Formation of Protoporphyrin from Haemoglobin *in vitro*

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1. The formation of protoporphyrin from red blood cells or purified haemoglobin in aqueous perchloric acid media without the prior isolation of haemin is described. The reaction is carried out in the absence of oxygen and in red light. Even traces of oxygen inhibit the reaction by oxidative destruction of protoporphyrin and by the oxidation of haem to haematin. 2. Perchloric acid releases iron and protoporphyrin from haemoglobin at similar rates, but the amount of protoporphyrin in the filtrate varies with the solubility of protoporphyrin in the concentration of perchloric acid used. The yield of protoporphyrin may reach 50–60%. Less than 5 µg. of haemoglobin/ml. can be detected by measuring the fluorescence of the porphyrin released. 3. A porphyrin other than protoporphyrin is obtained in small amounts. Its possible identity is discussed. 4. If sodium sulphite is present as a reducing agent the exclusion of oxygen is not required, but the porphyrin formed is more polar and more soluble in water than protoporphyrin. The presence of oxygen appears to be necessary for the formation of this polar porphyrin.

Methods for the preparation of protoporphyrin from blood involve the conversion of Hb† into haemin and treatment with a reducing agent and an acid in non-aqueous media (Falk, 1964, pp. 129–174). Water has been shown to inhibit the removal of iron from haem (Morell & Stewart, 1956). However, Clezy & Morell (1963) and Fischer & Zerweck (1924) were able to remove iron from haem in aqueous media without altering the porphyrin by first forming haemochromogens.

In this paper we describe some observations demonstrating the great ease with which protoporphyrin may be obtained directly from RBC or Hb in aqueous media provided that secondary oxidative reactions are prevented. Its yield depends on pH and on its solubility in the aqueous medium chosen. If oxidative reactions are prevented by the presence of sulphurous acid rather than the exclusion of oxygen a porphyrin is obtained from Hb in good yields that is closely related to protoporphyrin though not identical with it.

MATERIALS AND METHODS

Coproporphyrin III methyl ester, uroporphyrin methyl ester, haematoporphyrin and crystalline human Hb were purchased from Calbiochem, Los Angeles, Calif, U.S.A.,

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† Abbreviations: Hb, haemoglobin; CO-Hb, carboxy-haemoglobin; RBC, red blood cells.

protoporphyrin methyl ester was from Mann Research Laboratories, New York, N.Y., U.S.A., and coproporphyrin I standardized vials were from Sigma Chemical Co., St Louis, Mo., U.S.A. Haematoporphyrin was purified by extraction with ether and re-extraction into 0.04N-HCl. Protoporphyrin methyl ester was purified by column chromatography as described below. The other porphyrins were used without further purification. Reagent-grade chemicals and distilled deionized water were used. Organic solvents were purified as described by Falk (1964, pp. 119–121) except for anhydrous methanol, which was used without further purification. For iron measurements glassware was washed with HNO₃ and water redistilled from glass was used.

Spectra were recorded with a Cary model 14 spectrophotometer. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (wavelengths uncorrected). Infrared spectra were measured with a Perkin-Elmer model 421 spectrophotometer by using KBr pressings. Melting points were determined with a Fisher-Johns melting-point apparatus and were corrected.

Esters of porphyrins were prepared by dissolving the free porphyrin in a 5% (v/v) solution of conc. H₂SO₄ in anhydrous methanol (Falk, 1961). The solution was left for 20–24 hr. at room temperature (22–23°) or, for protoporphyrin, at 3°.

Protoporphyrin methyl ester was purified by a modification of the procedure of Lucas & Orten (1951) and Orten & Larsen (1964). Silicar 7G, thin-layer-chromatography grade (Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.), was suspended in chloroform and poured to form a column 13–15 cm. high × 2.5 cm. diam. After most of the chloroform had run through, the column was washed with petroleum (b.p. 35–60°). The porphyrin, dissolved in chloroform, was

then added and the column developed with chloroform-petroleum (b.p. 35–60°) (3:7, v/v). Enough pressure was maintained to keep the flow rate at 40–60 drops/min. Protoporphyrin methyl ester leaves the column first and could be recovered in pure form. The commercial sample of protoporphyrin methyl ester separated into six bands on the column. A band with a violet fluorescence moved almost as fast as the authentic protoporphyrin methyl ester and may have contaminated it to a small extent.

The methyl ester of protoporphyrin obtained from blood in our method separated into three bands, a band at the top of the column representing unesterified porphyrin, a green band that was probably a degradation product of protoporphyrin and the protoporphyrin ester band. Before passage through the column it had m.p. 219–221°; after passage it had m.p. 227–228°.

Free porphyrins were prepared by hydrolysing the esters in the dark in 4N-HCl for 24 hr. at room temperature ($22 \pm 2^\circ$). When protoporphyrin free from the decomposition products formed during hydrolysis of the ester (Falk, Dresel, Benson & Knight, 1956) was needed, it was extracted into ether, washed with water and 0.16N-HCl and then extracted with N-HCl.

The concentration of protoporphyrin in HClO_4 extracts was measured fluorimetrically against coproporphyrin standards. The ratio of coproporphyrin fluorescence to protoporphyrin fluorescence was determined by hydrolysing a known amount of pure protoporphyrin methyl ester and determining its fluorescence. A ratio of 1.25 was found, which is the same as that reported by Schwartz, Berg, Bossenmaier & Dinsmore (1960). When ethanol was present in the HClO_4 extract, the fluorescence of protoporphyrin was increased by 30%. This increase in fluorescence was taken into consideration when the concentration of protoporphyrin was calculated.

Reduced Hb was prepared from crystalline Hb by the method described by Wallach, Gail & Janeway (1965). Two columns containing mixed-bed resin AG 501 (X8) (D) (Calbiochem), 5 and 12 cm. high $\times$ 2.5 cm. diam., were prepared. A solution of 0.5 g. of $\text{Na}_2\text{S}_2\text{O}_4$ was added to the first column and one of 1.5 g. to the second. The columns were placed in tandem, the first column feeding in deoxygenated water to the second. A 1–2 g. sample of crystalline Hb was dissolved in 10–15 ml. of 0.02M-phosphate buffer, pH 7.6, and centrifuged. The Hb solution was then added to the second column and allowed to pass through the column at a flow rate of 20–30 drops/min. Before passing through the column the Hb solution was brown in colour and would not combine with O_2 or CO. After passage through the column the Hb solution was dull red; it turned light red on mixing with O_2 and cherry-red on mixing with CO.

The concentration of Hb used in the various experiments was determined by measuring the extinction of CO-Hb at 540 m μ . The CO-Hb was standardized by comparison with a sample of blood treated with CO and previously standardized against a cyanomethaemoglobin standard (Acuglobin; Ortho Diagnostics, Raritan, N.J., U.S.A.).

Iron was determined by a 1,10-*o*-phenanthroline method (Ballentine & Burford, 1957). A sample (1–8 ml.) of the HClO_4 extract was diluted to 10 ml. with water. Then 4 ml. of 3M-sodium acetate was added, which raised the pH to 4.5 ± 1.0 . Next 1 ml. of a 10% (w/v) solution of hydroxylamine hydrochloride was added and the extinction of the

solution was determined at 500 m μ in cuvettes with a 5 cm. path-length (sample *a*). Then 1 ml. of a 0.25% (w/v) solution of 1,10-*o*-phenanthroline was added and after a few minutes the extinction was again read at 500 m μ (sample *b*). The extinction of sample *a* and the extinction of a reagent blank including *o*-phenanthroline were subtracted from the extinction of sample *b*. The concentration of iron in the solution was obtained from the difference by reference to a standard extinction curve.

CO-Hb method for the preparation of protoporphyrin from blood or haemoglobin

Freshly drawn heparinized venous blood (10–20 ml.) was centrifuged for 5 min. at about 400g at 3°. The plasma and white-cell layer were removed by suction and the cells were washed three times with an equal volume of 0.85% NaCl. The desired volume of packed cells, usually between 0.5 and 10 ml., was added to 0.85% NaCl in a 500 ml. round-bottomed flask to give a total volume of 20–30 ml. The saline in the flask had been deoxygenated and the flask flooded with argon before the addition of the RBC. The volume of packed cells and the volume of saline depended on the final concentration of Hb desired.

The RBC were then deoxygenated on a vacuum pump at $30 \pm 3^\circ$. An arbitrary deoxygenation schedule was used that gave consistent results. It consisted of 2 min. of evacuation, 1 min. of equilibration with argon, 5 min. of evacuation, 2 min. of equilibration with argon, 5 min. of evacuation, 5 min. of equilibration with CO, 30 sec. of evacuation and 3 min. of equilibration with CO. During gassing and evacuation the RBC suspension was spread out to form a thin film by constant rotation in a rotary evaporator.

When crystalline human Hb was used, it was reduced as described and drained off the column into a 500 ml. round-bottomed flask that had been evacuated and flooded with argon. Then CO was bubbled through the solution with a Pasteur pipette until Hb was saturated.

Samples (10–20 ml.) of HClO_4 solutions, previously deoxygenated and saturated with argon, were placed into 25 ml. Corning low-actinic glass-stoppered tubes. Argon was bubbled through the HClO_4 solution as it was added to the tubes and for 10–15 sec. afterwards. The tube was then capped. With a Pasteur pipette in place and CO bubbling through the HClO_4 solution, 0.3–1.0 ml. of the suspension of cells or Hb solution was added to the tube. Bubbles of trapped CO formed and the tubes were capped when the bubbles had reached the lower end of the ground joint. When small amounts of RBC or Hb were used, bubbles of entrapped CO would not fill the tubes. To exclude as much oxygen as possible the tube was covered with Parafilm and the CO pipette slowly removed. The tube was then carefully capped with the glass stopper. When care was not taken to exclude oxygen in the above manner, lower yields of porphyrin were obtained.

After the RBC or Hb had been added, the tubes were inverted once or twice and left at room temperature for 1 hr. Yields of porphyrin did not vary when the incubation period was lengthened for up to 24 hr.

The tubes were centrifuged at 3° at about 400g for 5 min. with their stoppers on.

To remove traces of precipitated matter the HClO_4 solutions were transferred under red light to plastic centri-

fuge tubes and spun at 3° at about 17000g for 5 min. The supernatant solutions were poured back into clean red tubes and capped.

The concentration of porphyrin in the HClO₄ solution was determined by measuring the fluorescence of a 1:10 or 1:100 dilution in 0.3N-HClO₄ at an excitation wavelength of 400mμ and an emission wavelength of 600mμ. Samples were compared with a coproporphyrin standard (0.01 μg./ml.).

Sulphite method for preparing porphyrins from RBC or Hb

Blood or Hb was added to a 25ml. glass-stoppered low-actinic glass test tube containing 1.2ml. of 60% (w/v) HClO₄ and 17ml. of 5% (w/v) Na₂SO₃. The tube was inverted once and left at room temperature for 3 hr. It was then spun at 400g at 3°. The solution was decanted under red light into plastic tubes and spun in an angle-head centrifuge at 17000g at 3° for 5 min. After being mixed with 2ml. of conc. HCl (final concn. 1.2N), in red light, the supernatant was placed in clear glass tubes and irradiated with ultraviolet light (General Electric F20T12 BL black light tube, peak emission at 350mμ) for 30 min. Fluorescence was then determined at 400 and 600mμ for excitation and emission respectively.

Purification of porphyrin obtained in the sulphite method. A 100ml. sample of RBC extract in 1.2N-HCl was saturated with NaCl and extracted first with 10ml. and then twice with 5ml. of phenol, purified by the method of Barker, Smyth & Hogenkamp (1963). The combined extracts were twice washed with 2ml. of water, then mixed with 4vol. of peroxide-free ether and 0.15 vol. of water. Extraction with 0.15 vol. of water was twice repeated. The combined aqueous layers were twice extracted with 0.2 vol. of ether to remove residual phenol and gassed with nitrogen to drive out ether.

The aqueous layer from the phenol extraction was mixed with solvents in the proportions used for paper chromatography, e.g. 5ml. of 2,6-lutidine was added to 3ml. of aqueous extract, and the mixture placed on a lin.-diam. column of microcrystalline cellulose (Unibar; Analtech Inc., Wilmington, Del., U.S.A.). The following solvent systems were used: 2,6-lutidine-water (5:3 and 1:1, v/v), and butan-1-ol-pyridine-water (1:1:1, by vol.). The columns were developed with approx. 80ml. of solvent mixture. The *R_F* values on the column were the same as those obtained on microcrystalline cellulose thin-layer plates. The position of the porphyrin was determined by observation under ultraviolet light.

After development, the band of porphyrin was cut out and eluted with 0.1N-HCl while the column was still moist.

RESULTS

CO-Hb method

Porphyrins produced in the reaction. The porphyrins produced in the reaction can be distinguished by their solubility characteristics. The largest fraction, accounting for 99% of the total porphyrin at a perchloric acid concentration of 4N and for 80–90% at lower concentrations of perchloric

acid, is extractable into ether at pH 1.0–1.5. A second fraction is extractable into ethyl acetate in the pH range 1.0–3.0. A third fraction is not extractable by ether or ethyl acetate. It is the most variable and is quantitatively related to the amount of oxygen contaminating the reaction mixture. If extreme care is taken to exclude oxygen, this fraction approaches zero. If oxygen is not carefully excluded it may account for all of the porphyrins found. Attempts to esterify this fraction were unsuccessful and the residue when evaporated to dryness was green. It presumably consisted of oxidation products of protoporphyrin.

The ether-soluble fraction appeared to contain a single porphyrin after trace amounts of the ethyl acetate-soluble porphyrin were removed from ether with 0.16N-hydrochloric acid. The ether-soluble porphyrin was identified as protoporphyrin. Its methyl ester had m.p. 227–228°, in agreement with published figures (Schwartz *et al.* 1960; Falk, 1964, p. 175). Its chromatographic properties were identical with those of authentic protoporphyrin (Tables 1 and 2). Its visible absorption spectrum agreed with published data (Table 3). The infrared spectrum of its purified methyl ester, m.p. 227–228°, was compared with that of purified commercial protoporphyrin methyl ester, m.p. 222–223°. Both compounds gave identical tracings except in the region 2800–3000cm.⁻¹, where the peaks from our protoporphyrin were better resolved, probably owing to its higher purity (Fig. 1). The infrared spectrum showed the peaks assigned to protoporphyrin by Caughey, Alben, Fujimoto & York (1966), including the absorption maxima at 1625, 1295, 985 and 905cm.⁻¹ attributed to the vinyl groups.

The ethyl acetate-soluble fraction also appeared to consist of a single porphyrin, which we refer to below as porphyrin B. Its concentration varied from 0.4 to 3 μg./g. of Hb, depending on the concentration of perchloric acid used (Table 4). It was obtained from Hb as well as from blood.

The chromatographic properties of porphyrin B are shown in Tables 1 and 2 and its visible spectrum in Table 3. Its behaviour in a lutidine-water system suggests that it is a dicarboxylic porphyrin (Eriksen, 1953). The behaviour of its methyl ester suggests that it contains a polar group, probably a single hydroxyl group. It is ninhydrin-negative, showing that it is not attached to an amino acid or similar compound. Our sample of haematoporphyrin contained a small amount of an impurity that had similar chromatographic properties. Porphyrin B is probably 4(2)-monohydroxyethyl-2(4)-monovinyldeuteroporphyrin. A second possibility would be a tricarboxylic porphyrin containing a hydroxyl group, such as carboxymonohydroxyethylmonovinyldeuteroporphyrin. In Table 3 the

Table 1. *Chromatography of porphyrin methyl esters*

Solvent systems (1)–(3) were used with Eastman Kodak thin-layer Chromagram silica-gel sheets (ascending): (1) chloroform–decane (9:1, v/v); (2) kerosene–pentane–2,4-dione–methyl benzoate (12:7:1, by vol.); (3) water–acetonitrile–1,4-dioxan (2:7:1, by vol.) (Chu & Chu, 1966). Systems (4) and (5) were used with Whatman no. 1 paper (ascending). A double development was carried out in the same direction; (4) first development kerosene–chloroform (20:13, v/v); (5) second development kerosene–propan-1-ol (5:1, v/v) (Chu, Green & Chu, 1951). The R_F values for the impurities in haematoporphyrin are in parentheses.

System.....	$100 \times R_F$				
	(1)	(2)	(3)	(4)	(5)
Protoporphyrin (commercial)	50	43		68	94
Protoporphyrin (RBC)	49	43	46	70	94
Coproporphyrin	15			48	88
Uroporphyrin	1			0	16
Haematoporphyrin	0	5	(55), 62	0	39, (69)
Porphyrin B*	4	18	55	14	69

* See the text.

Table 2. *Chromatography of free porphyrins*

For systems (1) and (2), 2,6-lutidine–water (5:3, v/v), with NH_3 vapour, was used with Whatman no. 1 paper (ascending): (1) 5 hr. development; (2) 13 hr. development (Eriksen, 1953). For system (3) 0.1M-LiCl, with NH_3 vapour, was used (With, 1957).

System.....	$100 \times R_F$		
	(1)	(2)	(3)
Protoporphyrin (commercial)	68		0
Protoporphyrin (RBC)	70	87	0
Uroporphyrin		2	76
Porphyrin B		85	0

visible spectrum of porphyrin B is compared with those of the two porphyrins mentioned.

Properties of the reaction. Fig. 2 shows the liberation of porphyrin and iron when oxygen-free RBC or Hb are added to various concentrations of perchloric acid. When the concentration of perchloric acid is varied between 0.3 and 4.0N, porphyrin and iron are released at similar rates. The amount of iron detected at a given concentration of perchloric acid is five- to ten-fold greater on a molar basis than the amount of porphyrin detected.

The lower curve of Fig. 3 shows the results of experiments in which, at each of a series of perchloric acid concentrations, at least four different concentrations of RBC, containing from 9 to 65 mg. of Hb, were added. In spite of this variation essentially identical concentrations of porphyrin were present in solution at a given perchloric acid concentration, and similar concentrations were obtained when porphyrin was formed from crystalline Hb. Obviously the yield of porphyrin is limited by its solubility in the aqueous perchloric

acid solution and it is also apparent that the solubility increases with increasing concentrations of perchloric acid. The upper curve shows the increase in the amount of porphyrin in the solution when 1 ml. of ethanol is added to 11 ml. of perchloric acid solution before the addition of the RBC. Ethanol presumably increases the solubility of protoporphyrin in aqueous solutions, and this effect is clearly demonstrated when RBC are added to a solution containing a constant concentration of perchloric acid and increasing concentrations of ethanol: at a concentration of 1.0N-perchloric acid an increase of ethanol concentration from 0 to 33% (v/v) produces a 14-fold increase in the amount of porphyrin obtained. The curve is sigmoid, with the steepest increase of porphyrin yield between ethanol concentrations increasing from 8 to 25% (v/v). The excess of iron over porphyrin also decreases from five- to ten-fold to two- to three-fold in the presence of 8.3% (v/v) ethanol (Fig. 2). The discrepancy in the yields of iron and protoporphyrin in the absence of ethanol is therefore probably due to the insolubility of protoporphyrin in the aqueous medium. The protoporphyrin curve (lower curve in Fig. 2) approaches a plateau at about 1.0N-perchloric acid and increases linearly (on a logarithmic scale) at concentrations above about 1.5N-perchloric acid, possibly owing to a solvent or dehydrating effect of perchloric acid (Coll, Nauman & West, 1959). The initial rise of the protoporphyrin curve may perhaps be attributed to a pH effect on solubility.

Theoretically, 100% of the protoporphyrin from Hb should be obtainable at any concentration of perchloric acid between 0.3 and 4.0N if a small enough quantity of Hb is added. However, it is practically impossible to eliminate oxygen completely and the small amounts remaining made it

Table 3. Visible spectrum of protoporphyrin obtained by the present method (a) with literature values for protoporphyrin (b), and the visible spectrum of porphyrin B (c) compared with those of 4(2)-hydroxyethyl-2(4)-vinyldeuteroporphyrin (d) and carboxymonohydroxyethylmonovinyldeuteroporphyrin (e)

The ratios of band intensities, in parentheses, are calculated relative to band IV ($\lambda_{\max}$, 501 m μ in ether).

	$\lambda_{\max}$. (m μ)	Reference
2N-HCl	(a) 408 (b) 408	Lemberg & Legge (1949)
25% (w/v) HCl	(a) 411, 556.5, 600.5 (b) 411, 557.2, 602.5	Lemberg & Legge (1949)
Dioxan	(a) 406.5, 505, 539, 576.5, 604.5, 631.5 (b) 406, 504, 538, 575, 603, 631	Falk (1964, p. 232)
0.5N-HCl	(c) 405, 553, 595 (e) 405, 553, 595	Sano (1966)*
Ether	(c) 400.5 (11.6), 501 (1.0), 534.5 (0.75), 575 (0.46), 629 (0.36) (d) 401, 502, 533, 574, 628	Porra & Falk (1964)
Chloroform	(c) 405.5 (11.4), 504.5 (1.0), 539 (0.75), 573 (0.53), 627 (0.37) (d) 401 (12), 503 (1.0), 537 (0.66), 572 (0.45), 625 (0.25) (e) 406, 503, 538, 573, 626	Caughey <i>et al.</i> (1966) Sano (1966)

* Concentration of HCl not stated.

Fig. 1. Infrared spectra of samples of protoporphyrin methyl ester. (a) Sample prepared from RBC; (b) sample obtained commercially and purified.

difficult to obtain high yields from very low concentrations of Hb. Our best yields have been obtained with 4N-perchloric acid-8% (v/v) ethanol, and have been 50-60% of the theoretical amount as shown by the points in Fig. 2 at the perchloric acid concentration of 4N. Hb in concentrations of less than 5 μ g./ml. is easily detected in yields of about 30% by measuring the fluorescence of the released porphyrin.

The function of carbon monoxide is to exclude

Table 4. Concentration of porphyrin B as a function of the concentration of perchloric acid

RBC (0.5 ml.) containing 86mg. of Hb were added to 11.5 ml. of the HClO₄ solution. Total porphyrin and porphyrin B are expressed in terms of protoporphyrin.

Concn. of HClO ₄ (N)	Total porphyrin (μ g./g. of Hb)	Porphyrin B (μ g./g. of Hb)
0.4	19	0.4
0.8	72	1.3
1.5	145	2.5
3.0	740	3.0

oxygen, as shown by the fact that it can be replaced by argon. The amount of porphyrin released by perchloric acid is only a little lower when argon alone is used than when argon and carbon monoxide are combined (Table 5). The importance of iron being in the ferrous state is shown when methaemoglobin is used instead of reduced Hb (Table 5). If the RBC are not deoxygenated before being mixed with the perchloric acid solution, the porphyrin produced falls to near zero (Table 6). In the presence of oxygen the iron released is about 1000-fold greater than the porphyrin released on a molar basis. However, less iron is released in the presence of oxygen than in its absence, indicating that oxygen inhibits the formation of porphyrin in two ways, by oxidative destruction of the porphyrin and by the conversion of the ferrous iron of haem into the ferric iron of haemin (Lemberg, 1942). The release of ferric iron from

Fig. 2. Effect of HClO_4 concentration on the release of iron and porphyrin in the presence and absence of ethanol. A suspension of RBC in saline (0.5 ml.) containing approx. 12 mg. of Hb was added: (a) to 11.5 ml. of HClO_4 solution; (b) to a mixture of 10.5 ml. of HClO_4 solution and 1 ml. of ethanol. The Figure shows the release of porphyrin ($\mu\text{moles/g. of Hb}$) in the absence ($\bullet$) and in the presence ($\circ$) of ethanol, and also the release of iron ($\mu\text{moles/g. of Hb}$) in the absence ($\blacktriangle$) and in the presence ($\triangle$) of ethanol.

Fig. 3. Release of porphyrin from RBC and from crystalline Hb: $\bullet$, 0.5 ml. samples of RBC in saline containing 9–65 mg. of Hb were added to 11.5 ml. of HClO_4 solution; $\circ$, 12–18 mg. samples of reduced crystalline Hb were added to 12 ml. of HClO_4 solution; $\blacksquare$, RBC containing 12 mg. of Hb were added to 12 ml. of HClO_4 solution containing ethanol (8.3%, v/v).

haemin requires concentrated sulphuric acid (Falk, 1964, p. 33).

If concentrations of perchloric acid less than 0.3N are used difficulties arise from incomplete precipitation of protein. If concentrations above 4N-perchloric acid are used, a polar porphyrin is produced that has a visible spectrum markedly different from that of protoporphyrin. Inside this range of acid concentrations, the release of porphyrin from Hb appears to be a function of pH and acid concentration. Sulphuric acid, nitric acid

Table 5. Effect of using different types of oxygen-free RBC on the release of porphyrin

RBC containing 60 mg. of Hb were added to 12 ml. of HClO_4 solution.

Source and type of Hb	Porphyrin ($\mu\text{g./ml. of HClO}_4$ soln.)	
	0.8N- HClO_4	4.0N- HClO_4
RBC deoxygenated with argon and CO	0.6	17
RBC deoxygenated with argon alone	0.4	10
Crystalline Hb (carboxy-haemoglobin)	0.7	9
Crystalline Hb (methaemoglobin)	0	0.045

and hydrochloric acid, at 1.0N, can also release porphyrin when added to 0.3N-perchloric acid used as the precipitant. Trichloroacetic acid and metaphosphoric acid were without releasing effect. The lack of efficacy of metaphosphoric acid may be connected with the decreased acidity of the solution. Trichloroacetic acid is alone in that it has a definite inhibitory effect on the reaction independent of pH.

If the reaction is carried out in the presence of light there is an increase in the ether-insoluble fraction, probably owing to light-catalysed oxidation of protoporphyrin.

Sulphite method

When a suspension of RBC is added to a mixture of 5% (w/v) sodium sulphite in 0.6N-perchloric acid the exclusion of oxygen is no longer necessary. In fact, the situation is reversed and oxygen, instead of inhibiting the formation of free porphyrin, now promotes it. The porphyrin obtained, however, is not protoporphyrin or porphyrin B but a more polar porphyrin insoluble in many organic solvents. The characteristics of this product are briefly described below.

Solubility. The porphyrin produced in the sulphite method is not extracted from acid solution at pH 1.5–3.0 by ether, ethyl acetate, chloroform or butanol. It is slightly soluble in cyclohexanone and easily extractable with phenol. The porphyrin can be precipitated from solution by lead acetate.

Esterification. The unknown porphyrin, purified as described in the Materials and Methods section, was treated with methanol–conc. sulphuric acid (20:1, v/v) at room temperature. After 16 hr. none of the red fluorescence could be extracted by chloroform. If esterification did occur, the resulting product still had a high degree of polarity.

Table 6. *Effect of oxygen on the release of iron and porphyrin*

The oxyhaemoglobin was prepared from RBC washed with saline. It was not fully oxygenated. RBC containing 60 mg. of Hb were added to 12 ml. of HClO_4 solution.

Concn. of HClO_4 (N)	RBC (carboxyhaemoglobin)			RBC (oxyhaemoglobin)		
	Porphyrin ($\mu\text{g./ml. of HClO}_4$ soln.)	Porphyrin ($\mu\text{g./g. of Hb}$)	Iron ($\mu\text{g./g. of Hb}$)	Porphyrin ($\mu\text{g./ml. of HClO}_4$ soln.)	Porphyrin ($\mu\text{g./g. of Hb}$)	Iron ($\mu\text{g./g. of Hb}$)
1.0	0.7	245	100	0.003	0.9	80
2.0	1.5	550	850	0.01	3.0	200
3.0	5.5	2000	3000	0.08	28	580

Absorption spectrum. In 2N-hydrochloric acid the following absorption peaks were observed in the visible part of the spectrum: 595, 553.5, 525 and 405.5 $\text{m}\mu$. In 0.1N-potassium hydroxide peaks were observed at 612, 562, 537, 500 and 408 $\text{m}\mu$.

Fluorescence spectrum. In 1.2N-hydrochloric acid the maximum of activation was at 400 $\text{m}\mu$ and the maximum of emission at 600 $\text{m}\mu$. The fluorescence maxima of the polar porphyrin thus appear to be identical with those of protoporphyrin.

Paper chromatography. (1) In 2,6-lutidine-water (5:3, v/v) with ammonia vapour, on Whatman no. 1 paper (ascending) (Eriksen, 1953) it had R_F 0.055–0.072. (2) In 2,6-lutidine-water (1:1, v/v) (ascending) (Corwin & Orten, 1954) it had R_F 0.15–0.21. (3) In 0.1M-lithium chloride with ammonia vapour (descending) (With, 1957) it showed two spots, R_F 0.37–0.47 and 0.74–0.84.

The polar porphyrin is obtained from Hb as well as from RBC.

Properties of the reaction. Increasing the oxygen saturation of Hb increased the yield of porphyrin in the sulphite method. The moles of porphyrin produced were close to the moles of iron released (Table 7).

Other reducing agents can be substituted for sodium sulphite. Sodium hydrogen sulphite is as effective as sodium sulphite. Glutathione (1%, w/v) is only slightly less effective. Penicillamine (1%, w/v) and ascorbic acid (1%, w/v) were unable to protect porphyrin from oxidation, and a combination of ascorbic acid and sodium sulphite was much less effective than sodium sulphite alone.

The sulphite method appears to be as sensitive as the CO-Hb method in detecting small concentrations of Hb. A concentration of 5 $\mu\text{g.}$ of Hb/ml. is easily detected in yields of about 30%.

DISCUSSION

The nearly quantitative release of iron and protoporphyrin from haem or Hb in aqueous media has not been described before. The presence of a

Table 7. *Effect of oxygen on the porphyrin yield in the sulphite method*

The concentrations of the porphyrin are approximations since its molecular weight is not known.

Source of Hb	Porphyrin (μmole)	Iron (μmole)
RBC deoxygenated with CO	0.012	0.04
RBC	0.18	0.26
RBC saturated with oxygen	0.35	0.5

reducing agent to maintain the ferrous iron of haem in the reduced state is not required when oxygen is excluded. The fact that water has been found to be inhibitory (Morell & Stewart, 1956) may be connected with the presence of oxygen. The inhibition by oxygen occurs in the two ways described by Lemberg (1942): (1) oxidative destruction of protoporphyrin and (2) the conversion of the ferrous iron of haem into the ferric iron of haemin.

The ability to detect low concentrations of Hb by the methods described may be of value in measuring small amounts of Hb or other haemoproteins in tissues. The sulphite method might be more suitable for the purpose since saturation with oxygen is easier than exclusion of oxygen, and since the more polar porphyrin formed in this reaction is more soluble in aqueous media.

The second porphyrin (porphyrin B), as reflected by its lower solubility in organic solvents and its chromatographic behaviour, is more polar than protoporphyrin. Spectral and chromatographic data suggest that porphyrin B may be similar to or identical with 4(2)-monohydroxyethyl-2(4)-monovinyldeuteroporphyrin or carboxymonohydroxyethylmonovinyldeuteroporphyrin.

If porphyrin B is identical with monohydroxyethylmonovinyldeuteroporphyrin and is without optical activity (Paul, 1951; Granick, 1953), it may be assumed to be an artifact derived from the addition of water to one of the vinyl groups of protoporphyrin. The addition of water to the vinyl

groups of protoporphyrin under the influence of strong mineral acids is well known (Falk, 1964, p. 175). However, it is difficult to understand why the reaction should stop after the hydration of only one of the two vinyl groups and why the formation of porphyrin B was not more dependent on the concentration of perchloric acid (Table 4).

If porphyrin B is not a derivative of protoporphyrin the question arises whether it is a precursor of protoporphyrin. 4(2)-Monohydroxyethyl-2(4)-monovinyldeuteroporphyrin has been proposed as a possible intermediate in the reaction leading from coproporphyrinogen to protoporphyrin (Bogorad, 1960). Carboxymonohydroxyethylmonovinyldeuteroporphyrin would be the major product if the porphyrinogen intermediate proposed by Sano (1966), 2,4-bis-(carboxyhydroxyethyl)-deuteroporphyrinogen IX, was present in RBC.

Oxygen as well as sodium sulphite appeared to be necessary for the formation of the polar porphyrin. Acidified oxyhaemoglobin is a strong oxidizing agent (Lemberg, 1942) whereas sodium sulphite is a reducing agent. It is probable therefore that oxidoreductive reactions take place during the formation of the polar porphyrin. Keilin (1929) showed that alternate oxidation and reduction of ferriprotoporphyrin with ferricyanide and dithionite led to the formation of a 'haematin-c', which was later shown to contain three or four sulphonic acid groups (Zeile, 1932). An analogous reaction may be proposed for the formation of the polar porphyrin in the sulphite method, in which oxygen or peroxide acts as the oxidizing agent and sodium sulphite as the reducing agent.

A polar porphyrin, similar to or identical with that produced in the sodium sulphite-perchloric acid solution, was obtained by Zeynek & Kittel (1934) when they treated haematin or Hb with sulphurous acid and light in the absence of atmospheric oxygen. The porphyrin formed from haematin contained 1.5–1.9% S.

Negligible amounts of iron and porphyrin were produced from Hb in the CO-Hb method at pH 1.5, the pH of the sodium sulphite-perchloric acid mixture. This suggests that, in the sulphite method, the haem molecule is subject to changes that facilitate the release of iron. During the oxidation-reduction reaction the porphyrin molecule may be partially or completely reduced to the level of porphyrinogen, which has much less affinity for iron. Such a mechanism is supported by observations suggesting that porphyrinogens may be produced during the reaction. The fluorescence of the solution is less before than after irradiation. If small amounts of Hb (25 mg.) are added to a large excess of sodium sulphite-perchloric acid solution, no fluorescence is noticeable before irradiation and the solution is yellowish. After

irradiation fluorescence is present and the solution is pink. Porphyrinogens do not have the colour or fluorescence of porphyrins (Falk, 1964, p. 10). They are autoxidized to porphyrins by the catalytic action of light (Mauzerall & Granick, 1958).

The ease with which protoporphyrin can be formed from Hb raises the possibility that all or part of the 'free' porphyrin of normal RBC may be derived from Hb. Preliminary experiments have not confirmed earlier claims (Van den Bergh & Hyman, 1928) that no porphyrin is obtained from crystalline Hb by conventional methods in which washed RBC are extracted with a mixture of ethyl acetate and acetic acid. Using the method of Wranne (1960) we found 0.9–1.8 μ g. of porphyrin/g. of Hb when solutions of crystalline Hb were analysed, regardless of whether the Hb preparation was completely reduced or contaminated by methaemoglobin and whether it was of human or bovine origin. These amounts roughly correspond to those usually found in normal RBC.

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