formation of both isomers from biliverdin produced from pyridine protohaemochrome confirms the mass spectroscopic results.

DISCUSSION

On the basis of the present evidence it is not possible to distinguish between the pairs of isomers biliverdin IXα and IXγ and biliverdin IXβ and IXδ. Nonetheless, steric and electronic considerations suggest that the formation of the IXα or IXβ isomers on oxidation of the porphyrin ring would seem to be the more likely. Our evidence suggests that the β position is attacked preferentially when pyridine protohaemochrome is oxidized during coupled oxidation with a hydrogen doner. Although molecular orbital calculations[20] indicate a higher electron density at the α position of the porphyrin ring in the ground state the electron distribution in the activated state may be different. As well as this, π bonding from the central iron atom to the ligands may well alter the electron distribution in the co-ordinated compound.

Biliverdin IXβ (δ) is spectroscopically distinct from biliverdin IXα. However, the broadness of the peak in the red region of the spectrum makes structural assignments based on the visible spectrum unwise, particularly as oxidation of the vinyl groups during some preparative procedures occurs readily. Bilirubin IXβ (δ), on the other hand, shows a fairly sharp maximum at 402 mμ as compared with 450 mμ for bilirubin IXα and 435 mμ for mesobilirubin IXα. Although insufficient material was obtained after incubation of guinea pig liver homogenate with pyridine protohaemochrome to obtain a mass spectrum, the visible spectrum of the product strongly suggested it was biliverdin IXβ (δ). The use of large amounts of ascorbic acid by NAKAJIMA[18] for the induction of 'haem α methenyl oxygenase' together with the finding of other workers that the activity is heat-stable and dialysable suggests that oxidation of the non-physiological substrate, pyridine protohaemochrome, may at least partially

result from its non-enzymic coupled oxidation with ascorbic acid contained in the livers.

Formation or biliverdin IXβ (δ) *in vitro* strongly suggests that the physiological reaction is, however, enzymically controlled. Although coupled oxidation of haemoglobin *in vitro* produces a compound which appears to be largely biliverdin IXα yields were low and denaturation caused by oxidation of the cysteine residues in the protein caused much of the protein to precipitate.

Extension of the mass spectrometric method of isomer determination to bile pigments from natural sources reveals that after reduction the colouring matter of emu egg shells is indistinguishable from ox bile bilirubin. Traces of isomers other than the IXα are revealed as small peaks at *m/e* 374 and 360 and are also present in ox bile bilirubin. The intensity of these peaks suggests that these isomers contribute about 2–5 % of the total. Isomers other than the IXα have been detected in the early-labelled bilirubin fraction by PETRYKA[21] using the oxidation technique. This fraction is derived from hepatic sources as well as from ineffective haem utilisation during erythropoiesis[22]. However, the evidence of PETRYKA does not exclude formation of some of the IXβ or IXδ isomers during destruction of aged red cells.

Mesobilirubin derived from the phycobiliprotein R-phycoerythrin also appears to be of IXα type. In this case no other isomers could be detected. This would suggest a common oxidative mechanism for the degradation of porphyrin derivatives to bile pigments in both algae and animals if indeed the bile pigments of algae are formed, as recent work suggests[23], from metallo-porphyrin precursors. Possibly an enzyme of the mixed function oxygenase type such as that found by WISE AND DRABKIN[24,25] in dog placenta may have a more general role.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. P. S. CLEZY of the School of Chemistry, University of New South Wales for the NMR spectra reported here and Mrs. J. LANGLEY of the Mass Spectrometry Unit, University of Sydney for the mass spectra. Thanks are also due to Dr. R. LEMBERG of this Institute for the sample of Ceramium mesobilirubin and for helpful discussion. We are also grateful to the Kuring-gai Chase Koala Sanctuary, Sydney, for provision of the emu eggs. This work was supported by grants from the National Health and Medical Research Council (Australia), the Australian Research Grants Committee, and grant No. HE-07827 of the National Heart Institute, U.S. Department of Health.

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