The Chemical Structure of Pemptoporphyrin

BY S. SANO AND T. SHINGU

*Department of Public Health, Faculty of Medicine, Faculty of Pharmaceutical Sciences,
Kyoto University, Kyoto, Japan*

AND J. M. FRENCH AND ELIZABETH THONGER

*The Wellcome Research Wing, University Department of Medicine, Queen Elizabeth Hospital,
Birmingham*

(Received 2 March 1965)

1. Pemptoporphyrin dimethyl ester was further purified on an alumina column and then crystallized from chloroform and methanol with melting point 215–218°. The absorption spectra in the visible and ultraviolet with molar extinction coefficients are presented. 2. The nuclear-magnetic-resonance spectrum of pemptoporphyrin dimethyl ester in deuteriochloroform solution is reported, together with those of several other porphyrins. 3. One vinyl and one hydryl group were found at positions 2 and 4 (or 4 and 2) in the molecule. The structure of pemptoporphyrin is 2(4)-hydryl-1,3,5,8-tetramethyl-4(2)-vinyldeuterioporphyrin IX. 4. Reduction of pemptoporphyrin dimethyl ester with resorcinol gave deuterioporphyrin IX dimethyl ester.

French, England, Lines & Thonger (1964) first obtained the new porphyrin 'pemptoporphyrin' in the faeces of human subjects by using counter-current distribution. The mixture of porphyrin removed from faeces with 5% (w/v) hydrochloric acid yielded five distinct porphyrins, which were identified as coproporphyrin, deuterioporphyrin, mesoporphyrin and protoporphyrin together with pemptoporphyrin. However, the chemical structure of pemptoporphyrin is still unknown. From the absorption maximum at 404–405 m μ in the Soret band and its tube position in countercurrent distribution, it might be an intermediate between protoporphyrin and either mesoporphyrin or deuterioporphyrin. A single attempt to convert the postulated vinyl side chain of pemptoporphyrin into ethyl with methyl iodide according to the method described by Rimington (1938) resulted in a 40% yield of a porphyrin that on countercurrent analysis lay in a tube position intermediate between mesoporphyrin and deuterioporphyrin with a Soret peak at 402 m μ . Therefore it may be an intermediate between protoporphyrin and deuterioporphyrin.

In the present paper, further purification of pemptoporphyrin is described and its absorption spectra, molar extinction coefficient and melting point are recorded. The chemical structure of pemptoporphyrin was determined by using nuclear-magnetic-resonance (n.m.r.) spectrometry, which has recently been employed for characterizing porphyrins (Abraham, Jackson & Kenner, 1961;

Becker, Bradley & Watson, 1961; Caughey & Koski, 1962; Caughey & Iber, 1963). Detailed assignment of the spectral lines to the substituent groups in the molecules are presented. The n.m.r. spectra of the methyl esters of protoporphyrin IX, deuterioporphyrin IX, mesoporphyrin IX and haematoporphyrin IX are also presented.

EXPERIMENTAL

Porphyrins. Pemptoporphyrin was prepared from the faeces of a woman patient with intestinal malabsorption from a combination of adult coeliac disease and partial gastrectomy. Separation from other ether-soluble faecal porphyrins was achieved by the method described by French *et al.* (1964), except that to increase solubility the equilibration mixture for countercurrent analysis contained acetic acid (ether-acetic acid-0.05 N-HCl, 3:1:2, by vol.).

Crude pemptoporphyrin (about 30 mg.) was esterified by contact with 15% (w/w) HCl in methanol at 3° for 40 hr. and purified on an alumina column by using CHCl₃-methanol (200:1, v/v). The main band of pemptoporphyrin dimethyl ester was the fastest band obtained; green-brown porphyrin ran through slowly and some impure material remained at the top of the column. The main fraction so obtained was purified three times on an alumina column and the eluate was evaporated to dryness under reduced pressure at 35–40°. Pemptoporphyrin dimethyl ester was crystallized from CHCl₃ and methanol (Fig. 1). The m.p. was 215–218°. The yield was 7.5 mg.

Protoporphyrin dimethyl ester was prepared according to Ramsey's (1953) method. The m.p. was 231°.

Mesoporphyrin was prepared by reduction of proto-

porphyrin IX with colloidal palladium and hydrogen according to the method of Granick (1948). Mesoporphyrin dimethyl ester was prepared from mesoporphyrin. The m.p. was 216°.

Haematoporphyrin dimethyl ester was prepared according to the method of Sano, Nanzyo & Rimington (1964). The m.p. was 212–214°.

Solvents. Ether, ethyl acetate and acetic acid were freshly distilled to remove peroxide or unidentified oxidants. Chloroform was also distilled before use.

Alumina. Alumina standardized for chromatographic adsorption analysis according to Brockmann (E. Merck A.-G., Darmstadt, Germany) was used.

Absorption spectra. The absorption spectra in the visible and Soret regions were measured with a Hitachi recording spectrophotometer.

Nuclear-magnetic-resonance spectra. The n.m.r. spectra were obtained with a Varian Associates (Palo Alto, Calif., U.S.A.) spectrometer, model A60 system, operating at 60 Mc/sec. Spectra were scanned from low to high field at rates of 12–60 cyc./sec./min. In all cases the porphyrin samples were dissolved in deuteriochloroform (CDCl_3) at concentrations of approx. 0.1–0.24 M (20–22 mg. in 0.4–0.15 ml. of CDCl_3). Tetramethylsilane (TMS) was used as

an internal reference. The data are expressed in p.p.m. referred to 10.00 (τ values), according to the procedure of Tiers (1958).

RESULTS AND DISCUSSION

Absorption spectra of pemptoporphyrin dimethyl ester. The absorption spectra and molar extinction coefficient of pemptoporphyrin dimethyl ester are recorded in Table 1.

Paper chromatography of pemptoporphyrin dimethyl ester. Kerosene–chloroform paper chromatography (Chu, Green & Chu, 1951) of pemptoporphyrin dimethyl ester showed one spot at R_f 0.82. This suggests that no hydroxyl group is present in the molecule. Lutidine–aq. ammonia paper chromatography (Falk & Benson, 1953) of free pemptoporphyrin showed two ionizable carboxyl groups/mol.

Nuclear-magnetic-resonance spectrum of pemptoporphyrin dimethyl ester. Fig. 2(a) shows the n.m.r. spectrum of pemptoporphyrin dimethyl ester, and its chemical shifts are given in Table 2.

(a) Vinyl group. The signal of the vinyl group in the molecule was observed as the ABX type with typical J values of $AB = 2$ cyc./sec., $AX = 12$ cyc./sec. and $BX = 17$ cyc./sec. at 5.85–6.30 p.p.m. (4.15–3.70 τ) and 7.71–8.20 p.p.m. (3.29–1.80 τ). The former is assigned to the vinyl β -hydrogen (vinyl CH_2 , $\text{CH}=\text{CH}_2$) and the latter as the vinyl α -hydrogen (proximal, vinyl CH , $\text{CH}=\text{CH}_2$). The proton numbers calculated from the integrating curve were 2 and 1 respectively. This means that only one vinyl group exists at position 2 or 4 in the molecule. As a control protoporphyrin dimethyl ester was used: its two vinyl groups appeared at the same region with proton numbers 4 and 2 respectively.

(b) Ring H (the hydrid attached directly to the pyrrole ring). The ring H was observed at 8.67 p.p.m. (1.33 τ) as a quartet form with J values of 1 cyc./sec. coupled with the methyl group in the same pyrrole ring. The proton number calculated from the integrating curve was 1. Therefore one

Fig. 1. Crystals of pemptoporphyrin dimethyl ester ($\times 1000$)

Table 1. *Absorption bands of pemptoporphyrin dimethyl ester*

The ratios of band intensities, in parentheses, are calculated relative to band IV for neutral spectra and to band II for acid spectra.

Solvent		Maxima					Minima			
		I	II	III	IV	V				
CHCl_3	λ (m μ)	625	572	536	502	405	607	555	521	461
	$\epsilon_{0.1\text{mm}}$	0.420	0.645	1.04	1.365	16.8	0.108	0.192	0.387	0.258
		(0.31)	(0.47)	(0.76)	(1.0)	(12.3)	(0.079)	(0.14)	(0.28)	(0.189)
N-HCl	λ (m μ)	594	551	405				581		480
	$\epsilon_{0.1\text{mm}}$	0.507	1.55	33.3				0.413		0.079
		(0.327)	(1.0)	(21.4)				(0.266)		(0.0509)

Fig. 2. Nuclear-magnetic-resonance spectra of porphyrin esters: (a) pemttoporphyrin dimethyl ester (approx. 0.1M); (b) protoporphyrin dimethyl ester (approx. 0.1M); (c) deuterioporphyrin dimethyl ester (approx. 0.1M).

Fig. 2. Nuclear-magnetic-resonance spectra of porphyrin esters: (d) deuterioporphyrin dimethyl ester (approx. 0.27 M); (e) haematoporphyrin dimethyl ester (approx. 0.1 M); (f) mesoporphyrin dimethyl ester (approx. 0.1 M).

hydriyl group is attached directly to the ring at either position 2 or position 4 in the molecule. A control sample, deuteroporphyrin IX dimethyl ester, which has two ring H at positions 2 and 4, showed an absorption signal at 8.88 p.p.m. (1.12 τ).

(c) Ring CH₃ (the methyl groups attached directly to the pyrrole ring). The ring CH₃ at positions 1 and 3 in the molecule were observed at 3.36 p.p.m. (6.64 τ) and 3.40 p.p.m. (6.60 τ). The former signal showed a doublet with J values of 1 c/c./sec., which is largely attributable to the effect of ring H in the same pyrrole. The ring CH₃ at positions 5 and 8 were observed at 3.30 p.p.m. (6.70 τ), which was often overlapped with that of β -methylene of propionic acid. All the proton numbers were 12. The ring CH₃ of deuteroporphyrin dimethyl ester in positions 1 and 3 showed doublets, as with pemptoporphyrin.

(d) Methene. The methene protons were observed at 9.53 p.p.m. (0.47 τ , 1H), 9.56 p.p.m. (0.44 τ , 2H) and 9.68 p.p.m. (0.32 τ , 1H) as three signals (in multiple form). The proton number calculated was 4.

(e) α -Methylene, β -methylene and ester methyl of propionic acid ester. Signals of α -methylene (proximal) and β -methylene were observed at 4.13 p.p.m. (5.87 τ) and 3.08 p.p.m. (6.92 τ) as triplet forms respectively with J values of 7.1 c/c./sec. The proton numbers were 4 and 4. A signal of ester methyl was observed at 3.60 p.p.m. (6.40 τ) and the proton number calculated was 6.

(f) N-H. The peak at extremely high field at -4.62 p.p.m. (14.62 τ) results from protons bound to nitrogen at the centre of the ring. This might be explained by ring current of the porphyrin ring.

From these observations on n.m.r. spectra, the structure (I) of pemptoporphyrin is 2(4)-hydriyl-1,3,5,8-tetramethyl-4(2)-vinyldeuteroporphyrin IX.

(I)

Nuclear-magnetic-resonance spectra of other porphyrin esters. The resonance frequencies of all the substituent groups common to all the porphyrins (pemptoporphyrin, protoporphyrin, deuteroporphyrin, haematoporphyrin and mesoporphyrin), such as α -methylene, β -methylene and ester methyl of propionic acid ester, ring methyl, methene and NH group, are recorded in Table 2. Assignments of position as a result of special substitution of each porphyrin are described as follows.

(a) Protoporphyrin dimethyl ester (Fig. 2b). The signals of vinyl groups were observed as the ABX type with typical J values of AB = 2 c/c./sec., AX = 12 c/c./sec. and BX = 17 c/c./sec. at 5.88–6.33 p.p.m. (4.12–3.67 τ) and 7.63–8.23 p.p.m. (2.37–1.77 τ). The former signals show the vinyl β -hydrogens and the latter show the vinyl α -hydrogens with proton numbers 4 and 2.

(b) Deuteroporphyrin dimethyl ester (Fig. 2c). The signal of the two ring H in positions 2 and 4 was observed at 8.88 p.p.m. (1.12 τ). The signals of the ring CH₃ at positions 1 and 3 were observed at 3.57 (6.43 τ) and 3.59 p.p.m. (6.41 τ) as two doublets with J values of 1 c/c./sec., but the signals of the ring CH₃ at positions 5 and 8 were found at 3.42–3.47 p.p.m. (6.58–6.53 τ) as two single peaks. In a concentrated solution (22.5 mg./0.15 ml.) the positions of the peaks are shifted towards a high field with 15 c/c./sec. (Fig. 2d).

(c) Haematoporphyrin dimethyl ester (Fig. 2e). The methyl protons of the ring-substituted secondary alcohol at positions 2 and 4 give rise to two doublets at 1.67 p.p.m. (8.33 τ) and 1.76 p.p.m. (8.28 τ) with a J value of 6 c/c./sec. The methene proton of the secondary alcohol appeared at about 5.5 p.p.m. (4.5 τ). The OH proton was observed at 9.35 p.p.m. (0.65 τ , 2H). The absence of absorption at about 1.67 p.p.m. in pemptoporphyrin dimethyl ester demonstrates that pemptoporphyrin has no alcohol or ethyl group in the molecule.

(d) Mesoporphyrin dimethyl ester (Fig. 2f). The methyl and methylene protons of the ring-substituted ethyl groups give rise to one triplet at 1.79 p.p.m. (8.21 τ) with a J value of 7.1 c/c./sec. and 3.93 p.p.m. (6.07 τ) as one quartet.

Conversion of pemptoporphyrin dimethyl ester into deuteroporphyrin IX dimethyl ester. Reduction of the postulated vinyl group of pemptoporphyrin with resorcinol gave deuteroporphyrin IX. For this purpose, pemptohaem chloride or pemptohaem dimethyl ester was prepared by the insertion of iron into pemptoporphyrin or its ester according to the method of Warburg & Negelein (1932). A solution of ferrous acetate in acetic acid (prepared by refluxing 5 mg. of iron powder in 5 ml. of 85% acetic acid saturated with sodium chloride) was added dropwise to a solution of the porphyrin (7.1 mg.) refluxing gently in 5 ml. of acetic acid.

Conversion into the haem was virtually instantaneous. The haem was extracted at pH 4 into ethyl acetate, and any residual free porphyrin was removed by extraction with 1.5N-hydrochloric acid, then washed with water and evaporated to dryness under reduced pressure in a small test tube. Pemptohaem thus obtained was ground with 35mg. of resorcinol and then heated in an oil bath at 190–200° for 15 min., as described by Chu & Chu (1952). The dark-brown solid was treated for 1 min. directly with boiling acetic acid (5ml.) and concentrated hydrochloric acid (0.2ml.) treated with iron powder (5mg.). The resulting deuteroporphyrin was extracted into ethyl acetate, evaporated to dryness and esterified with 15% (w/w) hydrochloric acid in methanol, and then purified on the alumina column in a similar manner to that described for pemptoporphyrin dimethyl ester and crystallized from chloroform and methanol in a yield of 35%. The melting point was 213–214°. Absorption spectra, hydrochloric acid number and n.m.r. spectrum were in excellent agreement with those of authentic deuteroporphyrin IX.

It was clearly demonstrated that n.m.r. spectra are extremely useful for the elucidation of the structures of unknown porphyrins if they are pure crystallized samples. Infrared analysis of pemptoporphyrin was less useful in this case, for though the vinyl group was detected (at 985 and 900 cm.⁻¹) it was not quantitative. The reason why one of

two vinyl groups of protoporphyrin is selectively reduced, by intestinal bacteria presumably, is still unknown.

The authors thank Professor C. Rimington, F.R.S., for his continuous interest and helpful advice, which have made this collaboration possible. This work was supported in part (S. S.) by grants from the Rockefeller and Fujiwara Foundation and (J. M. F. and E. T.) by the European Office of Research (U.S. Army) under Contract no. DA-91-591-EUC-3359.

REFERENCES

- Abraham, R. J., Jackson, A. H. & Kenner, G. W. (1961). *J. chem. Soc.* p. 3468.
Becker, E. D., Bradley, R. B. & Watson, C. J. (1961). *J. Amer. chem. Soc.* **83**, 3743.
Caughey, W. S. & Iber, P. K. (1963). *J. org. Chem.* **28**, 269.
Caughey, W. S. & Koski, W. S. (1962). *Biochemistry*, **1**, 923.
Chu, T. C. & Chu, E. J. H. (1952). *J. Amer. chem. Soc.* **74**, 6276.
Chu, T. C., Green, A. A. & Chu, E. J. H. (1951). *J. biol. Chem.* **190**, 643.
Falk, J. E. & Benson, A. (1953). *Biochem. J.* **55**, 101.
French, J. M., England, M. T., Lines, J. & Thonger, E. (1964). *Arch. Biochem. Biophys.* **107**, 404.
Granick, S. (1948). *J. biol. Chem.* **172**, 177.
Ramsey, V. G. (1953). *Biochem. Prep.* **3**, 39.
Rimington, C. (1938). *Biochem. J.* **32**, 460.
Sano, S., Nanzyo, N. & Rimington, C. (1964). *Biochem. J.* **93**, 270.
Tiers, G. V. D. (1958). *J. phys. Chem.* **62**, 1151.
Warburg, O. & Negelein, E. (1932). *Biochem. Z.* **244**, 9.