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## Synthesis of Porphyrin *c*-Type Compounds from Protoporphyrinogen

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The porphyrin component of cytochrome *c* was first prepared by Hill & Keilin (1931). They named it porphyrin *c* and found it to be insoluble either in ether containing acetic acid or in chloroform. It could be transformed into haematoporphyrin. Theorell (1939) prepared natural porphyrin *c* with a molecular weight of  $890 \pm 10$  by hydrolysis of cytochrome *c* with hydrochloric acid. On heating protoporphyrin or haematoporphyrin with cysteine in hydrochloric acid, he also prepared a product resembling porphyrin *c*. He concluded that the prosthetic group of cytochrome *c* is bound to the apoprotein by thio ether bonds uniting cysteine residues to the vinyl side chains of protohaem (Theorell, 1938). Paul (1951) confirmed this result and prepared an optically active haematoporphyrin from cytochrome *c* through cleavage of these thio ether bonds with silver sulphate (Paul, 1950).

Zeile & Meyer (1939) hydrolysed cytochrome *c* with sulphuric acid under conditions that were presumed to exclude the possibility of addition between cysteine and the vinyl groups in the porphyrin. They obtained a dicysteine-porphyrin adduct and assumed it to be preformed in cytochrome *c*. Zeile & Meyer (1939) synthesized a porphyrin *c* type of compound by melting together L-cysteine hydrochloride and dibromoporphyrin. The natural and synthetic porphyrins *c* agreed in analytical data, absorption spectra and partition of their esters between ether and acid buffers, but their optical activities were very different.

Neilands & Tuppy (1960) prepared a crystalline synthetic porphyrin *c* by a modification of Zeile &

Meyer's (1939) method. This was split at a negligible rate by an enzyme from *Albizzia lophanta* (Hansen, Kjaer & Schwimmer, 1959) which cleaves a number of *S*-alkylcysteines having straight-chain alkyl groups but which has little action on those with branched chains. Neilands & Tuppy's (1960) finding would favour an  $\alpha$ - rather than a  $\beta$ -linkage in their synthetic product.

Although the above studies indicate that porphyrin *c* is a dicysteine-protoporphyrin adduct, they do not afford incontrovertible evidence on the point of attachment of the sulphur atoms to the side chains in positions 2 and 4 of the porphyrin ring (Fig. 1). Different isomers would result according to whether reaction of cysteine was with the  $\alpha$ - or  $\beta$ -carbon atoms of the vinyl side chains of protoporphyrin. Rigorous proof of the structure of the haematoporphyrin derived by cleavage of the C—S linkage would settle the issue but this has not been forthcoming. Paul's (1951) report of an optically active haematoporphyrin suggests the  $\alpha$ -isomer.

Recent biochemical evidence shows that the actual intermediates in the biosynthesis of haem are not porphyrins, but their reduction products, the porphyrinogens, which are hexahydroporphyrins. Sano & Granick (1961) demonstrated that protoporphyrinogen is an intermediate in the biosynthesis of protoporphyrin from coproporphyrinogen *in vitro*. They also observed that thiol compounds reacted with the vinyl side chains of protoporphyrinogen during its autoxidation in an acid medium under the influence of light, yielding a porphyrin *c* type of compound. This discovery opened up the possibility of synthesizing cyto-

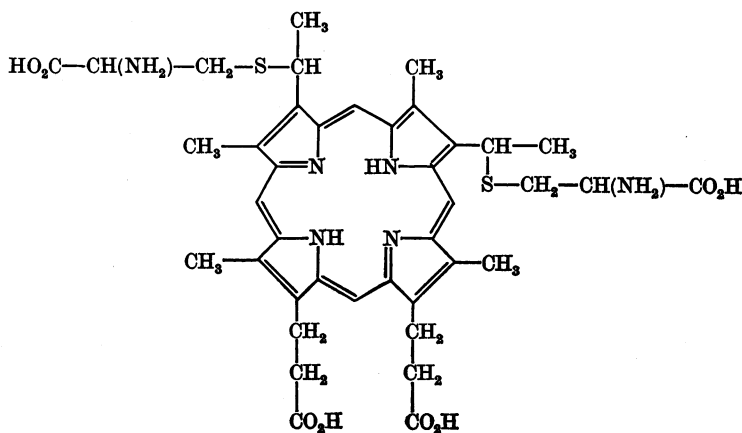


Fig. 1. Assumed structure of porphyrin *c*.

chrome *c*-like compounds and also offered an approach to the biosynthesis of cytochrome *c*. Studies in this direction were reported by Sano (1961).

The present paper describes the synthesis of various protomercaptoporphyrins and the investigation of their structures.

## MATERIALS AND METHODS

**Porphyrins.** Protoporphyrin IX was prepared by Fischer's (1953) method. No contamination by mesoporphyrin was observed. Haematoporphyrin IX hydrochloride, obtained from Laboratoires Roussel, Paris, was purified by fractionation with ethyl acetate and 0.05 *N*-HCl and was then esterified by contact overnight at 5° with methanolic 15% (w/w) HCl. Chloroform was added and the mixture washed with water until free from HCl. It was then evaporated to dryness and the residue was dissolved in a little chloroform and placed on the top of an alumina column (2 cm. × 50 cm.). The chromatogram was developed with chloroform-methanol (200:1, v/v) and the main fraction was rechromatographed three times more in a similar manner. The ester was then crystallized from chloroform-methanol. Haematoporphyrin IX was liberated from the ester by leaving in contact with 6 *N*-HCl overnight at room temperature and then evaporating the mixture to dryness over NaOH *in vacuo*. Coproporphyrin III tetramethyl ester was prepared from diphtheria toxoid broth (Gray & Holt, 1948). Uroporphyrin III octamethyl ester was prepared from turacin (Nicholas & Rimington, 1952). Mesoporphyrin IX was prepared by reduction of protoporphyrin IX with HI according to the method of Fischer & Orth (1943). Deuteroporphyrin IX was prepared according to the method of Fischer & Orth (1943). Diacetyldeuteroporphyrin IX was prepared according to the method of Lemberg & Falk (1951). Diformyldeuteroporphyrin IX ester was prepared from protoporphyrin IX according to the method of Sparatore & Mauzerall (1960).

**Porphyrinogens.** Protoporphyrinogen IX was prepared as follows. To protoporphyrin [10 ml. of 0.2% (w/w) solution in 0.02 *N*-KOH] was added under nitrogen, in dim

red light, freshly ground 3% (w/w) sodium amalgam (excess) and 3 drops of ethanol. Reduction was carried out for 2 min. at 80° (Sano & Granick, 1961), more ethanol being added if foaming occurred. Under the same illumination, the mixture was filtered on a sintered-glass filter which was washed with water; the colourless filtrate plus washings were adjusted to pH 7.2 with 50% (w/v) H<sub>3</sub>PO<sub>4</sub>. Five such preparations were combined and the porphyrinogen solution was diluted to 300 ml. with deaerated water. The yield, as determined by oxidation to protoporphyrin, was 65–70%. The protoporphyrinogen was oxidized for 30 min. in dim light in ether containing an equivalent amount of quinhydrone.

Haematoporphyrinogen, mesoporphyrinogen, uroporphyrinogen, deuteroporphyrinogen and coproporphyrinogen were prepared in the same way, but at room temperature. Ether/HCl fractionation followed by esterification of the porphyrin in each fraction, and kerosene-chloroform chromatography of the esters showed that no mesoporphyrinogen was formed during the reduction of haematoporphyrin. Paper chromatography of porphyrins was carried out in the lutidine-water system of Falk & Benson (1953) or the kerosene-chloroform system of Bogorad & Granick (1953). Deuterohaem was prepared according to the method of Schumm (1928).

**Solvents.** Ether, ethyl acetate and acetic acid were freshly distilled and free from peroxide or unidentified oxidants (Vogel, 1948). Dioxan was purified (Vogel, 1948) and free from peroxide. Polyphosphoric acid (80% P<sub>2</sub>O<sub>5</sub>, w/v) was obtained from British Drug Houses Ltd. Alumina was prepared for chromatography by stirring with ethyl acetate, keeping the mixture for 1–2 days, filtering and drying at 80°. Amberlite CG-50 (200–400 mesh) (Rohm and Haas Co.) was processed according to the method of Okunuki (1959). Infrared-absorption spectra were recorded on a Koken DS-301 infrared spectrophotometer.

Esterification of porphyrins was accomplished, unless otherwise stated, by leaving the material in contact with methanolic 15% (w/w) HCl for about 20 hr. in the dark. Chloroform was then added and the mixture shaken repeatedly with water, then with *N*-ammonia and finally once more with water. Porphyrin esters were hydrolysed by leaving them in contact with 7 *N*-HCl for 42–48 hr. in the

dark. The mixture was then dried over NaOH *in vacuo*. Periodate oxidation and determination of formaldehyde were performed according to the procedure of Frisell, Meech & Mackenzie (1954).

## RESULTS

### *Interaction of thiol compounds with protoporphyrinogen*

*Preparation of a porphyrin c-type compound from protoporphyrinogen in acid solution.* To 300 ml. of protoporphyrinogen solution prepared by the reduction of 100 mg. of protoporphyrin (see the Materials and Methods section) was added 300 mg. of L-cysteine hydrochloride dissolved in 10 ml. of water. The mixture was immediately diluted to 600–1000 ml. with acetic acid. The porphyrinogen solution readily autoxidized during 2 hr. even in the dark, but was kept overnight in the dark to ensure complete oxidation. The solution was adjusted to pH 3.5 with saturated aq. sodium acetate and diluted to 2 l. with water. Ether-soluble porphyrin was removed from this solution by several shakings with ether. The aqueous phase was diluted to 7 l. with water and was passed through a column (6.0 cm.  $\times$  20 cm.) of Amberlite CG-50 (H<sup>+</sup> form). The column was washed with 1 l. of water, then with 200 ml. of 0.2M-pyridine-acetic acid buffer, pH 5.0, to remove the excess of cysteine. The coloured part of the resin was separated from the colourless part and was suspended in a small amount of water. This suspension was slowly poured on a layer of fresh resin (2 cm.  $\times$  3 cm.), and porphyrin was eluted with aq. 30% (v/v) pyridine. The reddish effluent was collected and freeze-dried; the yield of porphyrin c-type compound was about 80 mg. The material was esterified with either diazomethane (Zeile & Meyer, 1939) or methanolic 20% (w/w) hydrochloric acid and the ester was extracted into ether at pH 6.0. The ether layer was washed with a small amount of water and porphyrin c-type ester was extracted with 0.1M-formate buffer, pH 3.0. More than 95% of the porphyrin ester present in the ether was extracted (Fig. 2). It was passed back into ethyl acetate and the solution was evaporated to dryness *in vacuo*. The material was then dissolved in a small amount of chloroform-methanol (200:1, v/v) and placed on an alumina column (3.0 cm.  $\times$  50 cm.). On development with the same solvent three bands were obtained. The first (minor) fraction (A) was reddish brown. The main component (B) was reddish pink. The slowest, diffusely flowing, band (C) was also a minor constituent. The yields of fractions A, B and C were 16, 79 and 5% respectively. The absorption spectra of these fractions were identical and showed maxima at 406, 504, 539, 572 and 626 m $\mu$  in chloroform-methanol (4:1, v/v). The

chloroform solution of fraction B was evaporated to dryness *in vacuo* and the residue crystallized as follows. It was dissolved in warm benzene-methanol (1:1, v/v) and filtered. An equal volume of light petroleum (b.p. 30–60°) was added and the solution was left in the cold. The crystals were dried over phosphorus pentoxide. The yield was 35 mg. (Found: S, 7.22. C<sub>44</sub>H<sub>56</sub>N<sub>6</sub>O<sub>8</sub>S<sub>2</sub> requires S, 7.44%). Pure c-type porphyrin was obtained by acid hydrolysis of the ester.

*Preparation of a porphyrin c-type compound from protoporphyrinogen in neutral solution.* A protoporphyrinogen solution, prepared by reduction of 100 mg. of protoporphyrin, was diluted to 300 ml. with 0.067M-phosphate buffer, pH 7.2, through which nitrogen had been previously bubbled. A solution of 300 mg. of L-cysteine hydrochloride in 10 ml. of distilled water was neutralized and added to the protoporphyrinogen solution, and the mixture was incubated at 37° in the dark. By next day autoxidation was complete. The porphyrin solution thus obtained was diluted with water, adsorbed on Amberlite CG-50 and treated in a manner similar to that described above. The ester prepared with either diazomethane or methanolic 20% (w/w) hydrochloric acid was fractionated from ether with 0.1M-formate buffer, pH 3.0, and further purified by alumina-column chromatography. Three bands were obtained. The yields of fractions A, B and C were 20, 75 and 5% respectively. Only the material in the main band was collected. The yield of c-type porphyrin tetra-

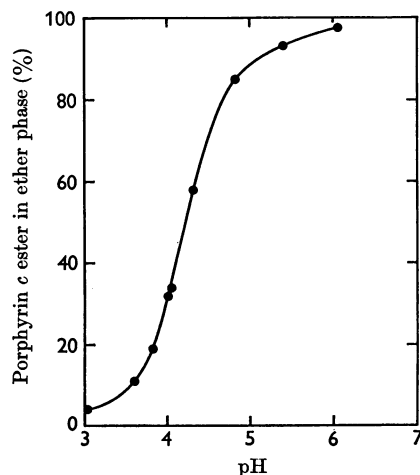


Fig. 2. Partition of c-type porphyrin tetramethyl ester, prepared from protoporphyrinogen in neutral solution, between ether and buffers as a function of pH. For pH range 3.02–3.60, 0.2N-tartaric acid–0.1M-sodium tartrate; for pH range 3.81–6.40, 0.2N-acetic acid–0.2M-sodium acetate; for pH 6.06, 0.2M-KH<sub>2</sub>PO<sub>4</sub>–0.2M-Na<sub>2</sub>HPO<sub>4</sub>.

methyl ester was 30–35 mg. (Found: S, 7.25.  $C_{44}H_{86}N_6O_8S_2$  requires S, 7.44 %).

To decide whether or not cysteine can react with protoporphyrinogen (fully reduced) or does so only during the autoxidation of protoporphyrinogen, the following experiment was performed. Protoporphyrinogen (1 mg.) in a Thunberg tube was diluted to 10 ml. with 0.067 M-phosphate buffer, pH 7.2, which had been previously saturated with nitrogen. Neutralized cysteine solution (containing 3 mg.) was added. The Thunberg tube was completely evacuated and the mixture incubated anaerobically at 37° for 2 hr. The colourless solution was then poured into a separating funnel containing 200 ml. of ether and 6 ml. of acetic acid-satd. sodium acetate (4:1, v/v) mixture. After vigorous shaking, the ether layer containing unchanged protoporphyrinogen was removed and the aqueous solution was oxidized by exposure to weak illumination for 2 hr., followed by storage in the dark overnight. Ether-insoluble porphyrin was obtained in 35 % yield. As a control, cysteine was added to protoporphyrinogen solution in the same concentration and the mixture immediately poured into the separating funnel containing ether and acetic acid-satd. sodium acetate as above. No ether-insoluble porphyrin was obtained. It may therefore be concluded that cysteine reacts partly with protoporphyrinogen (35 % yield of crude porphyrin *c*), but does so more readily during the autoxidation of protoporphyrinogen (85 % yield of crude *c*-type porphyrin).

Table 1 records the spectrophotometric constants of the porphyrin *c* tetramethyl ester prepared from protoporphyrinogen in both acid and neutral conditions; they are identical with published data for natural and synthetic porphyrin *c* ester (Theorell, 1939; Zeile & Meyer, 1939; Neilands & Tuppy, 1960). The  $R_p$  of the porphyrin *c* in a lutidine-ammonia solvent system indicated four ionizable carboxyl groups/mol. The spot gave a positive ninhydrin but a negative nitroprusside reaction. Kerosene-chloroform paper chromatography of the porphyrin *c* tetramethyl ester gave a single spot with  $R_f$  0.03.

*Reaction of other mercaptans with protoporphyrinogen.* Mercaptoethanol forms a porphyrin with two ionizable carboxyl groups/mol. and absorption maxima at 405, 552 and 596  $m\mu$  in *N*-hydrochloric acid. It is extractable from ether with 0.03 *N*-hydrochloric acid. The yield was more than 85 % in both acid and neutral conditions.

Glutathione reacts with protoporphyrinogen under the two conditions described above to form a porphyrin with six ionizable carboxyl groups/mol. The spectrum in *N*-hydrochloric acid was identical with those of porphyrin *c* and protomer-captoethanol-porphyrin. The yield was about 80 %.

Thioglycollic acid gives rise to two derivatives under acid conditions with an overall yield of 70 %. They were extractable from ether with 0.075 *N*- and 0.15 *N*-hydrochloric acid respectively. Each porphyrin was characterized by paper chromatography with lutidine-ammonia solvent, which indicated four ionizable carboxyl groups/mol. for the one (probably an addition product of two molecules of thioglycollic acid to one of protoporphyrinogen) and three ionizable carboxyl groups/mol. for the other (one molecule of thioglycollic acid to one of protoporphyrinogen). The ratio of these products was approx. 4:3. In neutral medium thioglycollic acid reacts more slowly and the yield of product was small (about 15 %). Tricarboxylic porphyrin was the main product.

*L*-Methionine and *S*-methyl-*L*-cysteine did not react with protoporphyrinogen under either acid or neutral conditions.

#### *Interaction of thiol compounds with haematoporphyrinogen*

*Reaction of cysteine with haematoporphyrinogen in acid and neutral solutions.* *L*-Cysteine hydrochloride reacted more smoothly with haematoporphyrinogen than with protoporphyrinogen in both acid and neutral solutions. Even in anaerobic and neutral conditions reaction occurred to the extent of 67–70 %. The porphyrin *c*-type compound was purified in the same way as the product from protoporphyrinogen.

*Reaction of other mercaptans with haematoporphyrinogen.* Glutathione gave a product similar to that obtained with protoporphyrinogen. The yield was 85 %. Thioglycollic acid reacted in 50–70 % (v/v) acetic acid or in *m*-orthophosphoric acid, yielding a tetracarboxylic porphyrin together with a trace of a tricarboxylic porphyrin. Di(thioglycollate)-haematoporphyrin adduct was extracted from ether with 0.15 *N*-hydrochloric acid. The yield was 80 %. *L*-Methionine and *S*-methyl-*L*-cysteine do not appear to react with haematoporphyrinogen under either acid or neutral conditions.

#### *Experiments with other porphyrinogens and with porphyrins and haems*

No reaction occurred between mercaptans and the following substances under either acid or neutral conditions: protoporphyrin, protohaem, mesoporphyrin, mesoporphyrinogen, deuteroporphyrin, deuteroporphyrinogen, coproporphyrin, coproporphyrinogen, uroporphyrin, uroporphyrinogen, haematoporphyrin, haematohaem and diacetyldeuteroporphyrin. However, condensation of thioglycollic acid and haematoporphyrin was accomplished by the use of polyphosphoric acid and gave a di(thioglycollate)-porphyrin adduct.

Table 1. Absorption bands of tetramethyl esters of porphyrin c-type compounds and of natural and synthetic porphyrin c

The substances used were A, tetramethyl ester of c-type porphyrin prepared from protoporphyrinogen and cysteine in acetic acid solution; B, tetramethyl ester of c-type porphyrin prepared in neutral solution; C, natural porphyrin c tetramethyl ester (Zeile & Meyer, 1939); D, natural porphyrin c (Theorell, 1938); E, synthetic porphyrin c (Neilands & Tuppy, 1960). The ratios of band intensities, in parentheses, are calculated relative to band IV for neutral spectra and to band II for acid spectra.

Substance	Solvent	Maxima					Minima				
		I	II	III	IV	V	610	555	524		
A	CHCl <sub>3</sub> -methanol (4:1, v/v)	λ (mμ)	626	572	539	504	406	610	555	524	465
		10 <sup>-4</sup> ε	0.372 (0.29)	0.624 (0.48)	0.868 (0.67)	1.297 (1.0)	15.40 (11.9)	(0.09)	(0.16)	(0.34)	(0.19)
A	3 N-HCl	λ (mμ)	598	554	408*	—	—	—	588	—	480
		10 <sup>-4</sup> ε	0.551 (0.374)	1.48 (1.0)	26.1 (17.6)	—	—	—	—	—	—
B	CHCl <sub>3</sub>	λ (mμ)	626	573	539	504	406	610	555	524	465
		10 <sup>-4</sup> ε	0.375 (0.32)	0.61 (0.52)	0.84 (0.71)	1.17 (1.0)	14.30 (12.1)	(0.09)	(0.18)	(0.33)	(0.22)
C	CHCl <sub>3</sub>	λ (mμ)	626	572.5	539	504	—	—	—	—	—
		10 <sup>-4</sup> ε	0.375 (0.28)	0.648 (0.47)	0.887 (0.66)	1.381 (1.0)	—	—	—	—	—
D	N-HCl	λ (mμ)	—	553	406	—	—	—	—	—	—
		10 <sup>-4</sup> ε	—	1.65	24.8	—	—	—	—	—	—
E	N-HCl	λ (mμ)	—	553	406	—	—	—	—	—	—
		10 <sup>-4</sup> ε	—	1.65	31.2	—	—	—	—	—	—

\* Maximum in N-HCl: 406 mμ.

Haematoporphyrin (1.3 mg.) and thioglycolic acid (13.4 mg.) were mixed well with a glass rod and 2-3 ml. of polyphosphoric acid was added. The mixture was kept at 60° for 15-20 min., then cooled and poured into 10 ml. of water. The porphyrin was extracted into ether and fractionated with hydrochloric acid. Most of the porphyrin was extractable with 0.5N-hydrochloric acid in which the Soret band was at 406 mμ. In ether solution absorption maxima in the visible region were at 627, 571, 532 and 500 mμ. Chromatography in lutidine-ammonia indicated a single porphyrin with four ionizable carboxyl groups/mol.

#### Studies on the structure of the porphyrin c-type compounds

*Infrared-absorption spectra of c-type porphyrin tetramethyl ester.* Specimens of porphyrin c tetramethyl ester prepared from cysteine and either protoporphyrinogen or haematoporphyrinogen under various conditions showed the same infrared absorption in Nujol (Fig. 3). The maxima due to the vinyl groups of protoporphyrin at 985 and 900 cm.<sup>-1</sup> had disappeared and a new absorption band due to NH<sub>2</sub> had appeared at 1641 cm.<sup>-1</sup>. This result indicates that cysteine had attacked the vinyl groups of protoporphyrinogen at either the α- or β-carbon position.

*Cleavage of porphyrin c-type compounds with silver sulphate.* Purified porphyrin c-type compound (20 mg.) was dissolved in distilled water (25 ml.). Acetic acid (10 ml.) and 0.32% (w/v) silver sulphate (25 ml.) were added and the mixture was kept at 80° for 25 min. (or at 60° for 1.5 hr.). The porphyrin formed was extracted into ether and fractionated by shaking with 0.1N-hydrochloric acid, which removed 90% of the total porphyrin. The yield was 65-70%. This material showed maxima in N-hydrochloric acid at 402.5, 550 and 596 mμ and in ether 499, 532, 570 and 622 mμ. Lutidine-ammonia paper chromatography indicated two ionizable carboxyl groups/mol. Therefore the porphyrin would appear to be identical with haematoporphyrin IX. Occasionally silver haematoporphyrin, with absorption bands at 530 and 554 mμ in ether, was obtained when applying this technique, but it was readily split by 6N-hydrochloric acid with liberation of the porphyrin. The haematoporphyrin was esterified by contact with dry methanolic 15% (w/w) hydrochloric acid overnight in the cold room. It was adsorbed from its chloroform solution on to the top of an alumina column (2 cm. x 50 cm.) and the chromatogram was developed with chloroform-methanol (200:1, v/v). A trace of fast-running porphyrin was eluted. The main fraction was then collected, evaporated to dryness *in vacuo*, and the ester was crystallized, m.p. 212°, from chloroform-

methanol. Kerosene-chloroform paper chromatography indicated a single porphyrin,  $R_f$  0.03, with two hydroxyl groups/mol. No trace contaminants were observed on the paper. The spectral properties of these haematoporphyrin dimethyl esters are compared in Table 2 with those of authentic haematoporphyrin dimethyl ester and those of iso-haematoporphyrin dimethyl ester (see below). The dimethyl ether dimethyl esters of these haematoporphyrins were prepared as follows: porphyrin dimethyl ester (5 mg.) was dissolved in dry methanolic 35% (w/w) hydrochloric acid (10 ml.) to which was added 1.0 ml. of conc. sulphuric acid, and the mixture was heated at 80° under reflux for 3 hr. and then kept overnight in the dark (Granick, Bogorad & Jaffe, 1953). Kerosene-chloroform paper chromatography of the product showed  $R_f$  0.89.

The dimethyl ether dimethyl esters originating from the porphyrin *c*-type compounds prepared from protoporphyrinogen or haematoporphyrinogen in neutral or acetic acid solution were identical in spectra, hydrochloric acid number, chromatographic behaviour and infrared-absorption spectra with authentic haematoporphyrin dimethyl ether dimethyl ester, as were also the corresponding dimethyl esters with one another and with authentic material.

*Examination of fraction A obtained during the purification of porphyrin c-type compounds.* The

brown-red porphyrin (fraction A) was converted into ether-soluble porphyrin by treatment with silver sulphate. The ether-soluble fraction was evaporated to dryness, esterified and chromatographed on an alumina column with chloroform-methanol (200:1, v/v). A porphyrin running diffusely through the column showed absorption maxima at 404, 503, 536, 572 and 626 m $\mu$  in chloroform. Kerosene-chloroform paper chromatography showed two spots. The major component had  $R_f$  0.03 (haematoporphyrin dimethyl ester) and the other small component had  $R_f$  0.23 (presumably monohydroxyethylmonovinyldeuteroporphyrin dimethyl ester). Fraction A would therefore appear to contain as a contaminant a monocysteinyethylmonovinyldeuteroporphyrin, but the amount was too small for determination of its sulphur content.

*Preparation of cysteine and serine from porphyrin c-type compounds.* Porphyrin *c*-type compound was treated with silver sulphate in dilute acetic acid as described above. After extraction of haematoporphyrin by ether, the yellow sediment in the aqueous layer was collected by centrifugation. It was suspended in 0.3N-hydrochloric acid and decomposed by hydrogen sulphide. After removal of silver sulphide by filtration and residual hydrogen sulphide by a stream of nitrogen, the solution was evaporated to small volume *in vacuo*. Ninhydrin and nitroprusside reactions were positive. Paper

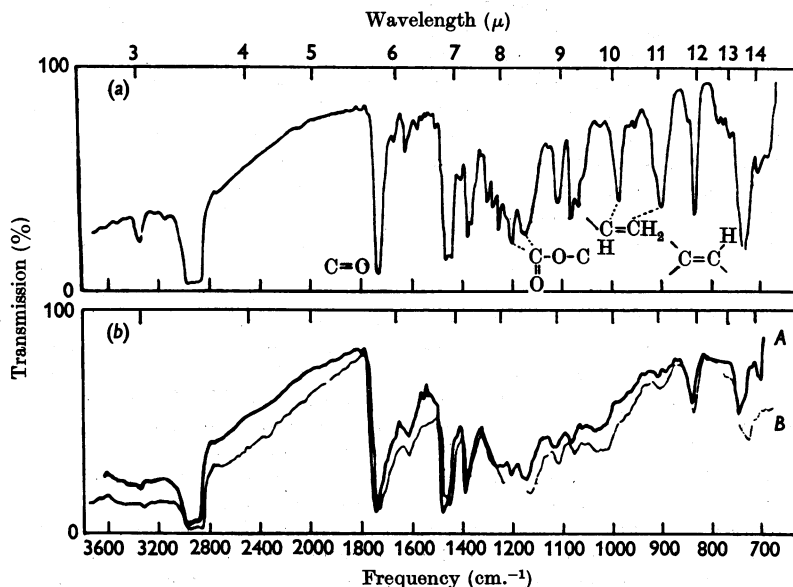


Fig. 3. Infrared-absorption spectra in Nujol of (a) protoporphyrin dimethyl ester and of (b): A, *c*-type porphyrin tetramethyl ester derived from reaction between protoporphyrinogen and L-cysteine in acetic acid solution, and B, *c*-type porphyrin tetramethyl ester derived from reaction between protoporphyrinogen and L-cysteine in neutral solution.

Table 2. Comparison of spectra of haematoporphyrin dimethyl esters prepared from porphyrin *c*-type compounds with authentic ester and with isohaematoporphyrin dimethyl ester

The substances used were: A, haematoporphyrin dimethyl ester prepared from porphyrin *c*-type compound obtained by reaction between protoporphyrinogen and cysteine in acetic acid solution; B, analogous material derived from the reaction in neutral solution; C, analogous material derived from the reaction between haematoporphyrinogen and cysteine in acetic acid solution; D, analogous material derived from the reaction in neutral solution; E, authentic haematoporphyrin dimethyl ester prepared for comparison; F, authentic haematoporphyrin dimethyl ester, quoted from Granick, Bogorad & Jaffe (1953); G, isohaematoporphyrin dimethyl ester (see the text). Wavelengths of maxima and minima are in  $m\mu$  and the ratios of the band intensities, in parentheses, are calculated relative to band IV. The solvent was chloroform except for substance F which was dissolved in pyridine.

Substance	Maxima					Minima			
	I	II	III	IV	V				
A	623 (0.29)	572 (0.46)	535 (0.66)	501	402.5 (13.5)	605 (0.08)	552 (0.17)	521 (0.31)	462 (0.19)
B	623 (0.30)	571 (0.46)	535 (0.64)	501	402.5 (12.9)	605 (0.07)	552 (0.13)	521 (0.27)	462 (0.17)
C	623 (0.29)	570 (0.45)	535 (0.61)	500	402.5 (12.1)	603 (0.06)	552 (0.13)	520 (0.25)	460 (0.16)
D	623 (0.29)	570 (0.45)	535 (0.62)	500	402.5 (12.0)	603 (0.06)	552 (0.12)	520 (0.26)	460 (0.17)
E	623 (0.29)	570 (0.46)	535 (0.63)	500	402.5 (12.2)	605 (0.07)	553 (0.12)	522 (0.25)	462 (0.15)
F	623 (0.29)	569 (0.45)	532 (0.61)	499	401 (11.8)	603 (0.05)	552 (0.09)	519 (0.23)	455 (0.14)
G	622 (0.29)	570 (0.45)	535 (0.62)	500	400 (12.0)	603 (0.06)	552 (0.12)	520 (0.25)	460 (0.15)

chromatography with the solvent butan-1-ol-acetic acid-water (12:3:5, by vol.) gave a spot with the same  $R_f$  as cysteine.

Hydrolysis with 6*N*-hydrochloric acid of the yellow sediment of silver-cysteine complex caused immediate liberation of hydrogen sulphide. The mixture gave positive ninhydrin but negative nitroprusside reactions. A portion of the solution was diluted with water, the silver chloride was filtered off and the filtrate was evaporated to about 2 ml. Detection of formaldehyde liberated during periodate oxidation of this solution indicated the presence of serine. These findings confirm the presence of cysteine in the porphyrin *c*-type compounds.

*Positions of the hydroxyl groups in the haematoporphyrin prepared from porphyrin c-type compounds.* It is unknown whether the addition of mercaptans to the vinyl side chains of protoporphyrinogen follows Markownikoff's rule. Evidence for addition is provided by the isolation of a haematoporphyrin (2,4-dihydroxyethyldeuteroporphyrin) when the porphyrin *c*-type compound is split by silver sulphate. Should the sulphur atom have been attached to the  $\alpha$ -carbon atom of the vinyl group, in conformity with the rule, cleavage would result in a haematoporphyrin having a secondary alcoholic function [2,4-di-( $\alpha$ -hydroxyethyl)deuteroporphyrin], whereas attachment to the  $\beta$ -carbon atom would yield an isohaematoporphyrin

[2,4-di-( $\beta$ -hydroxyethyl)deuteroporphyrin] possessing a primary alcoholic function. The haematoporphyrin derived from the porphyrin *c*-type compounds was examined in different ways, as described below, and compared with natural haematoporphyrin and with isohaematoporphyrin synthesized for the purpose (see below).

(i) Rate of dehydration of haematoporphyrin dimethyl ester prepared from porphyrin *c*-type compound: formation of protoporphyrin dimethyl ester. Since the rates of dehydration of a primary and of a secondary alcohol to a vinyl group might be expected to differ (Fieser & Fieser, 1944), the rate of dehydration of haematoporphyrin dimethyl ester prepared from porphyrin *c*-type compound was compared with that of authentic haematoporphyrin dimethyl ester. Haematoporphyrin dimethyl ester (1 mg.) from porphyrin *c*-type compound prepared in either acid or neutral conditions was dried at room temperature overnight *in vacuo* over phosphorus pentoxide and was then heated at 150° for 10 min. The product was dissolved in acetic acid-ether and the porphyrins were separated by shaking the washed ether first with 0.2*N*-hydrochloric acid and then with 2.5*N*-hydrochloric acid. Protoporphyrin in the latter fraction had absorption maxima at 408, 556 and 600  $m\mu$  and in chloroform at 508, 540, 576 and 632  $m\mu$ . The yield of protoporphyrin dimethyl ester calculated from the spectral absorption coefficient ( $10^{-3}\epsilon = 241$

at 408  $\mu$  in 2.5*N*-hydrochloric acid) was 35 %, identical with that obtained from an authentic sample of haematoporphyrin (cf. Granick *et al.* 1953). This is compatible with a secondary alcoholic function.

(ii) Infrared-absorption spectra. Samples prepared from porphyrin *c*-type compound were examined in chloroform and in Nujol and the spectra were identical with that of authentic haematoporphyrin dimethyl ester. The OH vibration band was located at 1070  $\text{cm}^{-1}$  in Nujol, which is evidence for a secondary rather than a primary alcoholic function (Granick *et al.* 1953).

(iii) Oxidation of haematoporphyrin dimethyl ester prepared from porphyrin *c* to diacetyldeuteroporphyrin dimethyl ester. Samples of haematoporphyrin dimethyl esters (5 mg.) were dissolved in 10 ml. of pyridine, and an excess of a slurry of chromic acid in pyridine (Poos, Arth, Beyler & Sarett, 1953) was added. The mixture was maintained at 60° for 2 hr., ether was added and the washed ether layer was extracted successively with 0.2*N*-, 1.0*N*- and 3.0*N*-hydrochloric acid. Unchanged haematoporphyrin was extracted into 0.2*N*-hydrochloric acid, monoacetylmonohydroxyethyldeuteroporphyrin into 1.0*N*-hydrochloric acid, and diacetyldeuteroporphyrin into 3.0*N*-hydrochloric acid, with yields of 1.7, 11.0 and 44 % respectively. The absorption maxima of the diacetyldeuteroporphyrin in chloroform were at 425, 518, 552, 588 and 640  $\mu$ . The infrared-absorption spectrum showed a strong carbonyl absorption band at 1660  $\text{cm}^{-1}$ . An oxime was prepared by heating this diacetyldeuteroporphyrin (2 mg.) in pyridine (10 ml.) with hydroxylamine hydrochloride (100 mg.) and anhydrous sodium acetate (100 mg.) for 20 min. in a boiling-water bath. The absorption maxima of the oxime in chloroform were at 405, 505, 537, 576 and 625  $\mu$ . The infrared-absorption spectrum of the oxime showed the OH vibration at 3660  $\text{cm}^{-1}$ . Diacetyldeuteroporphyrin prepared from dueterohaem behaved identically. This result strongly suggests linkage of the sulphur to the  $\alpha$ -carbon atoms. No diacetaldehydodeuteroporphyrin was obtained in the oxidation of haematoporphyrin prepared from different materials with chromic acid in pyridine.

The brown-red fast-running porphyrin (fraction A) was also oxidized with chromic acid. Monoacetylmonovinyldeuteroporphyrin was obtained, which was further characterized by paper chromatography, ultraviolet-, visible- and infrared-absorption spectra, and oxime formation. Evidence that, on the addition of cysteine to protoporphyrinogen, the main product is an adduct of two cysteine residues to the  $\alpha$ -carbon atoms of the vinyl groups of one protoporphyrinogen molecule may be

summarized as follows. Acid hydrolysis releases cysteine and serine; cleavage with silver sulphate affords a haematoporphyrin that is oxidized to diacetyldeuteroporphyrin; the sulphur content of the substance agrees with that calculated for the addition of two cysteine mol./mol.

*Is haematoporphyrinogen an intermediate in the reaction between protoporphyrinogen and mercaptans?* It has been shown above that mercaptans react with both protoporphyrinogen and haematoporphyrinogen, and in fact more smoothly with the latter. In each case a thio ether linkage results, but the mechanisms of reaction must differ. Though it is established that protoporphyrin readily becomes hydrated to haematoporphyrin in the presence of mineral acid (Schumm, 1927; Falk, Dresel, Benson & Knight, 1956), no corresponding experiments have been performed with protoporphyrinogen.

The possibility had to be entertained that in our experiments hydration of the vinyl groups of protoporphyrinogen occurred before reaction with mercaptan. The problem was approached by comparing the rate of haematoporphyrinogen formation from protoporphyrinogen with that of crude porphyrin *c*-type compound, from protoporphyrinogen and cysteine, under the same conditions. In neutral anaerobic solution the highest yield of haematoporphyrinogen (7–8 %) was obtained by maintenance of protoporphyrinogen at 37° for 2 hr., but with cysteine also present the yield of crude porphyrin *c*-type compound was 35 %. In air the yields were 15 and 70–80 % respectively. Similarly, protoporphyrinogen in 70 % (v/v) acetic acid kept for 1 hr. at 37° yielded 15 % of haematoporphyrinogen, but with cysteine also present more than 70 % was transformed into porphyrin *c*-type compound. This suggests that reaction occurs with protoporphyrinogen rather than with haematoporphyrinogen. However, it is also possible that selective removal of haematoporphyrinogen as it is formed could occur owing to reaction with cysteine so that the quantity of haematoporphyrinogen formed as an intermediate might ultimately represent a considerable portion of the protoporphyrinogen originally present.

*Incorporation of iron into c-type porphyrin.* Two methods were used. (a) Reduced-iron powder (0.1 g.) was boiled in 10 % (v/v) acetic acid (10 ml.) and the filtrate from this mixture was added dropwise to a solution of 6 mg. of porphyrin *c* in 2 ml. of 10 % (v/v) acetic acid. The mixture was kept at 70° for 20 min., when fluorescence had become very weak. Excess of iron was removed by placing the mixture on a column of Amberlite CG-50 ( $\text{H}^+$  form) and washing with 300 ml. of 10 % (v/v) acetic acid. The haem was extracted from the top of the column with the upper phase of butan-1-ol-acetic acid-water (4:1:5, by vol.) mixture and the

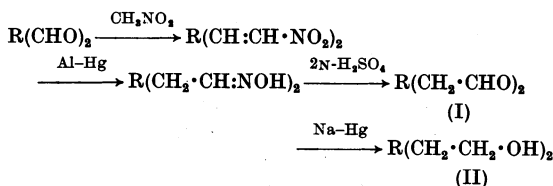


extract was shaken with *n*-hydrochloric acid several times to remove *c*-type porphyrin remaining with the haem. The *n*-butan-1-ol layer was then washed with water and freeze-dried. To a portion of this haem was added a 10% (v/v) solution of pyridine in 0.1*N*-sodium hydroxide (3.5 ml.) followed by a few crystals of sodium dithionite. The spectrum of the pyridine haemochromogen had maxima at 410, 521 and 550  $m\mu$ . The yield of *c*-type haem was 63%.

(b) *c*-Type porphyrin (1.03 mg.) was dissolved in 2 ml. of *m*-tris buffer (pH 8). Ferrous ammonium sulphate (10.14 mg.) and sodium dithionite (10.5 mg.) were added and the mixture was incubated anaerobically at 37° for 2 hr. The yield of *c*-type haem isolated as above was 29%.

*Synthesis of 2,4-di-( $\beta$ -hydroxyethyl)-1,3,5,8-tetramethylporphin-6,7-dipropionic acid [isohaematoporphyrin; 2,4-di-( $\beta$ -hydroxyethyl)deuteroporphyrin; II] and 2,4-diacetaldehyddeuteroporphyrin (I)*

The synthesis of these porphyrins was carried out as follows (R represents the porphyrin ring structure, the side chains being in the 2 and 4 positions):



Diformyldeuteroporphyrin dimethyl ester (20 mg.) was dissolved in boiling pyridine (30 ml.) containing piperidine (0.5 ml.). The temperature was lowered to 70° and a solution of nitromethane (2 ml.) in pyridine (6 ml.) containing piperidine (0.5 ml.) was added dropwise during 4 hr. The mixture was then diluted with about 30 ml. of ice-water and neutralized with 2.5*N*-hydrochloric acid. Porphyrin was extracted into ether which was washed with a small amount of water and shaken first with 0.1*N*-hydrochloric acid and then with 1.5*N*-hydrochloric acid. Most of the porphyrin was extractable with 1.5*N*-hydrochloric acid. It was returned to ether, the solvent was evaporated and the residue was weighed. The yield was 70%. This porphyrin was further purified by alumina-column chromatography with chloroform-methanol (200:1, v/v). The absorption spectrum of dinitrovinyldeuteroporphyrin dimethyl ester in chloroform showed maxima at 408, 508, 540, 576 and 630  $m\mu$ , and that in hydrochloric acid at 409, 557 and 600  $m\mu$ . The infrared-absorption spectrum showed strong absorption due to  $\text{NO}_2$  groups at 1548 and 1340  $\text{cm}^{-1}$ , and the CHO band of diformyldeuteroporphyrin ester at 1660  $\text{cm}^{-1}$  had disappeared.

Nitrovinylphenol, synthesized for comparison, had absorption maxima at 1558 and 1340  $\text{cm}^{-1}$ .

Freshly prepared aluminium amalgam (3 g.) was added to dioxan (20 ml.) containing dinitrovinyldeuteroporphyrin dimethyl ester (20 mg.) and the mixture was kept at room temperature in the dark until the red-purple colour had changed to pale brown (about 2 hr.). This porphyrinogen solution was filtered to remove the aluminium amalgam and was extracted with ether. The ether layer was washed with dilute iodine solution to oxidize the porphyrinogen. The porphyrin in the ether layer was then extracted with 0.1*N*-hydrochloric acid. This solution was made neutral to Congo red by the addition of sodium acetate, the porphyrin was extracted with ether and the ether solution was evaporated to dryness *in vacuo*. After purification on an alumina column with chloroform-methanol, the absorption maxima of the diacetaldehyddeuteroporphyrin ester oxime dissolved in chloroform were at 405, 504, 538, 572 and 627  $m\mu$ . The infrared-absorption spectrum showed no absorption due to  $\text{NO}_2$  groups but weak absorption at 3660  $\text{cm}^{-1}$  due to the OH of the oxime. The yield was about 10%.

Diacetaldehyddeuteroporphyrin dimethyl ester oxime (2 mg.) was hydrolysed by refluxing in a mixture of ethanol (200 ml.) and 2*N*-sulphuric acid (20 ml.) for 8 hr. at 80°. The mixture containing diacetaldehyddeuteroporphyrin was made neutral to Congo red with saturated sodium acetate and extracted with ether. The porphyrin had absorption maxima at 410, 508, 540, 575 and 630  $m\mu$ . The oxime shift is thus small (2–3  $m\mu$ ) in comparison with that of diacetyldeuteroporphyrin. The infrared-absorption spectrum of diacetaldehyddeuteroporphyrin differs from those of diacetyldeuteroporphyrin and diformyldeuteroporphyrin. The CHO absorption appears in the region of the carboxylic ester absorption (1735  $\text{cm}^{-1}$ ); in diformyldeuteroporphyrin it is at 1660  $\text{cm}^{-1}$ .

2,4-Di-( $\beta$ -hydroxyethyl)deuteroporphyrin (isohaematoporphyrin) was obtained by reduction with excess of sodium amalgam of the diacetaldehyddeuteroporphyrin (2 mg.) dissolved in 0.02*N*-potassium hydroxide. After 2 min. reduction the porphyrinogen was extracted into ether and oxidized with dilute iodine and the porphyrin was then extracted from ether with 0.1*N*-hydrochloric acid. After esterification with methanolic 10% (w/v) hydrochloric acid, the porphyrin ester was purified on an alumina column. Absorption maxima were at 400, 500, 535, 570 and 622  $m\mu$  in chloroform and at 402, 549 and 591  $m\mu$  in *n*-hydrochloric acid. Kerosene-chloroform paper chromatography showed it to have the same  $R_f$  (0.03) as haematoporphyrin dimethyl ester. The yield was 1.2 mg., giving an overall yield of isohaematoporphyrin of about 4%.

## DISCUSSION

Intensive investigation of cytochrome *c* has made it very probable that two cysteine residues are bound to positions 2 and 4 of the porphyrin ring by thio ether linkages, and the structure usually assigned to porphyrin *c* is that shown in Fig. 1. This contains four asymmetric carbon atoms, and confusion still exists about the optical activity of porphyrin *c* and of products derived from it. Should both sulphur atoms be attached to the  $\beta$ - rather than to the  $\alpha$ -carbon atoms of the side chains, optical activity would depend only on the configuration of the cysteinyl residues.

Earlier attempts (Zeile & Meyer, 1939) to cleave the thio ether linkages by acid hydrolysis led to a haematoporphyrin that was optically inactive, but when Paul's (1950) more gentle silver sulphate technique was used optically active products were obtained (Paul, 1951). Comparison has previously been made of natural materials with protoporphyrin-cysteine adducts prepared under rather drastic conditions such as melting together dibromoporphyrin with cysteine hydrochloride (Zeile & Meyer, 1939) or by heating together cysteine and either protoporphyrin or haematoporphyrin in hydrochloric acid solution *in vacuo* (Theorell, 1939). These synthetic products showed considerable similarity with natural porphyrin *c*, but certain discrepancies arose in their derivatives. These could have been due to optical isomerism but doubt still remained about the question of attachment of the sulphur atoms to the  $\alpha$ - or  $\beta$ -carbon atoms of the vinyl side chains. The position is well summarized in the paper by Paul (1951). Thus cytochrome *c* treated with silver sulphate gave a ferriporphyrin *c* that was converted by Grinstein's (1947) procedure in 3 min. into a haematoporphyrin dimethyl ester dimethyl ether (yield, 87 %) which had m.p. 144–145° and specific rotation  $-31.3^\circ$  in acetic acid. Chemically prepared ferrihaematoporphyrin, on the other hand, required 15 min. treatment to convert it into an optically inactive haematoporphyrin dimethyl ester dimethyl ether (yield, 28 %) with m.p. 148°. Although visible-absorption spectra and analyses agreed, non-identity was apparent from the depression of the mixed m.p. (132°) and from differences in infrared-absorption spectra. Paul (1951) considered positional isomerism to be unlikely since both products lost methanol at the same rate at 140° *in vacuo* to yield protoporphyrin. Tritylation experiments with the unesterified cytochrome ferriporphyrin supported the probability of the presence of secondary rather than of primary alcoholic groups. Paul's (1951) examination of the haematoporphyrin dimethyl esters derived from cytochrome *c* and authentic haematoporphyrin

respectively also revealed differences. That from cytochrome *c* had m.p. 214° and authentic material m.p. 213°, but the mixture melted at 198–200°.

In the present work advantage has been taken of the ready combination between mercaptans and autoxidizing protoporphyrinogen (Sano & Granick, 1961) to prepare porphyrin *c*-like substances and to examine the haematoporphyrin formed from them by cleavage with silver sulphate. Since the procedures involved are mild this should afford reliable information on the constitution. In addition, the structural isomer (isohaematoporphyrin) of haematoporphyrin has been synthesized in which the side chains at positions 2 and 4 carry primary alcoholic functions (hydroxyl groups attached to the  $\beta$ -carbon atoms). On oxidation, isohaematoporphyrin yields diacetaldehydodeuteroporphyrin, and haematoporphyrin yields diacetyldeuteroporphyrin. Oxidation of the porphyrin obtained by silver sulphate cleavage of our synthetic *c*-type porphyrin gave only diacetyldeuteroporphyrin, thus establishing attachment of the sulphur to the  $\alpha$ -carbon atoms of the vinyl side chains.

In a study of the addition of mercaptans to unsaturated compounds Mayo & Walling (1940) found that traces of peroxide lead to abnormal addition. Peroxide might very well be formed during the autoxidation of protoporphyrinogen, but the addition of quinol was without effect on the result; the addition appears to be an ionic rather than a free-radical reaction.

Just before this paper was submitted for publication, Popper & Tuppy (1963) published experiments on the combination of reduced protoporphyrin with cysteine. They confirmed the finding of Sano & Granick (1961) that a porphyrin *c*-like compound is formed from cysteine and protoporphyrinogen under mild conditions. Combination of protoporphyrinogen with bovine serum albumin was also reported.

## SUMMARY

1. The products formed in the reaction of protoporphyrinogen with L-cysteine, mercaptoethanol, reduced glutathione and thioglycollic acid have been purified and characterized by paper chromatography. L-Methionine and S-methyl-L-cysteine do not react with protoporphyrinogen or haematoporphyrinogen.

2. No reaction occurred between the above mercaptans and protoporphyrin, protohaem, mesoporphyrin, deuteroporphyrin, coproporphyrin, uroporphyrin (or their corresponding porphyrinogens), haematoporphyrin, haematohaem or diacetyldeuteroporphyrin. However, a thioglycollateporphyrin could be prepared from haematoporphyrin and thioglycollic acid in the presence of a condensing agent.

3. Haematoporphyrinogen reacted readily with thiol compounds, but evidence was obtained that haematoporphyrinogen is probably not an intermediate in those reactions in which protoporphyrinogen is the starting material.

4. The porphyrin *c*-like adduct formed from protoporphyrinogen and L-cysteine has been crystallized and its spectral properties in the visible and infrared regions have been recorded. Cleavage of the adduct with silver sulphate yields cysteine and a haematoporphyrin. On the basis of chromatography, visible and infrared spectral properties, rate of conversion into protoporphyrin and oxidation to 2,4-diacetyldeuteroporphyrin it is identical with authentic haematoporphyrin. This establishes that the sulphur atoms are attached to the  $\alpha$ -carbon atoms of the vinyl side chains.

5. Iron has been incorporated into the adduct by two different methods to produce a *c*-type haem.

6. The synthesis of isohaematoporphyrin [2,4-di-( $\beta$ -hydroxyethyl)-1,3,5,8-tetramethylporphyrin-6,7-dipropionic acid] from 2,4-diformyldeuteroporphyrin is described. 2,4-Diacetaldehydodeuteroporphyrin, an intermediate in this synthesis, and its oxime were characterized by their spectral properties in the visible and infrared regions.

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