The Prosthetic Group of Cytochrome Oxidase

1. PURIFICATION AS PORPHYRIN *a* AND CONVERSION INTO HAEMIN *a*

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Earlier publications from this Laboratory (Lemberg, Bloomfield, Caiger & Lockwood, 1955; Lemberg & Stewart, 1955) have described the preparation of porphyrin *a* from ox heart and the conversion of this porphyrin into haemin *a*.

Further studies on the structure of haematin *a* (Clezy & Barrett, 1961), its identification as the prosthetic group of cytochromes *a*, *a*₃ and *a*₁ and studies on the spectra of its various derivatives have required further improvement in the purification of porphyrin *a* and its preparation on a larger scale than hitherto. A preparation of porphyrin *a* of higher spectroscopic purity than previously described and a method for its conversion into haemin *a* without alteration are given in the present paper.

EXPERIMENTAL

Methods

Hydrochloric acid solutions. Throughout this paper the concentrations of aqueous HCl solutions are given in % (w/v) to conform with the convention in this field.

Ether. 'Technical' diethyl ether which did not contain added reducing agents such as quinol was used. Except where otherwise stated ethereal solutions were evaporated under vacuum in a water bath at 50°.

Acetone. Commercial acetone was used for extraction of lipids from mince and for acid-acetone extractions of haemins; for other uses including chromatography AnalaR-grade acetone was used.

Acetic acid. That designated 'specially treated' in the text was an AnalaR-grade reagent refluxed for 1 hr. after the addition of a small amount of FeSO₄ and then distilled; otherwise the AnalaR-grade reagent was used.

Cellulose powder. Whatman cellulose powder 'Standard Grade, Ashless Powder Chemically Prepared' (Balston Ltd., England) was used. Tubes 1½ in. × 48 in. or 1 in. × 48 in. were packed as indicated in the text with dry cellulose, the lower end being tapped frequently on a solid surface during packing to consolidate the powder. This procedure was more satisfactory than packing the cellulose as slurry in the solvent.

Light-absorption measurements. These were made with a Hilger 'Uvispek' spectrophotometer with the glass prism. The wavelength setting was adjusted by using the hydrogen lines at 486.1 and 656.3 mμ.

Paper chromatography of porphyrin *a*. Two solvent systems were used: 1. Propanol-kerosene (commercial grade, Shell Co. Ltd.) (1:5, v/v) on Whatman no. 1 paper,

the solvent front being allowed to ascend 10 cm. in a kerosene atmosphere. 2. Acetone-light petroleum (b.p. 60–80°) (1:3.5, v/v) on Whatman no. 1 paper strips 1.0–1.5 cm. wide suspended from the stopper in test tubes containing the solvent mixture. The solvent front was allowed to ascend 4–6 cm. Since porphyrin *a* dried on paper has a poor fluorescence the chromatograms were viewed under ultraviolet-blue light after being sprayed with kerosene.

Purification of porphyrin *a*

Preparation of defatted mince. About 18 kg. of lean ox-heart mince was washed for 30 min. in running tap water, soaked in 3% (w/v) NaCl for a further 30 min. and again washed with running tap water for 30 min. The strained mince was squeezed, with double layers of muslin, to remove as much water as possible and successively extracted for 1 hr. each time with 20 l. of acetone-water (4:1, v/v) (sp.gr. of mixture, 0.875), and 12.5 l. of acetone-ether (1:1), and kept overnight in 12.5 l. of acetone-water (4:1).

Extraction of haemins. After removal of excess of solvent, the mince was extracted once for 15 min. and twice for 30 min. with separate mixtures each containing 20 l. of acetone, 4 l. of water and 1 l. of conc. HCl. The extracted haemins were transferred to ether without delay by using 4% HCl to wash out the acetone. The ether from the combined solutions was evaporated under reduced pressure in six or seven 2 l. flasks immersed in hot water. A small amount of residual water could usually be poured off, leaving a fatty residue containing the haemins.

The haemins should not be kept in acetone-HCl any longer than necessary since the formyl group of formyl-haemins slowly condenses with acetone under these conditions (Clezy, 1959).

Precipitation of protohaemin and cryptohaemin *a*. The fatty haemin residue was shaken by hand for a few minutes at room temperature with 2 l. of ether and filtered through a no. 541 Eaton-Dikeman fluted paper (Eaton-Dikeman Co., Mount Holly Springs, Pa., U.S.A.) to remove undissolved protohaemin and most of the cryptohaemin *a*. The residue on the paper was washed with 1 l. of ether, and the combined filtrates were evaporated to 300 ml. under reduced pressure. At this stage the band of haemin *a* at 650–660 mμ was considerably stronger than the protohaemin band at 630–640 mμ when viewed in the hand spectroscope.

Acetone precipitation of lipids. The solution of haemins was dried for 15 min. over anhydrous Na₂SO₄ and filtered into 1200 ml. of acetone. The residue was washed with 300 ml. of acetone, and the filtrates were combined and made up to 1.8 l. with acetone. Two drops of a saturated aqueous solution of MgSO₄ were added, and the mixture was shaken and kept overnight at –15°. The precipitated

lipids were filtered off at -15° , the residue being washed with 200 ml. of acetone at -15° .

The haemins from four preparations treated as above and derived from a total of 65.5 kg. of mince were combined and dissolved in ether. The volume was brought to 2 l. by evaporation of the ether *in vacuo*, and the solution was dried over anhydrous Na_2SO_4 . The ethereal solution was evaporated to dryness *in vacuo* and dissolved in 1.1 l. of acetone. Two drops of saturated MgSO_4 solution were added, and the precipitated lipid was filtered off after being kept overnight at -15° . The residue was washed with 750 ml. of acetone at -15° . The haemins were transferred to 2 l. of ether and dried over anhydrous Na_2SO_4 .

Conversion of haemins into porphyrins. The approximate haemin (protohaemin + haemin *a*) content of the ethereal solution was calculated from the extinction at 550 $\text{m}\mu$ assuming that a solution in ether of 1 mg./ml. would have an extinction of 10 (specific extinction) at this wavelength. By this method 1280 mg. was found to be present in the ethereal solution.

This solution of haemins was then evaporated to dryness and dissolved in 12 l. of specially treated acetic acid (see above). Portions (500 ml.) were successively treated as follows. After CO_2 had been bubbled through each 500 ml. portion for 10 min., 900 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (14 moles/mole of haemin) dissolved in 10 ml. of conc. HCl were added at room temperature. The stream of CO_2 was continued for 5 min. when the solution, now greenish by reflected light, was poured into a 3 l. separating funnel containing about 1 l. each of ether and water plus sufficient sodium acetate to neutralize the solution to Congo red, and then shaken.

Removal of protoporphyrin and cryptoporphyrin a. The ethereal solution of porphyrins was washed twice with approximately an equal volume of water to remove much of the acetic acid and twice with small volumes of 7% HCl to remove the protoporphyrin and cryptoporphyrin *a* not previously precipitated as their haemins. Acetic acid then remaining in the ethereal solution was largely removed by washing twice with an approximately equal volume of 1% HCl and once with water.

Chromatography on cellulose-powder columns. The ethereal solution now contained lipid impurities, porphyrin *a*, traces of protoporphyrin and some protohaemin. The maxima of bands III and IV of the porphyrin *a* were at 558 and 517 $\text{m}\mu$ respectively. This solution was shaken gently with dil. aq. NaHCO_3 solution until the pH of the aqueous phase was 6.0–6.5. Any persistent emulsions were broken by gentle shaking with conc. $(\text{NH}_4)_2\text{SO}_4$ solution. The ethereal solution was kept at -15° overnight then filtered at -15° to remove ice crystals, which were washed with a little cold ether. A third of the preparation was chromatographed at a time on six columns (36 in. $\times$ 1.5 in.) of cellulose powder, each column bearing 16–20 mg. of porphyrin. The porphyrin *a* was applied to the columns in solution in ether–light petroleum (b.p. 60–80°) (1:1, v/v). This was followed by an equal volume of the same solvent mixture, which moved a yellowish lipid fraction, later rejected, well clear of the porphyrin *a* adsorbed on the top third of the column. The column was then developed with ether or 10% (v/v) acetone in ether, which eluted a broad band of porphyrin. The eluate was collected as six or seven separate fractions. The fractions having a ratio of bands III/IV of 1.90 or higher were combined and rechromatographed. From a

second or third chromatography, fractions with a ratio of bands III/IV as high as 2.45 were obtained. The pooled porphyrin *a* finally collected had a ratio of band III (558 $\text{m}\mu$)/band IV (518 $\text{m}\mu$) of 2.35. The yield at this stage, calculated by assuming a specific extinction of 27 at 558 $\text{m}\mu$ for pure porphyrin *a* (see below), was 297 mg. (from 65.5 kg. of mince). The specific extinction at 558 $\text{m}\mu$ of the prepared porphyrin *a* was 8.7.

Fractionation with hydrochloric acid. Ethereal solutions containing about 20 mg. of porphyrin *a* were evaporated to dryness under reduced pressure in a 2 l. flask so that the porphyrin was spread thinly on the walls of the flask. Each flask was then kept under vacuum at 60–80° for 30 min. after which the porphyrin *a* was dissolved in ether (a little acetone was sometimes necessary) and chilled. When all the porphyrin had been so treated it was extracted in the cold room (2°) into cold 17% HCl. Extraction at 2° minimizes the conversion of porphyrin *ax* into porphyrin *a\beta* (Lemberg & Stewart, 1955). The extract was washed with 2 vol. of ether and diluted with cold water so that it was 8% with respect to HCl. On thorough shaking with ether, porphyrin *ax* of increased purity went into the ethereal layer. The final yield was 210 mg. of porphyrin *a*.

Spectral characteristics. In solution in ether the porphyrin *a* had absorption maxima at 347 $\text{m}\mu$ (band I), 582 $\text{m}\mu$ (band II), 558 $\text{m}\mu$ (band III) and 518 $\text{m}\mu$ (band IV). The specific extinction at 558 $\text{m}\mu$ was 23. The following ratios of absorption maxima are useful in characterizing the porphyrin: band III/band I, 1.6–2; band III/band II, 1.92; band III/band IV, 2.36.

The millimolar extinction coefficient at 558 $\text{m}\mu$ for porphyrin *a* in ether was determined on two preparations similar to that described above by the copper titration method of Oliver & Rawlinson (1951) and found to be 21. This value is slightly higher than that given by Oliver & Rawlinson (1951), owing probably to the increased spectral purity of the present porphyrin *a*. From the molecular weight of 870 found for crystalline haemin *a* by Warburg, Gewitz & Völker (1955) the specific extinction of pure porphyrin *a* in ether at 558 $\text{m}\mu$ was calculated to be 27. Our product is therefore about 85% pure.

Paper chromatography of porphyrin a. This was done as described above. This technique was used to see whether porphyrin *a* existed in more than one chromatographic form. Several preparations, at different stages in the purification, exhibited two spots of about equal intensity. Both solvent systems gave these two spots. In the acetone–light petroleum system the spots had R_F values of about 0.60 and 0.45 and in propanol–kerosene of 0.28 and 0.18. The most highly purified preparations of porphyrin gave mainly the spot of higher R_F with a barely discernible second spot of the lower R_F . In one preparation the spot of higher R_F greatly predominated throughout the purification. It is difficult to judge whether the two spots observed are

due to different porphyrins or to the effect of contaminating lipids.

Interconversion of formyl porphyrins and haemins

Conversion of haemin a into porphyrin a. The method given above follows the principles discussed by Morell & Stewart (1956) for haemins generally. With ordinary AnalaR-grade acetic acid, however, removal of iron from haemin *a* produced variable amounts of porphyrin having its band III at 551–553 m μ and which could be separated from porphyrin *a* (band III, 558 m μ) on the cellulose column. This porphyrin was an alteration product of porphyrin *a*, the reaction being caused by an unidentified oxidant in the acetic acid which was active in the presence of Fe²⁺ ions and high proton concentration. The alteration was prevented by using the 'specially treated' acetic acid described above.

The altered porphyrin *a*, which we refer to below as 'porphyrin *a* acid', no longer contained a carbonyl group. It was largely separated from porphyrin *a* by cellulose chromatography and freed from traces of the formyl porphyrin as follows: 10 mg. of porphyrin *a* acid and 100 mg. of Girard T reagent, dissolved in 250 ml. of 95% ethanol, were heated together under reflux for 30 min. After the solution was cooled, the porphyrin was returned to ether and washed with water until the washings were free from water-solubilized porphyrin. The porphyrin in the ethereal layer was identical in spectral and paper-chromatographic behaviour with the carboxylic acid obtained from porphyrin *a* by the oxidation of the formyl group [see the next paper (Clezy & Barrett, 1961)].

Other formyl haemins, namely monoformyl- and 2-formyl-4-vinylferrodeuteroporphyrin (methyl esters), were also converted into porphyrins by using untreated acetic acid, FeSO₄·7H₂O (14 moles/mole of haemin) and conc. HCl. The resulting porphyrin esters were dissolved in ether and extracted with aq. 1% Na₂CO₃ solution. In each case the porphyrin so extracted was redissolved in ether by first adding dilute acetic acid and washed with water. These porphyrins were identical (spectra and *R_f* values) with the porphyrincarboxylic acids obtained by the CrO₃–H₂SO₄ oxidation of the corresponding formyl porphyrins (Barrett & Clezy, 1959).

Conversion of porphyrin a into haemin a. Porphyrin *a* purified as above was converted into the haemin by heating in specially treated acetic acid (see above) at 80° for 10 min. in the presence of 20 μ moles of FeSO₄·7H₂O/ μ mole of porphyrin and a few crystals of NaCl. The concentration of porphyrin in acetic acid was 1 mg./10 ml.; sufficient AnalaR Na₂CO₃ was added to this solution so that only the neutral spectrum could be seen. Care was specially required to exclude copper ions from this

reagent mixture. After being heated for 10 min. the mixture was cooled, diluted with ether (freshly washed with dil. aq. FeSO₄ to remove peroxides), and the acetic acid largely removed by three or four washes with dil. HCl. Traces of porphyrin *a* were then removed with several washes with small volumes of 20% HCl, and the haemin *a* solution was finally washed with 3% HCl.

The effect of the removal and replacement of iron on the molecule was tested by using porphyrin *a* having ratio of bands III/IV of 2.31. This porphyrin was converted into its haemin and then reconverted into the porphyrin. The ratio of the final porphyrin *a* produced was 2.24 and the band positions had not changed.

The haemin *a* prepared by reintroduction of iron into purified porphyrin *a* had absorption maxima (in ether washed with 3% HCl) at 662–663, 552 and 508 m μ . The following ratios of absorption intensities are useful in characterizing the haemin: $E_{552\text{ m}\mu}/E_{590\text{ m}\mu} = 1.97$ (to detect traces of copper complex absorbing at 590 m μ); $E_{663\text{ m}\mu}/E_{640\text{ m}\mu} = 1.15$ (to detect traces of protohaemin absorbing at 640 m μ). Since haemin *a*, like other haemins, has an absorption shoulder at 590–620 m μ , traces of the copper complex cannot be detected visually in the hand spectroscope. 'Native' haemin *a* is the term used below for haemin *a* purified without conversion into the porphyrin at any stage. The spectrum of haemin *a* prepared as above was compared with 'native' haemin *a*. The position of absorption bands was the same for the two haemins. $E_{552\text{ m}\mu}/E_{590\text{ m}\mu}$ was 1.93 for 'native' haemin *a* but $E_{663\text{ m}\mu}/E_{640\text{ m}\mu}$ was 1.06, indicating some contamination with protohaemin.

'Native' haemin *a* was prepared with very mild procedures to prevent any alteration which might affect its spectrum. The preparation was the same as for the porphyrin *a* preparation up to the stage of the second defatting. In solution in ether the haemin mixture was then chromatographed on cellulose-powder columns. A yellow lipid fraction preceded the haemins down the column and was discarded; some protohaemin, which is adsorbed more strongly to the cellulose, was retained on the columns. After elution of the haemin *a* with ether or ether plus acetone, the fraction containing the haemin *a* was evaporated under reduced pressure to an oily residue and partitioned between aq. acetone–HCl and acetone–light petroleum as described by Kiese & Kurz (1954). After passage three times through this countercurrent system there seemed to be no further separation of protohaemin from haemin *a* as judged from the relative intensities at 558 and 587 m μ of the haemochrome derivatives.

Quantitative measurements of protohaemochrome and haemochrome *a* with the formula

quoted by Lemberg & Benson (1959) showed a protohaemin contamination of the haemin *a* preparation of from 1 to 5% in different preparations. The specific extinction of the 'native' haemin *a* preparation was very low since the preparation contained much phospholipid and other lipid. These lipid impurities were much less readily removed from the haemin than from the porphyrin, largely because of the better chromatographic behaviour and solubility in HCl of the latter.

Porphyrin a from other tissues

Lemberg, Stewart & Bloomfield (1955) and Lemberg, Morell, Lockwood, Stewart & Bloomfield (1956) have measured the porphyrin derived from various rat and ox organs. Recent determinations on chicken heart and liver gave values of 20 and 21 mg./kg. wet wt. respectively, based on a specific extinction at 558 m μ of 27 for porphyrin *a*. The value for chicken heart is of the same order as that found for ox heart (19–22 mg./kg. wet wt.) or rat heart (24–27 mg./kg. wet wt.), but the value for chicken liver is unusually high compared with those for rat and ox livers, in which the concentration of porphyrin is less than 30% of that in their respective heart tissue.

The method of analysis on chicken tissues differed slightly from those done on other tissues by Lemberg, Stewart & Bloomfield (1955). Instead of minced tissues being used, the chicken tissues were homogenized in a high-speed blender with 0.1M-sodium acetate buffer, pH 5.6, and the residue containing the haematin *a* was recovered by centrifuging at 10 000 g. After resuspension in acetate buffer and recentrifuging the residue was extracted with cold acetone several times and then with acid-acetone. The method was therefore essentially the same as that used by the above authors. Porphyrin *a* of ratio band III/band IV of 2.4 was obtained from both chicken liver and heart.

Unlike animal tissues and bacteria, haematin *a* could not be extracted from yeast cells (*Saccharomyces cerevisiae*, *Torulopsis utilis*) by acetone-HCl even after the cells had been autolysed or lysed with toluene or CHCl₃. However, some success was achieved if the cells were lysed with pyridine and then extracted with acid-acetone. In this method pyridine was added to yeast paste to give a final concentration of 5% (v/v). After 5 hr. at 37° acid-acetone was added so that the acetone concentration was 80% (v/v), the water concentration 20% (v/v) and the pH 2.0. The haemin *a* and protohaemin were converted into the porphyrins, and the porphyrin *a* was purified by cellulose chromatography and by fractionation between HCl and ether. Porphyrin *a* with ratio band III/band IV of 2.24 was obtained, but because of gross contamination

of the crude preparation with lipids the yields were not quantitative.

The concentrations of haemin *a* and protohaemin have been measured as their porphyrins in green peas (picked before the phase of starch accumulation), dried peas and wheat germ. The method involved extraction of the homogenized material with aq. acetone-water (4:1, v/v) to remove chlorophyll pigments, extraction of the haemins with acetone-HCl, conversion into the porphyrins, extraction of protoporphyrin with 4% HCl, and extraction of porphyrin *a* with 20% HCl from ethereal solution. Porphyrin *a* with ratio band III/band IV of 1.6–1.8 was obtained and contained traces of chlorophyll and carotenoid pigments. Green peas yielded about 0.35 mg. of porphyrin *a* and 2.0 mg. of protoporphyrin/kg. dry wt., or 0.022 μ g. of porphyrin *a* and 0.13 μ g. of protoporphyrin/seed. In dried peas 0.25 μ g. of protoporphyrin/seed was found but no porphyrin *a* could be detected. Wheat germ yielded 2.0 mg. of porphyrin *a* and 16 mg. of protoporphyrin/kg. wet wt. It would seem from these results that, on ripening and drying of peas, haemin *a* is lost. The very low concentration of haemin *a* in these tissues and the difficulty of achieving good spectroscopic purity of the porphyrin *a* makes the chemical determination of porphyrin *a* in plant tissues inaccurate and difficult.

DISCUSSION

The preparation of porphyrin *a* given in this paper is an improvement over earlier methods in two main respects: (1) Chromatography on cellulose-powder columns showed that earlier preparations produced variable amounts of a porphyrin which we have now shown to be derived from porphyrin *a* during the conversion into porphyrin by oxidation of the formyl group to carboxyl. Although this alteration product can be largely separated by thorough chromatography, the present method avoids this complication by destroying the oxidant in the acetic acid which causes the alteration. This finding is applicable to all formyl porphyrins or haemins. (2) Heat treatment of porphyrin *a* after the chromatography stage allows consistent improvement in specific extinction at 558 m μ from 8–10 to 20–24 when the porphyrin is extracted into 17% hydrochloric acid. It would seem that some of the lipid is very closely bound to the porphyrin (possibly by van der Waals forces) and that heat treatment alters this combination. The chromatography step precedes the heat treatment and hydrochloric acid-fractionation step because otherwise the porphyrin *a* becomes too strongly adsorbed on the cellulose for efficient purification.

Although the ratio of band III/band IV given for

the porphyrin a in the final step of the preparation described here was 2.36, a number of fractions in this and other preparations had ratios from 2.40 to 2.45. Throughout this study we have assumed that the higher this ratio the greater the spectroscopic purity of the porphyrin a . This assumption is based on the fact that any alteration to the formyl substituent, which is probably the most labile group in the molecule, lowers this ratio. Lemberg & Falk (1951) have pointed out that, where a second electrophilic group is introduced on a pyrrole ring vicinal to the other, the ratio of band III/band IV is lowered. Thus our assumption could be invalid if a third electrophilic group was present on a pyrrole ring between the two others in intact porphyrin a and was destroyed. The evidence that this is not so is given in the next paper.

Warburg & co-workers (Warburg & Gewitz, 1951; Warburg *et al.* 1955) have prepared crystalline haemin a . Their method required as starting material 64 horse hearts. We have not been able to work on this scale; smaller preparations in our hands were unsuccessful and did not give crystalline haemin a . This may have been due to the undesirability of scaling down this preparation. The scale of our preparation, which can conveniently be reduced if desired, is particularly suitable for study of the spectral properties of haemin a and derivatives which require relatively small quantities of haemin a . Conversion into the porphyrin at an early stage, as in our preparation, offers several advantages. In particular, the characteristic absorption spectra of the porphyrins, with their sharp and high absorption bands, provides a greater sensitivity for the detection of alterations to the prosthetic group or in searching for the possible presence of prosthetic groups whose spectra are closely related and which might account for differences in cytochromes a and a_3 .

Our porphyrin a preparations show no evidence of containing two porphyrins with slightly different band positions. As pointed out by Morell, Barrett, Clezy & Lemberg (1959), the concentration of cryptohaemin a in ox heart is too low to represent the prosthetic group of either cytochrome a or a_3 . The two spots found on chromatography of our porphyrin a esters on paper, the slower-running of which largely disappears on purification, could conceivably indicate a second form of porphyrin a . However, the effect of purification and the probable effect of lipids on the chromatographic behaviour of porphyrin a make interpretation of these chromatograms uncertain. That there is only one type of prosthetic group for cytochromes a , a_3 and a_1 is shown by the above studies on mammalian tissues and by our finding, for cytochrome a_1 (Morell *et al.*

1959), that porphyrin a with spectral characteristics identical with those from these tissues could be extracted from *Proteus vulgaris*.

Our purest porphyrin a of specific extinction 20–24 has been calculated in this paper to be 85% pure. The residual impurity accompanies the porphyrin into 15 or 20% hydrochloric acid or into aqueous borate buffers. The lipophilic nature of porphyrin a or haemin a derived from the substituent long alkyl group or groups (see the next paper) adds to the difficulty of removing lipid impurity. Attempts to crystallize our material either as the porphyrin or haemin, with various solvents, were unsuccessful.

SUMMARY

1. A method of purifying porphyrin a of high spectroscopic purity and approximately 85% absolute purity from ox heart is described and the method given for its conversion into haemin a .

2. During the conversion of haemin a or other formyl haemins into their respective porphyrins, special treatment of the acetic acid solvent is often necessary to prevent oxidation of the formyl group to carboxyl.

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