The Prosthetic Group of Cytochrome Oxidase

2. CHEMISTRY OF PORPHYRIN *a*

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(Received 4 July 1960)

Early chemical studies relating to porphyrin *a*, the porphyrin derived from the prosthetic group of cytochrome oxidase, have been reviewed by Lemberg (1953), where the evidence is discussed in support of the presence in this molecule of a formyl group and an unsaturated side chain conjugated with the ring system. Analytical figures obtained from haemin *a* (Warburg & Gewitz, 1951) have suggested the presence of an alkyl side chain in haemin *a*, and this has been confirmed by the molecular-weight determinations by other workers (Lemberg, 1953; Granick, 1952). Warburg, Gewitz & Völker (1955) formulated this alkyl chain as an extended vinyl group.

Haemin *a* has been degraded by the resorcinol-melt treatment (Warburg & Gewitz, 1953; Warburg *et al.* 1955) to yield, after removal of iron, a deuteroporphyrin-like compound which was called cyto-deuteroporphyrin. However, bromine substitution indicated that cyto-deuteroporphyrin differed from a true deuteroporphyrin by containing three and not two unsubstituted β positions, and recently Marks, Dougall, Bullock & Macdonald (1959) have confirmed this and established the structure of cyto-deuteroporphyrin as I, by a direct synthesis of the molecule.

It is well established that a formyl group and an unsaturated side chain conjugated with the tetrapyrrolic ring system would be removed from a haemin by resorcinol-melt treatment, but the nature of the third substituent removed from haemin *a* during the conversion into cyto-deuterohaemin has not been determined.

Evidence has been presented (Barrett, 1959) for the presence of a hydroxyl group in porphyrin *a*. Since it has been established (Schumm, 1929) that α -hydroxyethyl groups are removed by the resorcinol-melt procedure, it was decided to investigate the possibility that this group was the third substituent removed during the formation of cyto-deuterohaemin. The results of this work together with other aspects of the chemistry of porphyrin *a* are given in this paper. Preliminary reports covering part of this work have been published elsewhere (Clezy & Barrett, 1959; Barrett & Clezy, 1959).

METHODS AND MATERIALS

Absorption spectra. Measurements were made with a Hilger Uvispek spectrophotometer or with a Beck-Hartridge reversion spectroscopy as indicated in the tables.

Solvents. Diethyl ether (not containing reducing agent) was used as supplied commercially. CHCl_3 was distilled to free it from trace metals. Acetone, used as a solvent for oxidation, was distilled over KMnO_4 . Pyridine was dried over KOH and distilled.

Chromatographic alumina. 'Aluminium oxide for chromatographic adsorption analysis' (British Drug Houses Ltd.) was used. This was washed with water until the washings were neutral to litmus paper, dried overnight at 60° and sieved through muslin.

Cellulose-powder chromatography. The preparation of the columns is described by Morell, Barrett & Clezy (1961). For 2–5 mg. amounts of porphyrin, columns 1 cm. $\times$ 40 cm. were used.

Paper chromatography. The method of Chu, Green & Chu (1951) with slight modifications was used. The methyl esters of the porphyrins were run on separate sheets of Whatman no. 1 paper in kerosene (commercial grade, Shell Co. Ltd.)–chloroform (4.0:2.6) solvent 1, and kerosene–propanol (5.0:1.0) solvent 2.

Introduction and removal of iron. Unless otherwise stated iron was introduced into porphyrins and removed from haemins by methods described in the previous paper (Morell *et al.* 1961).

Oxime reaction. A small amount of porphyrin was dissolved in pyridine–water (4:1, v/v). The solution was heated with excess of hydroxylamine hydrochloride for 20 min. in a boiling-water bath.

Bisulphite reaction. A small amount of porphyrin was dissolved in a pyridine–water (4:1, v/v) and to this solution was added a few crystals of NaHSO_3 . The mixture was shaken thoroughly and kept for $\frac{1}{2}$ hr. at room temperature (cf. Parker, 1959). The reaction is reversed by heat.

Acetate test for hydroxyl groups. This is described by Barrett (1959).

Chromium trioxide reagent for oxidation. The reagent (Jones reagent; Bowden, Heilbron, Jones & Weedon, 1946) was prepared as described by Djerassi, Engle & Bowers (1956).

Model porphyrins. Porphyrins, used as model compounds throughout this investigation, were prepared as follows: 2:4-diacetyldeuteroporphyrin dimethyl ester (Fischer & Zeile, 1929); monoacetyldeuteroporphyrin dimethyl ester (obtained as a by-product of the 2:4-diacetyldeuteroporphyrin preparation and separated from it by alumina chromatography); monoformyldeuteroporphyrin

dimethyl ester (Fischer & Wecker, 1942); chlorocruoroporphyrin (2-formyl-4-vinyldeuteroporphyrin) dimethyl ester (Lemberg & Parker, 1952); 2:4-diformyldeuteroporphyrin dimethyl ester (Lemberg & Parker, 1952). Monovinylmonoacetyldeuteroporphyrin was prepared by the partial reduction of diacetyldeuteroporphyrin with NaBH_4 and the subsequent dehydration of the monohydroxyethylmonoacetyldeuteroporphyrin formed at the reduction step. Monohydroxyethylmonovinyldeuteroporphyrin was prepared either by the reduction of monovinylmonoacetyldeuteroporphyrin or by partial hydration of protoporphyrin. Cryptoporphyrin *a* dimethyl ester was prepared by Miss J. Parker (Parker, 1959).

Porphyrim a. Porphyrim *a* used in this investigation was prepared by the methods described in the preceding paper (Morell *et al.* 1961).

Hydrochloric acid solutions. These are described in the preceding paper (Morell *et al.* 1961).

EXPERIMENTAL AND RESULTS

Acetal formation with carbonyl porphyrins

About 0.5 mg. of the porphyrin was dissolved in 5 ml. of methanol containing 3–4 drops of 10*N*-HCl, and the solution was boiled in a water bath for 5 min. After the solution was cooled, the porphyrin was returned to ether by using aqueous sodium acetate to neutralize the HCl. The ethereal solution was then washed free of acetic acid.

Porphyrins containing formyl groups conjugated with the ring system readily form dimethylacetals which show marked shifts of absorption maxima towards the blue end of the spectrum compared with the original formyl absorption bands (Table 1). Monoacetyl- and diacetyl-deuteroporphyrin exhibited little spectroscopic change when they were treated with methanolic HCl (see Table 1).

The acetal test allows distinction to be made between formyl and acetyl porphyrins, and since methylacetals can be hydrolysed by 5% HCl this group can be used to protect a formyl substituent in reactions carried out at neutrality.

Porphyrim *a* acetate

Porphyrim *ax* (8.5 mg., Lemberg & Stewart, 1955) was dissolved in 9 ml. of pyridine containing 1.5 ml. of acetic anhydride, and the mixture kept at room temperature for 3 days. The porphyrin was then returned to ether and the

pyridine removed by washing with 1% HCl. Methylation of the porphyrin carboxyl groups with diazomethane followed, and, after freezing out water from the ethereal solution at -15° , the porphyrin mixture was applied to a cellulose column in a solvent mixture containing 2 ml. of CHCl_3 and 4 ml. of light petroleum (b.p. 60–80°). The porphyrin was eluted with the same solvent mixture, by which porphyrin *a* ester acetate (early fractions) was separated from porphyrin *a* ester and degradation products. Paper chromatography in solvent system 2 was used to determine which fractions contained the acetate. A yield of 3 mg. of porphyrin *a* ester acetate was obtained; compared with the parent compound the acetate showed an average shift to the blue of its absorption bands of 2 $\text{m}\mu$. This spectral change was reversed on hydrolysis of the acetate.

From experiments in which the acetylation of porphyrin *a* β and porphyrin *ax* were carried out in parallel it was concluded that porphyrin *a* β acetylated more rapidly than did the α form. In general porphyrin *a* is not acetylated as readily as other formyl porphyrins containing α -hydroxyl groups which we have examined.

Oxidation of porphyrim *a* and its derivatives

The spectroscopic data relating to this series of experiments are shown in Table 2.

*Chromium trioxide oxidation of porphyrim *a* ester*. Porphyrim *a* dimethylester (1 mg.), dissolved in 5 ml. of acetone, was gassed with N_2 for 5 min., one drop of the $\text{CrO}_3\text{--H}_2\text{SO}_4$ reagent was added, and the oxidation allowed to proceed under N_2 for 1 min. The porphyrin was extracted into ether and the acetone washed out with water containing a little FeSO_4 . Extraction of the ethereal solution with an aq. 1% Na_2CO_3 soln. separated a small amount of an acidic oxidation product from the major non-acidic oxidation product.

The non-acidic oxidation compound showed carbonyl properties, and from an examination of the shift in spectrum (greater than for one formyl group) on oxime formation and from the band positions of its dimethylacetal (to the red of the acetal of porphyrim *a*) it was judged this compound contained a ketonic group as well as a formyl group. The acidic oxidation product obtained from porphyrim *a* also exhibited carbonyl properties (oxime reaction), and, moreover, it was demonstrated that this group was ketonic in character as negative bisulphite and acetal tests were obtained.

*Formation of porphyrim *a* acid from porphyrim *a* acetate*. Oxidation of porphyrim *a* acetate by the method given

Table 1. *Absorption spectra of carbonyl porphyrins before and after treatment with methanol-hydrochloric acid*

The solvent was ether. Spectra were determined with the spectrophotometer; Δ refers to the average shift in $\text{m}\mu$ of the four bands. Cryptoporphyrin *a* is a formyl porphyrin with one unsaturated side chain in which the double bond is conjugated to the ring (cf. Parker, 1959).

Porphyrin	Maxima (mμ)								Δ
	Before treatment				After treatment				
Monoformyldeuteroporphyrin	640.5	578	550.5	510.5	623	568	526.5	497	16
2:4-Diformyldeuteroporphyrin	648	593	557.5	521	625	570	528	498.5	25
Chlorocruoroporphyrin (2-formyl-4-vinyldeuteroporphyrin)	642	583	555	514.5	628	573	532	500.5	15
Cryptoporphyrin <i>a</i>	642	584	555	515	628	574	532	501	15
Porphyrim <i>a</i>	646	582	558	517	632	571.5	538.5	502.5	15
Monoacetyldeuteroporphyrin	634.5	576	543	505.5	633	576	542.5	503	1
2:4-Diacetyldeuteroporphyrin	639	586	546.5	512.5	638.5	584	545.5	510.5	1

Table 2. Absorption maxima of porphyrin *a*, porphyrin *a* alcohol, and the oxidation derivatives of porphyrin *a*. The solvent was ether. All measurements were made with the spectrophotometer.

The solvent was ether. All measurements were made with the spectrophotometer.																	
	Absorption maxima of porphyrin (m μ)				Absorption maxima of the dimethylacetal (m μ)				Absorption maxima of the acetate (m μ)				Oxime shift of original porphyrin-average of four bands				
	I		II		III		IV		I		II			III		IV	
	I	II	III	IV	I	II	III	IV	I	II	III	IV					
Porphyrin	646	582	558	517	631	571	538	503	0.88	645	581	556	516.5	2.0	7.6		
Porphyrin <i>a</i>	628	572	536	502	No acetal formed	No acetal formed				626	570.5	534	500	0.74	0.0		
Porphyrin <i>a</i> alcohol	642	579	552	511	1.79	No acetal formed				640	577	549	509	1.36	0.0		
Porphyrin <i>a</i> acid	648	590	558	519	1.25	636	582	542	0.71	No acetate formed					9.7		
Non-acid oxidation product of porphyrin <i>a</i> ester																	
Acidic oxidation product of porphyrin <i>a</i> ester	644	587	552	514	0.80	No acetal formed				No acetate formed				3.9			
Oxidation product of porphyrin <i>a</i> ester																	
Oxidation product of porphyrin <i>a</i> acid	644	588	552	514	0.78	No acetal formed				No acetate formed				3.7			

above yielded an acidic compound which showed no carbonyl properties. Hydrolysis of the acetate group, by keeping the material in 25% HCl overnight, gave a compound which can be formally derived from porphyrin *a* itself by the oxidation of the formyl group to a carboxylic acid group. This compound we propose to call porphyrin *a* acid.

*Oxidation of porphyrin *a* acid.* Oxidation of porphyrin *a* acid by the CrO₃ method gave a compound which was identical with the acidic oxidation product obtained from porphyrin *a* itself.

Oxidation of oximes of formyl porphyrins. Oxidation of the oximes of porphyrin *a*, hydrated porphyrin *a* (in which the double bond conjugated to the ring has been hydroxylated) and 2-formyl-4-hydroxyethyldeuteroporphyrin with CrO₃ gave compounds which showed a shift of their absorption bands towards the red. From a comparison of the relative shifts (Table 3), it is evident that two α -ketonyl groups are present in oxidized hydrated porphyrin *a* oxime.

*Oxidation of porphyrin *a* alcohol* (–CHO reduced to –CH₂·OH) by potassium permanganate. Porphyrin *a* alcohol (4.7 mg.) in 20 ml. of acetone, heated under reflux in a water bath, was oxidized with KMnO₄ (5 $\times$ molar proportion) in acetone. The KMnO₄ solution was added over 2–3 min. After cooling, the acetone solution was centrifuged to remove precipitated MnO₂, the porphyrin was extracted into ether, and the acetone removed by frequent washing with water. The porphyrin mixture was fractionated by chromatography on cellulose. Any re-formed porphyrin *a* or porphyrin *a* alcohol was eluted with ether or acetone; the new formyl porphyrin formed by the oxidation of the 'vinyl' group usually required the addition of methanol to the acetone to elute it and always travelled down the column behind porphyrin *a* and porphyrin *a* alcohol.

The porphyrin *a* was derived by oxidation of the hydroxymethyl group of porphyrin *a* alcohol, an oxidation possibly brought about by precipitated MnO₂, since we have shown (Barrett & Clezy, 1959) that hydroxymethyl groups are little affected by KMnO₄ in acetone. This was supported by the observation that the yield of porphyrin *a* was noticeably greater in preparations containing most lipid in which cases larger amounts of MnO₂ were generated. To avoid this type of oxidation porphyrin *a* alcohol can be acetylated.

We have shown elsewhere (Barrett & Clezy, 1959) that the presence of a formyl group in the porphyrin molecule inhibits the oxidation of a double bond conjugated to the ring, although R. Lemberg (unpublished work) has obtained from porphyrin *a*, in very small yield, a diformyl porphyrin by this method. However, prior reduction of the formyl group in porphyrin *a* is essential for a practical oxidation of the 'vinyl' group to a formyl group.

Reduction of the formyl group in porphyrin *a*

*With sodium borohydride (porphyrin *a* alcohol).* In a solution containing 1 mg. of porphyrin *a* in 20 ml. of ether was suspended 20 mg. of NaBH₄, and the mixture kept overnight. The excess of NaBH₄ was then decomposed with 0.5% HCl, leaving the reduction product, porphyrin *a* alcohol, in the ether. Unlike other carbonyl porphyrins which retain the four absorption bands of the neutral spectrum, porphyrin *a* on the addition of NaBH₄ to the ether shows a two-banded spectrum, reminiscent of that of a metal (eg. Na) complex. The dilute acid used to destroy the excess of reducing agent is sufficient to remove the

metal from the porphyrin *a* alcohol. The dimethylacetal of porphyrin *a* is not reduced by NaBH₄, porphyrin *a* itself being recovered after mild acid hydrolysis of the acetal group.

Wolff-Kishner reduction. Porphyrin *a* (20 mg.), moistened with 2-3 ml. of ethanol, was dissolved in 50 ml. of diethylene glycol. To this solution, 200 mg. of hydrazine sulphate and 400 mg. of KOH dissolved in 3 ml. of water were added, and the mixture was heated in an oil bath under a water condenser for 1 hr. The water was then run out of the condenser jacket, and the temperature in the reaction flask allowed to rise to 190-200° where it was maintained for a further hour. After cooling, the reaction

mixture was poured into 400 ml. of water, acidified with HCl and the porphyrin extracted into ether. The methyl ester of this porphyrin was purified by alumina chromatography. The yield was 30%.

By this procedure the formyl group in porphyrin *a* was reduced to a methyl group giving a product (I) which was spectroscopically similar to monohydroxyethylmonovinyldeuteroporphyrin. This reduction product exhibited no carbonyl properties (no oxime reaction) but was shown by acetylation to contain a hydroxyl group; this hydroxyl group could be oxidized by CrO₃-H₂SO₄ to a porphyrin (III) spectroscopically similar to monoacetylmonovinyldeuteroporphyrin (Table 4).

Table 3. *Absorption spectra of porphyrin oximes and their oxidation products*

The solvent was ether. Absorption spectra were determined with the spectrophotometer. Δ refers to the average shift of the four bands after oxidation.

Compound	Absorption maxima of porphyrin (mμ)				Ratio band III/ band IV	Δ (mμ)
	I	II	III	IV		
2-Formyl-4-hydroxyethyldeuteroporphyrin oxime	632	573	550	505	1.24	—
2-Formyl-4-hydroxyethyldeuteroporphyrin oxime oxidized	634	582	555	509	0.83	5
Porphyrin <i>a</i> oxime	638	578	547	509	1.46	—
Porphyrin <i>a</i> oxime oxidized	640	585	548	513	0.83	3
Hydrated porphyrin <i>a</i> oxime	632	576	543	518	—	—
Hydrated porphyrin <i>a</i> oxime oxidized	638	585	551	503	0.93	7

Table 4. *Absorption maxima of porphyrin *a* derivatives and model compounds*

The solvent was ether. All measurements were made with the spectrophotometer.

	Compound	Substituents at the 2, 4 and 8 positions of the porphyrin ring			Absorption maxima (mμ)				Ratio band III/ band IV
					I	II	III	IV	
I	Wolff-Kishner reduction product of porphyrin <i>a</i>	CH(OH)·R'	CH:CHR''	CH ₃	626	572	532	499	0.80
II	Monohydroxyethylmonovinyldeuteroporphyrin	or CH(OH)·CH ₃ CH:CH ₃	CH:CH ₂ CH(OH)·CH ₃	CH ₃	626	571	533	499	0.60
III	Oxidation compound of I	CO·R'	CH:CHR''	CH ₃	633	579	546	507	0.85
IV	Monoacetylmonovinyldeuteroporphyrin	or CO·CH ₃ CH:CH ₃	CH:CH ₂ CO·CH ₃	CH ₃	637	580	547	508	0.90
V	Resorcinol-melt product of the non-acid product of porphyrin <i>a</i> ester	CO·R'	H	H	—	—	545	505	1.0
VI	Resorcinol-melt product of III	CO·R'	H	CH ₃	633	575	544	504	0.95
VII	Monoacetyldeuteroporphyrin	or CO·CH ₃ H	H CO·CH ₃	CH ₃	633	576	543	505	1.10
VIII	Dehydration product of porphyrin <i>a</i>	CH:CHR'	CH:CHR''	CHO	648	584	562	520	2.0
IX	Dehydration product of porphyrin <i>a</i> acid	CH:CHR'	CH:CHR''	CO ₂ H	645	582	553.5	513	1.5
X	Dehydration product of porphyrin <i>a</i> alcohol	CH:CHR'	CH:CHR''	CH ₃ ·OH	633	579	540.5	505	0.80
XI	Porphyrin <i>a</i> hydrated	CH(OH)·R'	CH(OH)·R''	CHO	643	579	554.5	512	1.5
XII	Porphyrin <i>a</i> alcohol hydrated	CH(OH)·R'	CH(OH)·R''	CH ₃ ·OH	628	572	536	502	0.78
XIII	Monoformylmonohydroxyethyldeuteroporphyrin	CHO	CH(OH)·CH ₃	CH ₃	639	580	554	514	1.68
XIV	Cytodeuteroporphyrin*	—	—	—	619	566	530	496	0.53

* Derived from the haemin obtained from the resorcinol-melt treatment of haemin *a*. Solvent, chloroform.

Dehydration of porphyrin a and its derivatives

The classical method of dehydration by heating *in vacuo* was not successful with porphyrin *a* or its derivatives as much polymerization occurred. Hence other methods of dehydration were examined and the following procedures were found to dehydrate satisfactorily porphyrin *a* acid and porphyrin *a* alcohol. Porphyrin *a* itself appeared to be more resistant to dehydration than these two derivatives, but the second procedure yielded a dehydration product from porphyrin *a*. Spectroscopic data are given in Table 4.

Dehydration by heating in pyridine with toluene-p-sulphonyl chloride. Porphyrin (1 mg.) was heated in 4 ml. of pyridine containing 20 mg. of toluene-*p*-sulphonyl chloride on a boiling-water bath for 7 hr. The porphyrin was then extracted into ether and the pyridine removed by repeated extraction with 1% HCl.

Dehydration by heating with toluene-p-sulphonic acid in benzene. Porphyrin ester (1 mg.) was heated in 5 ml. of benzene containing 2 mg. of toluene-*p*-sulphonic acid for 6 hr. For porphyrins such as haematoporphyrin, which contained no electronegative side chains, dehydration was complete in this time. With porphyrin *a* and its derivatives the benzene solution after cooling was poured on to a cellulose column, the column was washed with light petroleum and then ether, the latter eluting porphyrin *a* from which the hydroxyl group has been removed.

By these methods porphyrin *a* acid and porphyrin *a* have been dehydrated to give compounds which no longer contain hydroxyl groups and which show a shift in absorption maxima of 2–3 μ towards the red end of the spectrum. Porphyrin *a* alcohol can also be dehydrated to a compound which shows a similar spectroscopic shift but this compound still contains a hydroxyl group, the hydroxymethyl group formed by the reduction of the formyl group in porphyrin *a*.

Although it seems that α -hydroxyethyl porphyrins are dehydrated more readily under acidic than basic conditions, the ease of this reaction is governed primarily by the nature of the other substituent groups. In a series of porphyrins, (I) monohydroxymethyl-, (II) monovinyl-, (III) monocarboxy-, (IV) monoacetyl-, and (V) monoformyl-monohydroxyethyldeuteroporphyrin, the relative rate of dehydration can be indicated as $I = II \gg III = IV > V$. Thus, in accord with expectations, the ease of dehydration decreases as the electronegative nature of the substituent increases.

Hydration of vinyl-substituted porphyrins

The porphyrin (2 mg.) was treated with 1 ml. of HBr in acetic acid [50% (w/v) HBr, British Drug Houses Ltd.] in a stoppered tube overnight. To this was then added 10 ml. of 5% HCl and the solution, after gassing with N_2 , was left at room temperature for 24 hr. The hydrated porphyrin was then extracted into ether over an aq. 10% sodium acetate soln.

As would be expected both chlorocruoroporphyrin and porphyrin *a* were hydrated more slowly than protoporphyrin.

Resorcinol-melt treatment of haemin a derivatives at 200°

In an unpublished investigation carried out in this Laboratory we have observed that α -ketonyl and carboxyl groups in conjugation with porphyrin ring are not removed in the resorcinol melt if this is carried out below 200°,

whereas hydroxyethyl, hydroxymethyl, formyl and vinyl groups are thereby removed from the porphyrin. This greater stability of the acetyl groups has been utilized in the following experiment.

A mixture of haemin and five times its weight of resorcinol was heated at 190–195° for 15 min. After cooling, the reaction mixture was dissolved in ether, and this solution was washed well with 1% HCl which removed yellow by-products. After the ethereal solution had been dried (–15° overnight), the solvent was removed *in vacuo* to leave the crude haemin which was converted into the corresponding porphyrin by using $FeSO_4$ in acetic acid (100 moles of $FeSO_4$ /mole of porphyrin). The crude porphyrin was purified by acid fractionation and by alumina chromatography of the dimethyl esters.

The non-acidic compound obtained by the oxidation of porphyrin *a* dimethyl ester, and that (III, Table 4) obtained by the oxidation of the Wolff–Kishner reduction product of porphyrin *a* were converted into their respective haemins and subjected to the resorcinol-melt treatment. Both (V and VI, Table 4) yielded porphyrins spectroscopically similar to monoacetyldeuteroporphyrin (VII, Table 4) but differed significantly from this model porphyrin in R_F values (solvent 1; compounds V and VI, 0.52; compound VII, 0.49; solvent 2; compounds V and VI, 0.68; compound VII, 0.55) and in HCl number (compounds V and VI, approx. 12; compound VII, 4).

Comparison of the relative rate of formation of porphyrin $\alpha\beta$ from porphyrin $\alpha\alpha$ and the hydroxylation of protoporphyrin in hydrochloric acid

It has been shown by Lemberg & Stewart (1955) that porphyrin *a* is transformed into the β form spectrally identical with porphyrin $\alpha\alpha$ by standing in dil. HCl. Since the vinyl groups of protoporphyrin are hydrated under these conditions, we considered the possibility that the $\alpha \rightarrow \beta$ transformation was due to the hydroxylation of a double bond, in one of the alkyl side chains, not conjugated to the porphyrin ring. The respective porphyrins (2 mg.) were kept in 20 ml. of 2, 5 and 20% HCl at room temperature for 1–4 days. The porphyrins were extracted into ether, and the ether solution was analysed for the content of porphyrin $\alpha\beta$ in the one series and for monovinylmonohydroxyethyldeuteroporphyrin and haematoporphyrin in the other. Porphyrin $\alpha\beta$ was readily formed from porphyrin $\alpha\alpha$ in 2 and 5% HCl, more slowly in higher concentrations. The hydroxylation of the vinyl group was similarly more efficient at the lower concentrations of HCl. However, there was a very marked difference in the rate of formation of porphyrin $\alpha\beta$ and the hydroxylation of the vinyl groups. After 24 hr. in 5% HCl there was a 50% conversion into porphyrin $\alpha\beta$ and by 3 days this was complete, whereas after 14 days in 5% HCl only 10% of the protoporphyrin had been hydrated. Though protoporphyrin is not a strict model the large difference in rates suggest strongly that the addition of water to a double bond in an alkyl side chain is not the explanation of the $\alpha \rightarrow \beta$ transformation.

DISCUSSION

Analytical determinations on haemin *a* (Warburg & Gewitz, 1951) indicated the presence of at least six oxygen atoms in this molecule. Five of

these atoms are accounted for by the two propionic acid side chains and by the formyl group, and it seemed likely that the sixth oxygen atom provided an activating influence which resulted in the loss of the third and unknown substituent during the conversion of porphyrin *a* into cytochrome c. We have therefore attempted to characterize the group containing this sixth oxygen atom.

The magnitude of shift of absorption bands to the blue upon oxime formation can be taken as a measure of the number of carbonyl groups in the porphyrin molecule. From Table 5 it is apparent that the oxime shift observed with porphyrin *a* is of the same order as those found for other monoformyl porphyrins and is smaller than for diformyldeuteroporphyrin and diacetyldeuteroporphyrin. This indicates that neither a second formyl group nor an acetyl group conjugated with the ring system is present in porphyrin *a*. Moreover, there is additional evidence for excluding from porphyrin *a* an acetyl group conjugated with the ring system. Porphyrin *a* is readily reduced by sodium borohydride but porphyrin *a* dimethylacetal is spectroscopically unaltered by this reagent. It has been shown above that acetyl porphyrins do not form acetals under the conditions quoted in this paper and yet are readily reduced by sodium borohydride; that no spectroscopic change occurs when porphyrin *a* dimethylacetal is treated with borohydride argues against the presence in the porphyrin *a* molecule of an acetyl group conjugated with the ring system. In addition, we have shown in experiments with model compounds that acetyl groups are substantially unaffected by the resorcinol-melt procedure when it is carried out below 200°. Thus it can be concluded that the formyl group is the only carbonyl substituent, conjugated with the tetrapyrrolic ring system in porphyrin *a*.

Experiments reported in this paper show, however, that a ketonyl group in conjugation with the porphyrin ring system can be introduced into porphyrin *a* and porphyrin *a* acid by oxidation with chromium trioxide. Since these ketonyl compounds no longer contained hydroxyl groups (acetylation test) we assume that the new carbonyl group was formed by the oxidation of the hydroxyl

group shown to be present in porphyrin *a* (Barrett, 1959). In addition it has been demonstrated in this paper that this hydroxyl group can be removed from porphyrin *a* and its derivatives by dehydration procedures, the resulting compound showing shifts in absorption maxima to the red end of the spectrum indicating the formation of a carbon-carbon double bond conjugated with the porphyrin ring system. An examination of the spectrum of porphyrin *a* shows that it lies 4 m μ (average of the four bands) towards the red compared with the spectrum of monoformylmonohydroxyethyldeuteroporphyrin. This difference of 4 m μ can be accounted for entirely by the presence, in addition to the hydroxyalkyl and formyl groups, of the group —CH:CHR known to be present in porphyrin *a*.

The synthetic work of Marks *et al.* (1959) has established the structure of cytochrome c as (I) and shown that the unsubstituted β -pyrrolic carbon atoms present in this molecule occur at positions 2, 4 and 8. Earlier Lemberg (1953) from the spectroscopic properties of porphyrin *a* deduced that the formyl group and the unsaturated side chain must occupy opposite pyrrolic rings. This

Table 5. Absorption maxima of carbonyl porphyrins and their oximes in pyridine

Determinations were made with a Beck-Hartridge reversion spectroscope. Δ refers to the average shift of the four bands.

Porphyrin	Porphyrin bands (m μ)				Oxime bands (m μ)				Δ
	638.4	583.4	551.5	513.0	630.0	578.2	540.9	504.8	
Monoformyldeuteroporphyrin	648.3	594.6	560.5	525.4	638.9	586.7	548.3	512.5	10.7
2:4-Diformyldeuteroporphyrin	642.7	587.6	556.3	517.2	635.8	583.1	544.9	509.6	7.6
Chloroeruoroporphyrin	634.8	580.0	545.7	506.9	623.4	574.3	534.3	500.6	9.6
Monoacetyldeuteroporphyrin	638.9	587.8	552.7	515.5	625.5	576.6	537.1	503.5	13.0
2:4-Diacetyldeuteroporphyrin	645.0	588.0	560.5	519.0	637.2	580.4	551.6	513.0	7.6

deduction is substantiated by the lowering of the ratio of band III/band IV of porphyrin *a* on hydration of the double bond (see Table 4). The formyl group and unsaturated side chain must then occupy positions 4 and 8. The hydroxyl group in porphyrin *a* must be so placed that on oxidation a keto group is produced which is conjugated with the porphyrin ring system. This limits its placement to either the γ -carbon atom of the extended vinyl chain or the α -carbon atom of the grouping at position 2. A decision between these two alternatives can be made on the available spectroscopic evidence. If the hydroxyl group were placed on the γ -carbon atom of the unsaturated chain, oxidation to a keto group or dehydration to produce a double bond would increase the electronegativity of this group. This would tend to raise the ratio of band III/band IV due to the presence of the formyl group in the opposite pyrrolic ring. However, oxidation of the hydroxyl group or its removal by dehydration lowers the ratio of band III/band IV in porphyrin *a* or porphyrin *a* acid (see Tables 2 and 4) indicating that the new electrophilic group has been introduced into a ring adjacent to the pyrrole ring carrying the formyl or carboxyl acid groups. Thus the hydroxyl group in porphyrin *a* is placed on the α -carbon atom of the group situated at position 2. On the basis of this evidence porphyrin *a* can be formulated as (II) or (III).

Piatelli (1960) has distinguished between these possibilities by identifying the pyrrolic acid (IV) as one of the oxidation products of porphyrin *a* nitrile, obtained by the dehydration of porphyrin *a* oxime. This indicates that the formyl group present in porphyrin *a* occurs on one of the pyrrolic rings carrying a propionic acid side chain and this, coupled with the structure given by Marks *et al.* (1959) for cytodeuteroporphyrin, has led us to propose (see Lemberg, Clezy & Barrett, 1959) formula (III) as the structure for porphyrin *a*.

The Wolff-Kishner reduction of porphyrin *a* gave a compound spectroscopically similar to monohydroxyethylmonovinyldeuteroporphyrin. Oxidation of this compound by the Jones reagent gave a ketonyl porphyrin spectroscopically similar to monovinylmonoacetyldeuteroporphyrin. The production of both these compounds is in accord with structure (III) for porphyrin *a*. When this carbonyl porphyrin was subjected to the resorcinol-melt procedure another carbonyl porphyrin was obtained, which, although resembling monoacetyldeuteroporphyrin spectroscopically, differed from it in R_f and had a significantly higher HCl number than the model porphyrin. A ketonyl porphyrin of similar type was obtained by resorcinol melt of the non-acidic oxidation product of porphyrin *a* ester. Hence it must be assumed that the ketonyl porphyrins obtained from porphyrin *a* by the above procedures are not simple acetyl porphyrins, and it follows that the chain carrying the other group in porphyrin *a* itself is not simply an α -hydroxyethyl group. This would indicate that at least some of the 12-16 carbon atoms, which form the long alkyl side chain postulated by Warburg to account for his analytical figures from haemin *a*, are present in position 2. Warburg *et al.* (1955) had previously concluded that the unsaturated side chain was extended to include these extra carbon atoms since, after hydrogenation, haemin *a* no longer yielded crystalline material when subjected to the Schumm melt. Piatelli (1960) also has found evidence that points to the unsaturated side group in porphyrin *a* not being a simple vinyl group since the pyrrolic acid (V) was not amongst the oxidation products of hydrogenated porphyrin *a*. It would then seem that porphyrin *a* has two extended carbon chains, and this molecule can be represented by (III), where R' and R'' are the two alkyl side chains the constituents of which have yet to be determined.

The formulation of the carbon-carbon double bond as $\text{—CH:CHR}'$ needs further confirmation. The appearance of an α -ketonyl group on this side chain after oxidation of hydrated porphyrin *a* oxime and the production of a formyl porphyrin by oxidative cleavage of this double bond both require the presence of an α -CH group at position 4, but there is no such evidence to confirm the presence of a hydrogen on the β -carbon atom. Also the carbon β to the porphyrin ring at position 2 may not simply be a methylene group, although at least one hydrogen must be so placed to account for the dehydration experiments.

In other reports from this Laboratory (Lemberg, 1955; Lemberg & Stewart, 1955) mention has been made of the $\alpha \rightarrow \beta$ conversion whereby the naturally occurring porphyrin *ax* of high HCl number is transformed by the action of dilute hydrochloric acid into porphyrin *ab* of low HCl number. The

reason for this transformation is not yet understood but some progress has been made in determining the position of the groups involved in this conversion. The $\alpha \rightarrow \beta$ transformation does not occur in simple porphyrins so that it must be associated with one of the substituents at positions 2, 4 or 8, on the tetrapyrrolic ring. The formyl group is excluded, as porphyrin α alcohol can exist in either the α or β form. Since the formyl porphyrin obtained by oxidative scission of the unsaturated side chain in position 4 of porphyrin α alcohol can undergo the $\alpha \rightarrow \beta$ conversion, the locus of this transformation must lie in the hydroxyalkyl side chain at the 2 position of the ring system. Since α -hydroxyethyl porphyrins are known not to give this type of transformation, this is further evidence that this chain is extended beyond two carbons.

Both in partition studies and on chromatography porphyrin $\alpha\beta$ behaves as a more polar compound than porphyrin $\alpha\alpha$. That these properties are due to hydration of a double bond, isolated from the porphyrin ring, is unlikely, since the $\alpha \rightarrow \beta$ transformation persists after potassium permanganate treatment of porphyrin α . The comparative rates of formation of porphyrin $\alpha\beta$ and the hydration of the vinyl groups of protoporphyrin in hydrochloric acid, although the latter compound is not a strict model because of conjugation of the double bonds to the ring system, support this conclusion.

The high HCl number of porphyrin α has been

assumed to be due to the alkyl side chains present in this molecule. However, this cannot be the full explanation of this property as cryptoporphyrin α , a formyl porphyrin containing a large alkyl side chain (Parker, 1959), has an HCl number comparable with those of other monoformyl porphyrins. Further, the β form of porphyrin α has an HCl number of 5, which is very similar to that of monoformylmonohydroxyethyldeuteroporphyrin. Thus the high HCl number of naturally occurring porphyrin α must be a characteristic of the α form of this compound and not directly a result of the carbon chains in the molecule.

Morrison & Stotz (1959) and Morrison, Connelly, Petix & Stotz (1960), from a spectroscopic study of the pyridine haemochromes or haemin α and its derivatives, have concluded that a formyl group and a double bond conjugated with the porphyrin ring system are not sufficient by way of electro-negative substituents to account for the spectroscopic properties of this molecule (cf., however, Lemberg *et al.* 1959). In particular they draw attention to the difference of 8.6 m μ (Lemberg & Falk, 1951) between the pyridine haemochrome of haemin α oxime and chlorocruorohaemin oxime, and suggest this is due to a keto group in the haemin α molecule, since Lemberg & Falk have shown that acetyl haemins do not react with hydroxylamine. Morrison *et al.* (1960) find support for the presence of a keto group in haemin α from the fact that dimedone, which reacts specifically with alde-

hydes, yields a derivative with haemin *a* having a pyridine haemochrome at 558 m μ , whereas reduction of haemin *a* with sodium borohydride, which would reduce all carbonyl groups present, gives a haemin with a haemochrome at 552 m μ . These authors claim support for a second carbonyl grouping in haemin *a* from infrared spectroscopy. To explain these findings they propose that haemin *a* contains at position 2 the grouping $\text{—}\overset{\alpha}{\text{C}}\text{H}(\text{OH})\cdot\overset{\beta}{\text{C}}\text{O}$.

It does not appear possible to reconcile such a formulation with the evidence put forward in this paper. In particular, it is difficult to explain the dehydration of porphyrin *a* and its derivatives in terms of such a structure. The absorption maxima of our dimethylacetal of porphyrin *a* lie about 2 m μ to the red of the corresponding bands of porphyrin *a* alcohol. This is an analogous situation to the dimerized-reduced haemin *a* comparison of Morrison *et al.* (1960). However, as mentioned earlier in this paper, porphyrin *a* dimethylacetal is quite stable to sodium borohydride, which indicates that any spectroscopic difference between it and the alcohol is not due to the presence of a carbonyl group in the molecule. Further, porphyrin *a* and chlorocruoroporphyrin have a very different orientation of substituents, and this may be the explanation of some of the spectroscopic differences between the two haemins reported by Morrison *et al.* (1960).

As is shown in Table 2, the acetylation of porphyrin *a* and its derivatives result in a shift of absorption maxima towards the blue. This change is reversed upon hydrolysis of the acetate. Morrison *et al.* (1960) have also observed this type of spectroscopic shift when studying the pyridine haemochrome of haemin *a* and its acetate. This change must involve more than a simple acetylation of a hydroxyl group since model porphyrins hydroxylated on a carbon α to the ring system, e.g. haematoporphyrin or 2-formyl-4-hydroxyethyldeutero-porphyrin, do not exhibit this spectroscopic shift on acetylation. This shift on acetylation also occurs in the β form of porphyrin *a* and is therefore not contingent on this transformation. An explanation of this alteration and of the $\alpha \rightarrow \beta$ conversion must await the elucidation of the full structure of the substituent at the 2 position.

SUMMARY

1. Porphyrin *a* can be oxidized to a carbonyl derivative and dehydrated to a compound which contains a second carbon-carbon double bond. Since both these new functional groups are in conjugation with the pyrrolic ring system it is concluded that porphyrin *a* has a hydroxyl group on a carbon α to the porphyrin ring.

2. Evidence is presented which indicates that the α -hydroxyalkyl side chain is situated at the 2 position of the ring system and is of greater length than two carbon atoms. It has been shown that this side chain contains the locus of the $\alpha \rightarrow \beta$ transformation of porphyrin *a*.

3. From evidence presented in this paper porphyrin *a* can be described as 1:3:5-trimethyl-2-(α -hydroxyalkyl)-4-(β -alkylvinyl)-6:7-di-(β -carboxyethyl)-8-formylporphyrin.

We wish to thank Dr R. Lemberg, F.R.S., for his interest and encouragement throughout this work. We acknowledge support from the National Health and Medical Research Council of Australia.

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