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The Porphyrin Component of Cytochrome c and its Linkage to the Protein

KARL-GUSTAV PAUL

Biokemiska avdelningen, Medicinska Nobelinstitutet, Stockholm, Sweden

Hill and Keilin [1] found that digestion of yeast cytochrome c with a hydrogen bromide-glacial acetic acid mixture gave an ether soluble porphyrin. It was identical with hematoporphyrin, to judge from its spectrum and its loss of water upon heating *in vacuo*. The authors also found that sulphur dioxide in concentrated hydrochloric acid released a porphyrin from cytochrome c which had a spectrum intermediate to the meso- and protoporphyrin types. This porphyrin, "porphyrin c", was soluble in water at all pH values and could not be taken into ether. Zeile [2] and Zeile and Piutti [3] prepared derivatives of protoporphyrin by introducing nitrogen bases in its side chains 2 and 4. Some of these products or their iron complexes behaved in certain respects as cytochrome c or porphyrin c, *e. g.* as regards solubility, spectrum, and reactivity with hydrogen bromide-glacial acetic acid. The dimethyl ester of mesoporphyrin, obtained from cytochrome c hematoporphyrin by means of reduction with hydrogen iodide, gave no melting point depression with mesoporphyrin ester from blood ferri protoporphyrin [4]. Theorell [5, 6], employing an improved technique, prepared porphyrin c with a molecular weight of 890 $\pm$ 10. The ratios total-N : amino-N : S : COOH : Fe (subsequently introduced) were found as 6 : 2 : 2 : 4 : 1. Since there was no nitrogen in excess of what could be accounted for by the amino and pyrrol nitrogen atoms, the bonds between porphyrin and protein could not be mediated by nitrogen.

Porphyrin c gave a sulphide upon melting with solid potassium hydroxide. The nitroprusside reaction for free sulphhydryl groups was negative as well as the polarographical test for a disulphide. It was therefore considered as probable that the sulphur atoms were present in thio-ether bonds. Porphyrin c did not take up any hydrogen in the presence of palladium. The ninhydrine test was positive. It was therefore concluded that porphyrin c was the di-

cysteine adduct of protoporphyrin. The theory was confirmed by the isolation of l-cystine from porphyrin c [7]. The yield of cystine was 40 % of the theoretical for two cysteine adducts. The suspicion that porphyrin c could be an artifact, formed during the acid hydrolysis of cytochrome c [8], could be rejected when it was proved that a twenty-fold lowering of the cytochrome concentration during the hydrolysis did not influence the result [9].

Zeile and Meyer [9] compared two preparations of porphyrin c, obtained in different ways. The "natural" porphyrin c was prepared by hydrolysis of cytochrome c with sulphuric acid, while the "synthetic" porphyrin c was obtained by melting together l-cysteine hydrochloride and di-bromoprotoporphyrin. Both preparations, isolated via their methyl esters, were free from ash and of the elementary composition required by protoporphyrin-di-cysteine. They agreed completely as regards spectra and partition of their esters between ether and acid buffers. Neither preparation could be crystallized. Their optical activities were, however, quite different, $[\alpha]^{17}_{\text{white light}}$ being — 172° for the natural and + 27° for the synthetic porphyrin c in 0.1 % hydrochloric acid. Also the yields of hematoporphyrin from their iron complexes by hydrogen bromide — glacial acetic acid treatment differed, 30 % of the theoretical for the natural and up to 92 % for the synthetic porphyrin c. The hematoporphyrin from the iron complex of natural porphyrin c was optically inactive.

The above mentioned investigators employed methods, which included treatment with a strong acid for the liberation of the porphyrin from the protein. This is to be noticed, since mineral acids are known to catalyze the addition of thiols to alcoholic structures in side chains [10]. There is also an equilibrium between proto- and hematoporphyrin in hot hydrochloric acid [11]. The splitting of cytochrome c with a silver salt [12] involves milder conditions. It seemed therefore to be of interest to re-examine the structure of the cytochrome c porphyrin. The splitting procedure also opened a possibility to determine the positions of the thio-ether bonds in the side chains 2 and 4 of the cytochrome c porphyrin.

EXPERIMENTS

Material

Cytochrome c with an iron content of 0.41 % was prepared from cow hearts [13]. This material was used for most experiments. A part of the sample was further purified by electrophoresis at pH 10.45 and 7.7 [14]. It was dialysed against 0.1 % ammonia until negative sulphate reaction. Finally it was lyophilized, redissolved in a small volume of redistilled water and electrodialyzed. The filtered solution was found to contain 89.2 μg iron and 3.24 mg nitrogen (micro-Kjeldahl) per ml, which corresponds to 145 nitrogen atoms per one iron atom. The dry weight was 21.04 mg per ml, giving an iron content of 0.424 %. The ash content was 0.027 and 0.071 mg after drying and ignition in the gas flame of 0.10 and 0.20 ml respectively.

The light absorption at the wave-length for the top of the α-band of reduced cytochrome c is frequently used for the determination of the concentration of cytochrome c in solutions and to follow spectrophotometrically reactions in which cytochrome c is involved. The value reported in the literature for the molar absorption of the α-band of reduced cytochrome c vary considerably, however [15]. For this reason we considered it to be of interest to re-determine the molar absorption with the Beckman spectrophotometer (type DU). Suitably diluted samples (0.05—0.15 ml to 10.0 ml with $M/15$ phosphate buffer pH 6.8) were reduced in the cuvettes and their absorptions measured with the slit 0.01 mm. On the basis of the iron content the molar absorptions (β) * were found as 6.36×10^7 and 1.76×10^7 $cm^2 \times mole^{-1}$ for the maximum of the α-band and the minimum between the α-and the β-bands respectively. The value 6.36×10^7 has been found by the author for several cow heart cytochrome c preparations. It is advisable to make determinations at every mμ within 548—552 mμ to find the maximum, since the position of the top of the α-band varies a little from one preparation or instrument to another.

The partition of nitrogen between the split products

3.00 ml of the purest preparation, containing 6.94×10^{-4} gramatoms of nitrogen, were digested at 60° with 3.0 ml silver sulphate solution (800 mg salt per 100 ml salt solution) and 0.6 ml glacial acetic acid. After 80 min the mixture was cooled, the protein precipitated with 42 ml acid acetone (1 ml 5 N sulphuric acid to 100 ml acetone) and centrifuged down. The precipitate was redissolved in 3 ml distilled water + 0.4 ml glacial acetic acid and reprecipitated with 30 ml acid acetone. This procedure was repeated once more. The combined acetone solutions were evaporated to about 5 ml and rinsed over into a 10-ml volumetric flask with 3 ml acetic acid and water. The total nitrogen content of the fraction, determined on aliquots of 3 and 6 ml, was found to be 0.367 mg. If complete splitting of the cytochrome c is assumed, this means that the ratio of nitrogen to iron was 5.4 in the ferri porphyrin fraction. No amide-nitrogen was found in this fraction.

The total nitrogen content of the protein fraction was found to be 6.67×10^{-4} gramatoms. The recovery of nitrogen (protein + ferri porphyrin) was thus 6.93×10^{-4} gramatoms.

Elementary composition of the ferri porphyrin from cytochrome c

Cytochrome c with 0.41 % iron was used for these experiments. Of this preparation 1.44 g, corresponding to 75 mg ferri porphyrin (calculated as ferri hematoporphyrin chloride) was treated as above with silver sulphate and

* $\beta = \frac{1}{e} \times \frac{1}{d} \times \ln \frac{I_0}{I}$, where c = concentration in moles per ml solution, d = optical depth of the solution in cm, I_0 and I = intensities of incident and transmitted light respectively.

acetone. Only one acetone extraction was made. The acetone was removed *in vacuo* and the ferri porphyrin flocculated from the acetic acid — water mixture by the addition of sodium acetate. The suspension was left in the cold room overnight to complete the precipitation, then centrifuged down and washed with water, 0.1 M HCl and once more with water. After drying at 60° for three hours and overnight in a desiccator the material weighed 68 mg.

Before any further purification was attempted, the preparation was examined for amino acid residues [16]. 9.1 mg were hydrolyzed for 24 h with 2.2 ml 20 % hydrochloric acid. After the removal of the acid by repeated evaporation to dryness and the addition of a few drops of 4 M sulphuric acid, the filtered hydrolysate was run in a two-dimensional paper chromatogram with the systems phenol-water 4 : 1 and lutidine-collidine-water 1 : 1 : 1 (volume ratios). No amino acids or cysteic acid could be detected with ninhydrine *.

The rest of the material was dissolved in 10 ml methanol, added in small portions with immediate filtration and evaporation to dryness after each addition. The dry material was ground with ether for 30 min and collected on a glass filter. After careful washing with water, the preparation was dried by suction and in a desiccator. The weight was 53.7 mg. To convert any ferri porphyrin hydroxide, which might be present, to the chloride, the preparation was eluted from the filter with 0.01 ml conc. hydrochloric acid in 5 ml acetone. The weight after drying in a current of air and *in vacuo* was 50.5 mg.

The spectrum in methanol is given in Fig. 1.

Analyses:	C	H	N	Fe	Ash
Found (%)	59.8	5.0	7.6	8.1	12.7
Calc. for hematohemin ($C_{34}H_{36}O_6N_4FeCl$)	59.4	5.3	8.1	8.1	11.6

Nitrogen was determined according to Dumas, which method often will give too low values for pyrrol nitrogen. Total conversion of the iron to Fe_2O_3 was assumed for the calculation of the ash weight.

Conversion of the ferri porphyrin to porphyrin

In the same way as described above 46 mg ferri porphyrin were obtained from 1.00 g cytochrome c, the grinding with ether being omitted. We found it, however, to be preferable to substitute the acetate for sodium bicarbonate for

* Dr. S. Paléus gave valuable assistance with the paper chromatogram, which is gratefully acknowledged.

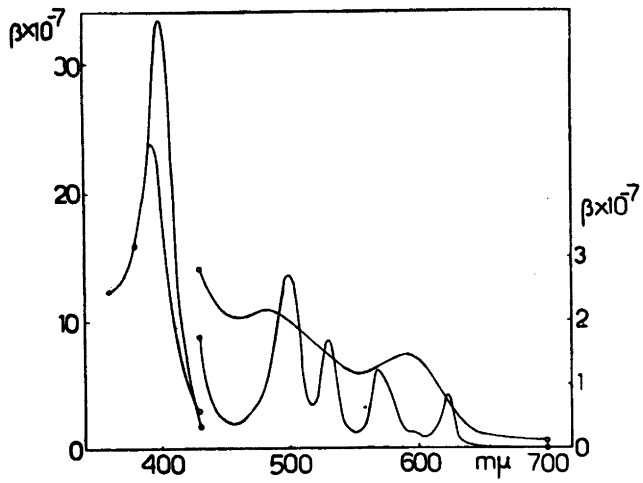
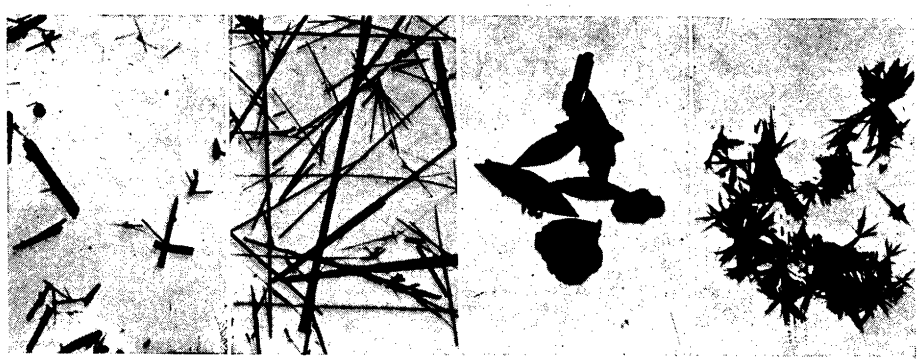


Fig. 1. Hematohemin c in methanol (O—O).

$\beta_{393} = 23.9 \times 10^7\ cm^2 \times mole^{-1}$.
$\beta_{483} = 2.19$ » » »
$\beta_{590} = 1.46$ » » »

Tetramethyl hematoporphyrin c in dioxane (●—●).
$\beta_{400} = 34.9 \times 10^7\ cm^2 \times mole^{-1}$.
$\beta_{499} = 2.88$ » » »
$\beta_{530} = 1.79$ » » »
$\beta_{568} = 1.24$ » » »
$\beta_{623} = 0.83$ » » »
Left ordinate scale for 360—430 mμ, right scale for 430—700 mμ.

the flocculation. Maximal flocculation occurred when 1/5 of the acetic acid had been neutralized (pH 4). The dried ferri porphyrin was divided into four equal parts, which were separately dissolved in 10 ml methanol each. 0.5 g ferrous sulphate was added, and dry hydrogen chloride was vigorously bubbled through the solution for three minutes [17]. After that time no absorption band of the "hemin" type could be detected. The solution was cherryred and showed the acid type spectrum of a porphyrin. The combined solutions were taken into a separatory funnel with chloroform-water-acetate. The chloroform solution was washed three times with 7 % sodium chloride solution, filtered through a chloroform-moistened, double paper and evaporated to dryness. The commonly used washing of the chloroform solution with dilute ammonia was omitted, since it was found to develope a brown discoloration of the porphyrin.



A B C D

Fig. 2. *A = tetramethyl hematoporphyrin c (fraction I), m. p. 144°.*
B = » » » ») m. p. 145°.
C = trimethyl » » (fraction IIB).
D = tetramethyl hematoporphyrin, m. p. 148°.
All specimens were crystallized from chloroform-methanol. Magnification 72 ×.

The material was further purified by chromatography on alumina * grade IV [18]. It was adsorbed on the top of the column from its chloroform ** solution and the chromatogram was developed with chloroform-methanol 200 : 1. Two porphyrin coloured zones appeared, the lower of which (fraction I) descended twice as fast as the upper (fraction II). No other fractions were seen. Obviously the preparation was rather pure, since the brown region at the top of the column, which is generally seen, did not appear.

Fraction I. The total weight was 35 mg. The material crystallized very easily from chloroform-methanol with m.p. 144° (Fig. 2 A), after rechromatography on alumina grade I + II (taken directly from the package) with chloroform as eluant 145° (Fig. 2 B).

Fraction II. No crystals were obtained from the commonly employed solvents, so the material was rechromatographed on alumina grade IV with chloroform-methanol 500 : 1 as eluant. This revealed three fractions (IIA, IIB, and IIC). IIA, brownish and very small, was discarded. IIB and IIC were porphyrin coloured. Both fractions crystallized from chloroform-methanol. IIB appeared at first as very thin equilaterals of m. p. 110°, which

* Savory and Moore Ltd (Aluminium oxide for chromatographic analysis standardized according to Brockmann).

** Chloroform to be used for the chromatograms was washed once with potassium carbonate solution, twice with water, dried and distilled over calcium chloride, and stored in a dark bottle. It was used within two days. Methanol a. g. was employed without pretreatment.

gradually changed in shape (Fig. 2 C). The rhomboedric crystals from fraction IIC, of the same appearence as fraction I, m. p. 144° (Fig. 2 A), melted indefinitely at 202—206°, after drying at 56° for one hour *in vacuo* at 203—206°. Attempts were made to reach a more definite melting point by chromatography on various adsorbents and with various eluants but without success. The spectra of fractions IIB and IIC agreed with the hematoporphyrin dimethyl ester spectrum.

Ferri hematoporphyrin chloride was prepared as described before [19]. For the conversion of it to the corresponding methylated porphyrin it was found, however, that three minutes did not suffice for complete reaction under conditions identical with those employed for the cytochrome ferri porphyrin as regards quantities of reactants *etc.* Not until after fifteen minutes had the sample aquired the pure porphyrin colour. The yield of tetramethyl hematoporphyrin was also low (49 mg from 200 mg ferri hematoporphyrin chloride, = 28 % as compared to 87 % for the cytochrome porphyrin). The difference in reactivity between the two ferri porphyrins was confirmed in several experiments with different preparations. After washing and chromatography as described above, the methylated hematoporphyrin crystallized from chloroform-methanol with m. p. 148° (Fig. 2 D). No change in the shape of the crystals or their melting point was achieved by repeated chromatography.

A ground mixture (1 : 1) of the two substances (m. p. 145° and 148° respectively) melted at 132°.

The visible spectra of the two substances agreed (Fig. 1 and Table 1). Their infrared spectra * are given in Fig. 3.

Analyses:	C	H	CH_3O
Cytochrome porphyrin;			
Fraction I	69.9	7.0	18.6
» IIB	—	—	13.9
» IIC	—	—	13.7
Hematoporphyrin;			
Fraction I	69.8	6.9	18.4
Calculated for			
Hematoporphyrin dimethyl ether dimethyl ester ($C_{38}H_{46}O_6N_4$)	69.7	7.1	19.0
Hematoporphyrin dimethyl ester ($C_{36}H_{42}O_6N_4$)	69.0	6.8	9.9
Hematoporphyrin monomethyl ether dimethyl ester ($C_{37}H_{44}O_6N_4$)	69.3	6.9	14.5

* This result is a part of a more general investigation of the infrared spectra of porphyrins and porphyrin derivatives by H. Theorell and K.-G. Paul (to be published shortly).

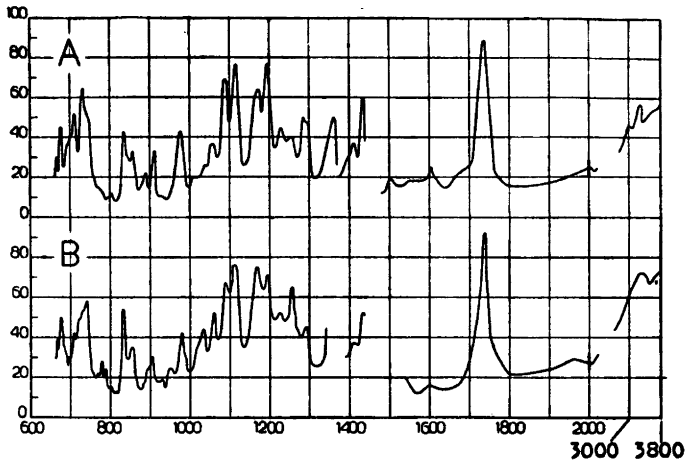


Fig. 3. Infrared spectra of tetramethyl hematoporphyrin c (A) and tetramethyl hematoporphyrin (B). Specimen/paraffine oil = 1/2.5. Thickness of mull in the cuvette 0.025 mm. Abscissa; cm^{-1}. Ordinata; Absorption in per cent.

Optical activity of cytochrome porphyrin and hematoporphyrin

Because of the low intensity of the transmitted light from the relatively concentrated porphyrin solutions which had to be used, a 2 kW carbon-carbon arc was employed as light source. The light was filtered through a 5 cm cuvette with pure water, through Schott Gen. filter nr BG 21 (gray) and in some experiments also through Schott Gen. filter RG 1 (red). A Hilger polarimeter with a 5.00 cm microtube, taking 0.3 ml, was used for the readings.

Determinations on the cytochrome porphyrin (fraction I) were made on two solutions of the same preparation. In the first experiment 2.775 mg substance were dissolved in 1.00 ml purified dioxane [20]. The rotation of this solution was $-0.137° \pm 0.009°$ (S.D.) ($-0.03°$ to $-0.21°$, $n = 30$) with the blank value for pure dioxane in the tube $-0.002° \pm 0.007°$ ($0.08°$ to $-0.07°$, $n = 30$). The rotation due to the substance was thus $-0.135° \pm 0.011°$, which gives the specific rotation $-97.3° \pm 7.9°$. In the second experiment 14.812 mg were dissolved in 1.00 ml glacial acetic acid. Because of the very high light absorption the red Schott filter was not used. The rotation was found as $-0.323° \pm 0.030°$ ($-0.19°$ to $-0.46°$, $n = 9$). When this solution was diluted with one volume of glacial acetic acid (both Schott filtra) the rotation was $-0.184° \pm 0.012°$ ($-0.07°$ to $-0.29°$, $n = 32$). The blank value with glacial acetic acid in the tube was $-0.076° \pm 0.015°$ ($0.02°$ to $-0.14°$, $n = 16$). The actually determined angles were thus after corrections for the

Table 1. Spectra of the tetramethyl compounds of hematoporphyrin and hematoporphyrin c (Beck-Hartridge reversion spectroscope).

Solvent	Tetramethyl hematoporphyrin	Tetramethyl hematoporphyrin c
5 % HCl	593.8	593.1
	573.9	572.3
	550.3	549.7
Aqueous pyridine	623.9	623.7
	568.3	568.3
	535.5	533.4
	500.4	499.7
Chloroform	622.5	622.5
	567.5	567.7
	535.5	533.5
	501.2	499.4

blank $-0.247° \pm 0.034°$ and $-0.108° \pm 0.019°$, giving the specific rotations $-33.3° \pm 4.6°$ and $-29.2° \pm 5.1°$ respectively.

For the determination of the optical activity of the tetramethyl compound of hematoporphyrin 7 mg were dissolved in 1 ml glacial acetic acid. The determined angle was $0.019° \pm 0.020°$ ($-0.10°$ to $0.15°$, $n = 16$) for the solution and $0.026° \pm 0.020°$ ($-0.12°$ to $0.13°$, $n = 16$) for the blank, giving the rotation due to the substance $-0.007° \pm 0.028°$. Thus no significant optical activity could be demonstrated for the synthetic porphyrin. This is in agreement with earlier observations [9].

The positions of the hydroxyl groups of the cytochrome c porphyrin

The loss of water from cytochrome c ferri porphyrin was registered in the following way. 1 ml of a solution of cytochrome ferri porphyrin in methanol, containing about 0.04 mg per ml, was pipetted into each of five Thunberg tubes. Another series was prepared in the same way with hematohemin. The tubes were then dried in a current of air, leaving the substance as a film on the glass walls, evacuated, and immersed in an oil bath of + 140° C. After certain times a pair of tubes were removed, one from each series. They were cooled, and 5.0 ml of a mixture of pyridine and 0.1 *M* NaOH (1 : 3) + some

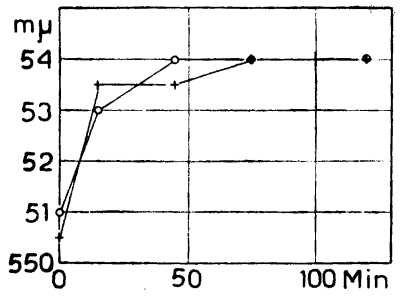


Fig. 4. Shift in wavelength for the top of a mixture of pyridine hemochromes upon heating of the corresponding ferri porphyrins (cf. text).
+ hematohemin c, O hematohemin.

dithionite were added. The spectrum from 545 to 560 mμ of the mixture of the hemochromes was determined. The shift in wave-length of the top of the absorption band, due to the formation of vinyl groups, was plotted against time of heating (Fig. 4).

Tetramethyl hematoporphyrin is converted to protoporphyrin dimethyl ester when heated *in vacuo* at 135—150° C for 5—10 min.[21]. The same was found for the tetramethyl porphyrin from cytochrome c: 9.79 mg of fraction I, previously dried at 56° *in vacuo* over P_2O_5 for one hour, were heated at 140° for 10 min. The loss of weight was 0.94 mg, corresponding to the removal of 1.96 moles of methanol per mole of tetramethyl porphyrin. The residue was chromatographed on alumina grade IV with methanol-chloroform 200 : 1 as eluant. The first fraction that left the column weighed 7.0 mg. It was red, crystallized from chloroform-methanol with m. p. 226°. A freshly prepared sample of protoporphyrin dimethyl ester (m. p. 228°)[17] gave no melting point depression (229°). The spectra of the two substances in chloroform agreed completely. Another three fractions, green, red, and brown respectively, all very small and of about equal strength, appeared on the column but were not isolated. Thus the tetramethyl porphyrin from cytochrome c gives protoporphyrin dimethyl ester upon heating, and behaves in that respect as tetramethyl hematoporphyrin.

The two experiments described above indicate that the two porphyrins in question have their hydroxyl groups bound in the same way. The behaviour of the cytochrome porphyrin upon tritylation should give further evidence for the positions[22]. To 34.0 mg cytochrome ferri porphyrin in 2.2 ml anhydrous pyridine were added 30.3 mg triphenylmethyl chloride (2.2 moles per mole ferri porphyrin) in 1.4 ml anhydrous pyridine. The trityl chloride had previously been recrystallized from acetyl chloride and dried under protection against moisture. The mixture was left in an ice-bath for two hours and then at room temperature overnight. After the addition of 2 ml of water and stand-

ing for another two hours at room temperature the solution was evaporated to dryness on a waterbath (bath temperature not exceeding + 50°, vapour temperature + 27°). The residue was dissolved in acid acetone, the solution filtered, dried, and the residue re-dissolved in methanol. After washing several times with light petroleum and standing overnight under a layer of light petroleum the methanol solution yielded a sediment of darkbrown material with an iron content of 6.47 %. This iron content corresponds to the addition of 0.7 triphenylmethyl groups per ferri porphyrin. The precipitate was insoluble in ammonia. Since the carboxyl groups of ferri protoporphyrin chloride have to be neutralized to 90 % before dissolution in an aqueous medium is possible [23], it is likely that the tritylation has attacked the carboxyl groups. It was easily soluble in pure methanol.

The dimethyl ester of the porphyrin would be the best material for the tritylation studies. However, it has as yet not been possible to develope a suitable procedure for the removal of iron from the cytochrome ferri porphyrin. The yield is very poor (< 10 %) by various methods, and as a rule several fractions of about equal magnitude appear. A few mg, obtained from 40 mg ferri porphyrin by means of the ferrous acetate procedure and subsequent esterification with diazomethane and chromatography, melted at 214°, Hematoporphyrin dimethyl ester (m. p. 213°) gave a melting point depression (198—200°).

DISCUSSION

The cytochrome c ferri porphyrin is easily soluble in alcohols, and under certain conditions it can also be obtained in an aqueous medium (excessive washing of its solution in ether-acetic acid with dilute hydrochloric acid). It might therefore be questioned whether some extra, hydrophilic group(s) might be present. The value 5.4 nitrogen atoms per one iron atom could be an indication thereof. However, this is probably not the case. The ratio of nitrogen to iron was determined in a single experiment, and with the rather large volumes of solvents, which had to be used, it is quite possible that some foreign nitrogen could have entered the solution. It seems also to be difficult to attribute this extra nitrogen to some position in the molecule. Also the "synthetic" hematohemin is more hydrophilic than the proto-, meso-, and deuterohemins. An indication thereof can be seen in the R_f-values of these four substances in paper chromatography with the system methyl ethyl ketone / aqueous buffer of pH 3, which were found as 0.85, 0.95, 0.9, and 0.9 respectively [24]. Accordingly it is not necessary to suppose any hydrophilic groups in addition to the hydroxyl and carboxyl groups.

The solubility in alcohols depends no doubt upon the hydroxyl groups, since the other three substances, mentioned above, are sparingly or not at all soluble in alcohols. This property of hematohemin c * offers a convenient method for the removal of impurities, *e. g.* in isotope experiments [12,25].

Hematohemin c is obviously an isomer of hematohemin. The non-identity of the two substances is evident from 1) their different reactivities during the methanol-hydrogen chloride treatment 2) the melting point depression 3) the optical activity of hematohemin c and 4) their infrared spectra.

The addition of hydrogen bromide to the vinyl groups of ferri protoporphyrin chloride should proceed in such a way that bromine becomes attached to the secondary carbon atoms of the side chains 2 and 4 [26]. This direction of the reaction is favoured by the glacial acetic acid being a strongly polar solvent, and also by the traces of iron which are liberated from the ferri porphyrin. No peroxide is present, and the solubility of oxygen in the hydrogen bromide solution (sp. g. 1.4) is probably low. In his later papers on hematoporphyrin Hans Fischer also gave the structure of hematoporphyrin as possessing two secondary alcoholic groups. The vinyl groups are equally available to the hydrogen bromide from all sides. Random configuration is probable, and the racemic form will thus appear.

When cytochrome c is treated with hydrogen bromide in glacial acetic acid, hematoporphyrin is obtained [1]. This hematoporphyrin is optically inactive [9], in contrast to hematoporphyrin c. If the reasonable assumption is made that the two secondary carbon atoms in question are optically active already in the intact cytochrome c molecule, the silver splitting procedure must imply conditions, which retain this activity and prevent racemization. This protection could be effected by the silver ions. Sulphides are known to form addition compounds with salts of heavy metals, "probably as a result of the donor activity of the sulphur" [27]. In that way the covalent carbon — sulphur bond could become more polar, and thereby exert a directing influence on the hydroxyl groups.

The conversion of an α-hydroxy ethyl group to a vinyl group should proceed much easier than the corresponding reaction with a β-hydroxy ethyl group. Hematohemin has two and hematohemin c at least one α-hydroxy ethyl group, as is evident from its optical activity. Since the dehydration of the two substances proceeds at the same rate (Fig. 4), it is likely that also hematohemin c has two α-hydroxy ethyl groups. The difference between the pyridine

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* To avoid confusion with the iron complex of porphyrin c (= the di-cysteine adduct of protoporphyrin) the name *hematohemin* c is suggested for the ferri porphyrin, which is liberated from cytochrome c by the procedure with silver salt and acid acetone.

proto- and hematohemochromes is 7 mμ, which is exactly the double of the terminal value in our experiment. No experiment has been made to explain the discrepancy. The essential thing is, however, that hematohemin and hematohemin c reacted in the same way. The same conclusion applies of course also to the removal of methanol from tetramethyl hematoporphyrin c by heating.

Helferich [22] has recently reviewed the subject of tritylation. According to this review secondary as well as primary hydroxyl groups can react with trityl chloride in pyridine. However, there is a considerable difference in tritylation velocity between the two kinds of hydroxyl groups, primary alcohols being tritylated much easier. When the reaction is carried out in pyridine with only a slight excess of trityl chloride and at room temperature, the difference is in fact of such an order of magnitude that Helferich concluded: "If an hydroxyl group does not react with trityl chloride in pyridine, it is not a primary hydroxyl group. No instance of an unreactive primary hydroxyl group has yet been reported." With due reservation given to the fact that the experiences on tritylation reactions have been aquired mainly in the field of carbohydrate chemistry, the negative result of the tritylation attempt in this case is interpreted as strongly supporting the idea that the two hydroxyl groups are not bound to the primary carbon atoms of the side chains 2 and 4.

The spectra in visible light of the isomers of coproporphyrin do not differ from each other, and the same applies to the uroporphyrins. The meso- and coproporphyrin spectra are also very similar [28]. It is therefore most unlikely that in the present case an α-β-isomery (or of course the difference between the optically active form and its racemic form) would reflect in the spectra in visible light.

Contrary to what is found for the spectrum in visible light the infrared spectrum can be expected to be influenced by the structural difference between the tetramethyl compounds of hematoporphyrin c and hematoporphyrin [29]. The sharp peak at about 1735 cm^{-1}, found also in the spectra of the free proto-, meso-, deutero-, and hematoporphyrins, is caused by the carbonyl group of the carboxyl group [29]. Accordingly it is not to be expected that the two spectra should differ at this frequency. The structure —C—O—C—, present in ethers and esterified carboxyl groups, gives a band [29] at 1110—1250 cm^{-1}. The bands at 1115, 1170, and 1195—1200 cm^{-1} are found also in the spectra of the dimethyl esters of proto-, meso-, deutero-, and hematoporphyrins, whereas the band at 1090 cm^{-1} is absent in them. It *might* therefore be due to the ether structure at the side chains 2 and 4. Differences between the two curves in Fig. 3 are found in the regions 1 000—1 100 and 1 200—1 300 cm^{-1}. These two spectra will be discussed more detailed together with the spectra of some other porphyrin derivatives elsewhere [30].

Fischer and Müller presented in 1924 a detailed study of tetramethyl hematoporphyrin [21]. When prepared from ferri protoporphyrin chloride by bromination and subsequent substitution of the bromine for methoxyl groups without hydrolysis in water, the substance could appear in four different types A—D with the melting points 185°, 178.5°, 140°, and 110°. The crystals varied, A being needles, B double pyramides, C cubes, and D of miscellaneous forms (bundles of needles, prisms etc). A and D were crystallographically identic. It was possible to convert type A to type D by recrystallization from chloroform-methanol and *vice versa*. Types C and D could be crystallized from the mother liquor of type B crystals. With reference to Willstätter's formula for hematoporphyrin (one hydroxy ethyl and one hydroxy vinyl side chain) Fischer and Müller concluded that the differences between the crystallographical types were due to structural isomery. This is rather startling since their experiments on the removal of methanol by heating *in vacuo* gave a compound, which was identic with ooporphyrin (= protoporphyrin) ester. This result fits well with Küster's hematoporphyrin formula (two hydroxy ethyl groups), which also was referred to. Accordingly it is possible that the different melting points depend upon some other factor, *e. g.* the structure of the crystals.

In the present investigation, where the tetramethoxyl derivative was prepared in a different way and subsequently chromatographed, only one form of the crystals appeared. Its crystallographical examination will be undertaken later on. In any case there is a structural difference between the tetramethyl compounds of hematoporphyrin and hematoporphyrin c.

From the results presented here it is evident that hematohemin c possesses two α-hydroxy ethyl groups. It is most unlikely that the warming in 3 *M* acetic acid and the subsequent treatment with acetone etc. could have introduced any artifacts. Thus Theorell's [6] formula with the cysteine residues attached to the secondary carbon atoms of the side chains 2 and 4 has been conclusively confirmed. It is interesting to note that optical activity, exerted by the asymmetric carbon atoms of amino acids in native proteins, is found also in the case of the asymmetric carbon atoms of the porphyrin. As far as we are aware this is the first case when optical activity has been demonstrated in an isolated, chemically identified porphyrin, free from adducts, which are optically active themselves.

The question about the optical activity of porphyrin c of various origins is not yet clear. A consistent hindrance in earlier investigations was that the available method (hydrogen bromide in glacial acetic acid) for the preparation of hematoporphyrin from either porphyrin c or cytochrome c also possibly converts a certain amount of hematoporphyrin to protoporphyrin, which subsequently via the bromine adduct can give the ordinary hematoporphyrin.

Similar troubles were met with in the acid hydrolysis of cytochrome c, where possibilities for the formation of adducts between porphyrin and cysteine were at hand (α-position with hematoporphyrin, β-position with protoporphyrin) [8,14,31]. The consistent value for the optical activity of porphyrin c, obtained by hydrolysis of cytochrome c with dilute sulphuric acid at various concentrations of the pigment [9], indicates that a resynthesis probably did not occur. However, since in this case the optically active asymmetric carbon atoms of the cysteine residues influenced the rotation, no conclusive evidence as regards α- or β-position of the thio-ether bonds were obtainable. The attempt of Zeile and Meyer [9] to prepare porphyrin c by melting together dibromo protoporphyrin with l-cysteine hydrochloride ought to give a substance with the sulphur bonds in α-positions. The optical activity of this preparation was however, quite different from that found for the preparation from cytochrome c (*cf.* page 390). The experiment can, unfortunately, not be exactly reproduced since no information as regards reaction temperature or time is given. For the complete interpretation of published data concerning porphyrin c, the "synthetic" as well as the different kinds of "natural", it would be necessary to study in detail the equilibrium between hemato- and protoporphyrin in acids of various concentrations and a tvariou stemperatures, and also to elucidate the difference in the mode of action between sulphuric acid and hydrochloric acid on cytochrome c. A more profitable investigation would perhaps be to study the result of the silver digestion on the different types of porphyrin c. Experiments of this kind are being done.

The preparation of an isomer of hematoporphyrin with β-hydroxy ethyl side chains would be of interest in connection with another question. It has been stated [32] that hematohemin does not combine with the apoenzyme of horse radish peroxidase. This statement has been disputed [33] as well as confirmed [34]. When hematohemin c is brought to react with the apoenzyme under suitable conditions a recombination to holoenzyme with the same activity as "synthetic" protohemin horse radish peroxidase takes place [35].

SUMMARY

1. The ferri porphyrin, which is liberated from cytochrome c by treatment with silver salt and precipitation with acid acetone of the protein moiety, is an isomer of hematohemin. The name *hematohemin c* is suggested for this isomer.

2. Hematohemin c is readily converted to the dimethyl ether dimethyl ester of hematoporphyrin c by the procedure designed by Grinstein. There is

a significant difference between hematohemin c and hematohemin in this respect, hematohemin reacting more sluggishly.

3. The non-identity of the two tetramethyl compounds is evident from the above-mentioned difference in reactivity, the melting point depression given by them, and their infrared curves.

4. Hematoporphyrin c is optically active. As far as the author is aware this is the first case, when optical activity has been demonstrated for an isolated, chemically identified, simple porphyrin.

5. Hematohemin and hematohemin c are dehydrated at the same rate upon heating. Tetramethyl hematoporphyrin c is converted to protoporphyrin dimethyl ester by heating *in vacuo*. According to the literature tetramethyl hematoporphyrin gives the same product under the same conditions.

6. The hydroxyl groups of hematohemin c do not seem to react with triphenylmethyl chloride.

7. It is concluded that the side chains 2 and 4 of hematohemin c are α-hydroxy ethyl groups.

8. The two cysteine residues in cytochrome c are linked to the secondary carbon atoms (α-positions) of the side chains 2 and 4.

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