THE CRYPTOPORPHYRIN OF HEART MUSCLE

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SUMMARY

Cryptoporphyrin *a* has been isolated from ox-heart muscle in a yield of about 2–3 mg/kg fresh muscle, and has been obtained as crystalline methyl ester.

It has been shown that it is closely related to chlorocruoroporphyrin, but differs from it by a greater molecular weight (700–740 as against 562) and other properties. The increment of the molecular weight is probably present as a large saturated alkyl side chain ($R = C_{12}H_{25} - C_{15}H_{31}$) which has no influence on the spectrum, similar to that found by WARBURG AND GEWITZ[13] in haemin *a*.

Cryptoporphyrin *a* is, however, spectroscopically quite dissimilar from porphyrin *a* and closely resembles chlorocruoroporphyrin. Like this, it contains one formyl group and one unsaturated side chain with its double bond in conjugation with the porphyrin ring, in the same relative position on proximal pyrrole rings. The unsaturated group of chlorocruoroporphyrin is vinyl, that of cryptoporphyrin *a* vinyl or $-CH = CHR$.

Cryptoporphyrin *a* is not formed from protohaem compounds during the isolation, nor has any evidence been found for it being formed from haemin *a*. It is therefore unlikely to be derived from cytochrome oxidase or cytochrome *a*, both of which yield porphyrin *a* and may well be derived from a so far unknown haemoprotein in heart muscle which has cryptohaem *a* as its prosthetic group.

INTRODUCTION

In 1932, NEGELEIN[1] reported the isolation from pigeon breast muscle of a crystalline porphyrin which he called cryptoporphyrin. Its analytical composition was claimed to be very similar to, if not identical with, that of Spirographis porphyrin (chlorocruoroporphyrin), the component of chlorocruorin, a blood pigment of Sabellid worms. He reported that cryptoporphyrin could also be obtained from crystalline, but not from recrystallised blood haemin. He concluded that cryptohaemin occurred widely in animal tissue, and that it was perhaps the prosthetic group of the respiratory ferment or of cytochrome *a*.

By re-introducing iron into the porphyrin, NEGELEIN obtained a haemin which in its reactions with hydroxylamine showed the characteristics of a haemin bearing a formyl group as a side chain. Its haemochrome (haemochromogen) had two absorption bands.

In two later papers[2,3] NEGELEIN retracted his earlier finding when he isolated

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another haemin with a single-banded haemochrome (now known as haemin *a* or cytohaemin). He now considered cryptoporphyrin as an artifact, giving two different explanations for its formation, (1) by the action of light on the solution of protoporphyrin in hydrochloric acid, and (2) from haemin during the removal of iron by the ferrous acetate–hydrochloric acid method.

In contrast to NEGELEIN, ROCHE AND BÉNÉVENT[4] maintained that the haemin giving the two-banded haemochrome was the original one, and that the haemin with the single-banded haemochrome was derived from it by alteration. Their work has, however, not been confirmed by the later investigators.

LEMBERG AND FALK[5] found that irradiation of protoporphyrin in hydrochloric acid solution gave a product which was spectroscopically similar to, but not identical with NEGELEIN's cryptoporphyrin.

A re-investigation of cryptoporphyrins was undertaken because a porphyrin similar to NEGELEIN's cryptoporphyrin was consistently obtained in small amounts as a by-product in the preparation of porphyrin *a* (the prosthetic group of cytochrome oxidase and cytochrome *a*) from heart muscle[6,7]. Some of the differences between porphyrin *a*, cryptoporphyrin *a* and a cryptoporphyrin *p* derived from blood preparations have been tabulated by LEMBERG[6], the results having been obtained during the course of the present investigation. Although more recent work has shown that the formation of cryptoporphyrin from blood is more complex than was then anticipated, and that not one, but several cryptoporphyrins *p* are obtained depending on the conditions of the isolation, these porphyrins all differ from cryptoporphyrin *a* in not having a formyl side chain and also in spectral properties, melting points and molecular weight. The cryptoporphyrins *p* will form the subject of another paper.

This paper deals with the isolation of cryptoporphyrin *a* from ox-heart muscle, the purification and crystallisation of its methyl ester, and its relationship to chlorocruoroporphyrin. Some evidence is also presented which indicates that cryptoporphyrin *a* is not an artifact formed from haemin *a* and that therefore a haemoprotein with cryptohaemin *a* as prosthetic group may well exist in heart muscle.

MATERIALS AND METHODS

Absorption spectra: A Hilger "Uvispek" photoelectric spectrophotometer, model H700 was used. Its wavelength scale was adjusted using the hydrogen lines at 486.1 mμ and 656.3 mμ for standardisation. Measurements were carried out in 1 cm cells covered with a coverslip.

Solvents: These were of A.R. quality. Chloroform was freed from traces of zinc by redistillation.

Melting points: A Griffin and Tatlock electric micro-melting point apparatus with curved thermometer embedded in a copper block was used and the readings were corrected, using specially purified substances of known melting point. The melting point was best observed in reflected light with the crystals mounted on a thin microscope coverslip placed on top of the copper square covering the hole through the copper block. The sample was rapidly heated to a temperature 50° below the melting point and then very slowly when approaching the melting point.

Conversion of haemins to porphyrins: The 2 methods used were: (a) The $FeSO_4$–HCl method (LEMBERG, BLOOMFIELD, CAIGER AND LOCKWOOD[8], p. 440) at 80°, with

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the molar ratios of haemin: Fe^{++}: HCl of 1:50:500. (b) The modified $FeSO_4$–HCl method (MORELL AND STEWART[9]) at room temperature, with molar ratios of haemin: Fe^{++}:HCl of 1:350:4,250.

Separation of the porphyrins by fractionation: The ethereal solution of the porphyrins was extracted first with half its vol. of 3 % HCl (w/v). 2 extractions were sufficient to remove 95 % of the protoporphyrin. Further extractions with 3 % HCl removed increasing amounts of cryptoporphyrin *a*. The main cryptoporphyrin *a* fraction was obtained by extracting repeatedly with 8 % HCl (w/v). These extracts still contained almost as much protoporphyrin as cryptoporphyrin *a*. Porphyrin *a* was obtained free from protoporphyrin and cryptoporphyrin *a* when the remaining ether solution was extracted with 25 % HCl (w/v). The porphyrins were taken back into ether by neutralisation with sodium acetate. Several extractions with fresh ether were found necessary for quantitative extraction. This method yielded firstly the fraction in 8 % HCl which was used for the isolation of cryptoporphyrin *a* (see below), and then gave a fraction which contained only 1 or 2 porphyrins, so that a spectrophotometric analysis at 1 or 2 wavelengths would allow estimation of the amounts of each porphyrin. It was found[7] that analysis of the crude porphyrin mixture prior to HCl fractionation using 3 wavelengths, while giving reasonably accurate values for the 2 major constituents, protoporphyrin and porphyrin *a*, did not give reliable values for the minor constituent, cryptoporphyrin *a*. Sometimes negative values for cryptoporphyrin *a* were found while subsequent analysis after fractionation established its presence.

Estimations of the porphyrins after HCl fractionation: Protoporphyrin and porphyrin *a* were estimated from optical density (A) measurements at 538 mμ and 557 mμ respectively, using the specific extinction coefficients given by LEMBERG AND PARKER[7]. Cryptoporphyrin *a* was estimated in the 3 % and in the 8 % HCl fractions by the spectrophotometric method of LEMBERG AND PARKER[7] using a very slightly modified formula:

$$\text{mg cryptoporphyrin } a = 0.0566\, E_{555} - 0.0099\, E_{538}$$

where E represents A/cm, x ml of solution.

The accuracy of this estimation was determined with the 3 % HCl extracts by esterification and separation of the porphyrin esters by repeated alumina chromatography. The purified cryptoporphyrin a ester was then estimated from the optical density at 555 mμ using the value for the specific extinction given by LEMBERG AND PARKER[8]. The yield of isolated cryptoporphyrin *a* was 0.27 mg/kg of fresh ox-heart muscle, compared with 0.34 mg as estimated with the above formula, while the formula (a) of LEMBERG AND PARKER[14] gave only 0.01 mg.

Removal of acetic acid from ethereal porphyrin solutions: Acetic acid must be removed before esterification with diazomethane. The most convenient method found was to spray water through a bulb perforated with fine holes into the ethereal solution contained in a large separating funnel. The dilute acetic acid solution could be continuously run off through the bottom of the funnel.

Esterification of the porphyrins: This was carried out in ethereal solution with diazomethane prepared from nitrosomethylurea[10].

Separation of the porphyrin esters by chromatography on alumina: The alumina column was made by packing a glass tube (2.4 cm × 68 cm) to a height of 45 cm with alumina (B.D.H., for chromatographic adsorption analysis) added as a slurry in ether,

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tapping frequently to ensure homogeneity*. The porphyrin esters were added in etheral solution and were eluted with ether.

Paper chromotography of porphyrin esters: The method of CHU, GREEN AND CHU[11] was used with chloroform–kerosene (2.6:4) in an atmosphere of chloroform. The porphyrin esters were applied to the base line of Whatman No. 1 filter-paper cylinders in the form of a band and the solvent front allowed to ascend to a height of 10 cm.

Paper chromotography of the porphyrin-free acids: The method of KEHL AND STICH[12] was used with a mixture of equal volumes of 2,6-dimethylpyridine and water as developing solvent in an atmosphere of ammonia at 37°.

Hydrogenation of the porphyrin esters: This was carried out in glacial acetic acid with platinum black or colloidal palladium at 20°, similar to the method used by WARBURG AND GEWITZ[13] for the hydrogenation of the unsaturated side chain of haemin *a*. The main flask of the Warburg vessel contained 10 mg of the catalyst in 2.0 ml glacial acetic acid, the side arm approximately 1 mg porphyrin ester dissolved in 0.5 ml glacial acetic acid. Hydrogen uptake was measured manometrically.

Preparation of diazoacetic ester adducts of porphyrin oximes: Diazoacetic ester in ether solution was added to a small amount of porphyrin oxime. The mixture was heated under nitrogen at 60°, allowing the ether to evaporate. The vessel was stoppered at once, transferred to an oven kept at 60° and allowed to remain in it for 20 h. After cooling, the residue was dissolved in ether and the porphyrin diazoacetic ester adduct extracted with 10% (w/v) HCl. It was then taken back into ether with sodium acetate, and its absorption spectrum was measured in chloroform.

Chlorocruoroporphyrin dimethyl ester: This substance was prepared from protoporphyrin according to LEMBERG AND PARKER[14].

Monoformyl deuteroporphyrin esters: These substances were synthesised from deuteroporphyrin (FISCHER AND WECKER[15]). The separation of the 4-monoformyl and the 2-monoformyl deuteroporphyrin esters was effected by repeated fractional crystallisation from chloroform-methanol (LEMBERG AND PARKER, unpublished).

The 4-formyl ester crystallised in the form of plates melting at 275–277°, while the 2-formyl ester was obtained in the form of needles melting at 252–253°.

The mixed m.p. showed a depression. The m.p. of the 4-monoformyldeuteroporphyrin ester was higher than that recorded by FISCHER AND WECKER[15] (260°) which probably still contained some 2-monoformyl compound.

Porphyrin a: Method B 3 of LEMBERG, BLOOMFIELD, CAIGER AND LOCKWOOD[8] was used for its preparation and the method of LEMBERG AND STEWART[16] for purification. The ratio of band III to band IV was 2.0.

Protoporphyrin dimethyl ester: The method of GRINSTEIN[17] was used. Traces of dioxyprotoporphyrin and cryptoporphyrin *p* were removed by chromatography of the ester on alumina. The m.p. of the recrystallised ester was 230–232°.

Isolation of cryptoporphyrin a

The first steps of isolation were similar to those used in method B 3 by LEMBERG, BLOOMFIELD, CAIGER AND LOCKWOOD[8] for the isolation porphyrin *a*. The heart muscle mince was prepared as described in the above paper and stored at −15° in

* Recent samples of this alumina were found to have increased in adsorptive power. It was found necessary to decrease this by washing with alcohol and acetone containing 5% of water and drying at 37°.

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500 g lots, this being found a convenient amount to work up at one time. After thawing, the mince was washed with water until the washings were colourless. This ensures that no cryptoporphyrins *p* are formed. The mince was extracted with 1 l of neutral 80 % acetone–20 % water at room temperature for 30 min with mechanical stirring, and then with ether until the extract was no longer coloured (two 1 l lots were usually sufficient). The main purpose of this pre-extraction with organic solvents was to remove emulsifying agents which retard later stages in the isolation. It could be omitted without significantly affecting the yield of protoporphyrin, cryptoporphyrin *a* or porphyrin *a*.

The haemins were then extracted with 2 l of acid–acetone–water (80 % acetone, 20 % water, 0.7 % (w/v) HCl) for 16 h at 0°. In some expts. the extraction was carried out at room temperature (see below). The haemins were transferred to ether as described by LEMBERG *et al.*[8] and converted to their porphyrins by method (a), see METHODS. The porphyrins were separated by fractionation with HCl (see METHODS), and the amounts in each fraction estimated (see METHODS).

The average yield of cryptoporphyrin *a* in 20 expts. was 1.6 mg/kg fresh muscle. The range was 0.9 to 4.4 mg/kg fresh muscle. In later expts. fairly consistent yields of between 2 and 4 mg/kg fresh muscle were obtained. The yield of porphyrin *a* was about 20 mg, smaller than the yield of 30 mg reported by LEMBERG *et al.*[8], that of protoporphyrin being about 60 mg/kg. Variations in yield of cryptoporphyrin *a* of up to 40 % have been found in expts. in which starting material and method of preparation were the same; they indicate that the isolation and estimation techniques are not entirely reproducible.

Purification: The ether solution obtained from the 8 % HCl extract containing the bulk of cryptoporphyrin *a* was washed free of acetic acid and esterified as described under METHODS. The etheral solution of the esters was concentrated to about 100 ml by distilling under reduced pressure and chromatographed on an alumina column as described under METHODS. Preliminary drying of the porphyrin solution must be avoided since the esters tend to precipitate on the walls of the containing vessel if allowed to stand even a short time, and separation of the precipitated esters is difficult.

Protoporphyrin ester migrated through the column as a diffuse pink band followed by a second diffuse band containing cryptoporphyrin *a* ester. A third more concentrated band was eluted more slowly; it contained some cryptoporphyrin *a* ester, together with another porphyrin ester resembling protoporphyrin spectroscopically. As the fractions were eluted, samples were examined under the spectroscope for the characteristic band III at 537 mμ of protoporphyrin and at 555 mμ for cryptoporphyrin. Other samples were run on paper chromatograms (see METHODS).

Cryptoporphyrin *a* ester had an R_F value of 0.65 and migrated as a yellow band showing weak red fluorescence under u.v. light. Protoporphyrin ester had an R_F value of 0.80 and gave a pinkish-brown band with strong fluorescence. The third porphyrin ester migrated as a broad diffuse band of R_F value 0.29, also showing strong red fluorescence. An almost complete separation of cryptoporphyrin *a* ester and protoporphyrin ester was affected by a single run on alumina. The small intermediate fractions containing a mixture of both were re-chromatographed.

It was not possible to separate mixtures of cryptoporphyrin *a* ester and the proto-like ester of R_F 0.29 by the repeated alumina chromotography. A complete separation was, however, achieved by allowing the ether solution to evaporate slowly

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at room temperature. Cryptoporphyrin *a* ester precipitated on the walls of the vessel and could be freed from the other ester by washing with ether. It was then added to the chloroform solution of the main fraction.

The pure solutions of cryptoporphyrin *a* ester in ether were combined and allowed to crystallise at —15° overnight. Large oval leaflets were obtained, sometimes showing optical twinning. Recrystallisation from chloroform–ether raised the m.p. from 240–245° to 254–257°. At this stage the crystal form was generally rectangular or large pentagonal plates with no evidence of twinning. Constant m.p. was attained at 259–260°, the crystals being diamond-shaped with one of the directions of extinction making a very small angle (1–5°) with one of the sides of the diamond. Fig. 1 gives a diagrammatic representation of the various crystal forms.

Absorption spectra: The absorption maxima of cryptoporphyrin *a* dimethylester, of its hydrochloride and of its oxime are given in Table I, and compared with those of chlorocruoroporphyrin. The absorption curves of both esters in the visible range are given in Fig. 2. Both reveal the close similarity of the two spectra. However, the specific extinctions of cryptoporphyrin *a* are distinctly lower than those of chlorocruoroporphyrin. If the molar extinctions of the two porphyrins are assumed to be the same, a mol. wt. of about 740 is calculated for cryptoporphyrin *a* from its specific extinction at 559 mμ (band III) and about 700 from the sum of the two maximal

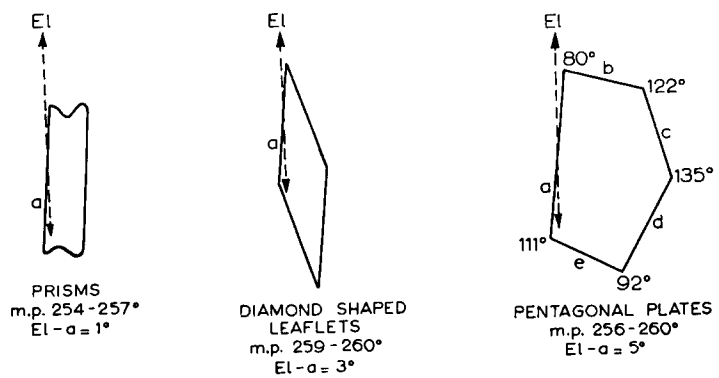

Fig. 1. Crystal forms of cryptoporphyrin dimethyl ester.

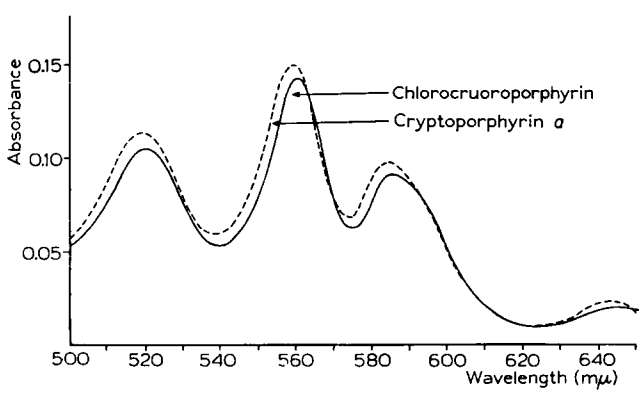

Fig. 2. Absorption curves of cryptoporphyrin *a* (- - - -) and chlorocruoroporphyrin dimethyl (———) esters in chloroform.

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TABLE I

ABSORPTION SPECTRA OF CRYPTOPORPHYRIN *a* AND CHLOROCRUOROPORPHYRIN

		Cryptoporphyrin a					*Chlorocruoroporphyrin natural***			
	Bands	*I*	*II*	*III*	*IV*		*I*	*II*	*III*	*IV*
Dimethyl ester in chloroform	max. (mμ)	642.5	584	559	519*		644	584	558.5	518.5**
	Ratio to band IV	0.21 :	0.86 :	1.32 :	1		0.21 :	0.91 :	1.40 :	1
	$E^{1\%}_{1cm}$	31.0	127	194.5	147.5		38.0	167 synthetic	255§***	182
						max. (mμ)	644.5	585	560	520
						Ratio to band IV	0.22 :	0.89 :	1.40 :	1
						$E^{1\%}_{1cm}$	38.7	157.5	250	179
Hydrochloride in 10% HCl max. (mμ)		613.5		563.5		In 20% HCl	615.2	564.3§		
Oxime in chloroform	max. (mμ)	636.5	580	548.5	510.5		634	579	546	511
	Ratio to band IV	0.28 :	0.55 :	0.85 :	1		0.27 :	0.56 :	0.93 :	1

* Measured in Hilger "Uvispek" spectrophotometer.
** LEMBERG AND FALK, in Beckman spectrophotometer.
*** Based on STERN AND WENDERLEIN[23] value of $E^{1\%}_{1cm} = 255$ for band III.
§ LEMBERG AND PARKER.

TABLE III

THE DIAZOACETIC ESTER SHIFT GIVEN BY THE OXIMES OF CRYPTOPORPHYRIN *a*, CHLOROCRUOROPORPHYRIN AND PORPHYRIN *a* COMPARED WITH THAT OF PROTOPORPHYRIN

Porphyrin Oximes of	*Absorption maxima (mμ) and ratios of intensities (in parentheses)*				*Diazoacetic adduct Absorption maxima (mμ) and ratios of intensities (in parentheses)*				*Shift of absorption maxima*			
	I	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*
Cryptoporphyrin *a*	636.5 (0.28)	580 (0.55)	548.5 (0.85)	510.5 (1)	634.5 (0.28)	578 (0.56)	546 (0.84)	509.5 (1)	2.0	2.0	2.5	1.0
Chlorocruoroporphyrin	635.5 (0.27)	578 (0.56)	546.5 (0.93)	510 (1)	633 (0.28)	577 (0.57)	545 (0.81)	508 (1)	2.5	1.0	1.5	2.0
Porphyrin *a*	639 (0.25)	578 (0.96)	553 (1.36)	513.5 (1)	637 (0.25)	577.5 (0.87)	550.5 (1.14)	512 (1)	2.0	0.5	2.5	1.5
Protoporphyrin	632	577	544	506.5	626.5	573	539.5	504.5	5.5	3.5	4.5	2.0

extinctions of bands III and IV, whereas that of chlorocruoroporphyrin is only 562.

Cryptoporphyrin *a* ester oxime was prepared according to LEMBERG AND FALK[5]. Insufficient material was available for its crystallisation. Compared with the absorption bands of the porphyrin ester, those of the oximes of both chlorocruoroporphyrin and cryptoporphyrin are shifted towards shorter wave lengths, and the rhodotype spectra (III > IV > II > I) have been converted to aetiotype (IV > III > II > I). This reaction proves the presence of one carbonyl side chain, but does not allow differentiation between formyl and acetyl.

The absorption spectrum of cryptoporphyrin *a* is, however, altered by $NaHSO_3$ in dilute cold pyridine, a reaction typical of formyl-substituted porphyrins such as chlorocruoroporphyrin and porphyrin *a*, but not given by acetyl-porphyrins and cryptoporphyrins *p* (LEMBERG, unpublished).

Cryptohaemin a: The haemin was obtained by introduction of iron into the dimethylester by the usual ferrous acetate–dil. HCl method. The absorption spectra of the haemochrome and that of its oxime are given in Table II, together with the corresponding chlorocruorohaem compounds.

If dithionite was used as reducing agent, a faint band at 553 mμ could be seen in the hand spectroscope; in the spectrophotometer the maxima were found at 579 and 530 mμ, with a slight flattening of the curve at 554–556 mμ. With ascorbic acid as reducing agent only the two main bands were discernible.

The most noticeable difference (7 mμ) between cryptohaemochrome *a* and chlorocruorohaemochrome is in the position of the weak second band which is in marked contrast to the similarity of the haemochrome spectra of the two oximes. In this regard it should be noted that the position of the cryptohaemochrome spectrum of NEGELEIN agrees with that of cryptohaemochrome *a* rather than with that of chlorocruorohaemochrome.

The formation of the oximes was carried out according to LEMBERG AND FALK[5]. It occurs under these conditions only with the haemochromes of formyl, not of acetyl porphyrins and the observed shift (20 mμ) of band I is additional evidence for the presence of a formyl group in cryptoporphyrin *a*.

Number of carboxyl groups: Lutidine–water paper chromatography (see METHODS) showed cryptoporphyrin *a* to be a dicarboxylic acid, migrating with an R_F of 0.85, similar to protoporphyrin (R_F, 0.86) and somewhat lower than porphyrin *a* (R_F, 0.92).

Evidence for the presence of a vinyl or substituted vinyl side chain: The presence

TABLE II

ABSORPTION SPECTRA OF CRYPTOHAEMOCHROME *a* AND CHLOROCRUOROHAEMOCHROME AND OF THEIR OXIMES

Solvent: 50% pyridine in water.

	Max. (mμ) Cryptohaem		*Chlorocruorohaem**	
	I	*II*	*I*	*II*
Haemochrome	581	531	582	538
Haemochrome after treatment with hydroxylamine	561	531.5	561.4	529.5

* From synthetic chlorocruoroporphyrin dimethyl ester m.p. 279–282°. (LEMBERG AND FALK[5]). gave bands at 583 mμ and 545 mμ; but this still contained some protohaemin.

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of such a group was tested for by treating the oxime of cryptoporphyrin *a* ester with diazo-acetic ester (see METHODS). RIMINGTON *et al.*[18] used a similar method for determining the number of unsaturated side chains in porphyrin *a*. They prepared first the diazo-acetic ester adduct and then treated this with hydroxylamine. Their results were inconclusive since diazoacetic ester can react with the formyl as well as with the unsaturated group. Unambiguous results can only be obtained by masking the formyl group (*i.e.* by first converting it into oxime group) before the reaction with diazoacetic ester.

Table III shows the shift of the absorption maxima of the oximes of cryptoporphyrin *a*, chlorocruoroporphyrin and porphyrin *a* as well as of protoporphyrin caused by their reactions with diazoacetic ester.

Since cryptoporphyrin *a*, chlorocruoroporphyrin and porphyrin *a* give a shift approximately half that given by protoporphyrin with two vinyl groups, it can be assumed that each has one reactive vinyl or substituted vinyl group. The presence of a vinyl group in chlorocruoroporphyrin has been confirmed by its synthesis[15]. WARBURG AND GEWITZ[13] have demonstrated by hydrogenation expts. the presence of one unsaturated side chain in porphyrin *a*.

Hydrogenation of the vinyl or substituted vinyl group of cryptoporphyrin a. The unsaturated group of cryptoporphyrin *a* ester could be hydrogenated without reduction of the formyl group by the technique described under METHODS, using platinum oxide as catalyst.

The reaction was accompanied by the formation of a platinum complex, and the amount of hydrogen taken up did not correspond to that calculated for the hydrogenation of one unsaturated bond. The reaction was continued until uptake of hydrogen stopped. The porphyrin ester and its platinum complex were then transferred into ether, the free porphyrin recovered by extraction with 20 % (w/v) HCl and brought back into ether with sodium acetate. After esterification the porphyrin ester was crystallised from ether and recrystallised from chloroform–ether in the form of rather thick oval leaflets. The yield was 8 % and the material was not sufficient for a melting point determination.

No metal complex was formed when palladium was used as catalyst, but some hydrogenation of the formyl group as well as of the vinyl group occurred, giving rise to by-products. These by-products containing hydroxymethyl or methyl groups instead of formyl could, however, be easily detected by their absorption spectrum, and separated from the dihydroporphyrin esters by chromatography on alumina (see METHODS). Ether eluted small amounts of a porphyrin resembling deuteroporphyrin spectroscopically. 25 % (v/w) chloroform in ether eluted the hydrogenated formyl compound, and chloroform–ether (1 : 1) the hydroxymethyl compound. The yield of the hydrogenated formyl compound was 5.6 % by this method.

Dihydro-chlorocruoroporphyrin ester was prepared similarly in a yield of 3.5 % using palladium as a catalyst. It was crystallised from chloroform-ether. It should be noted that this hydrogenation product, with intact formyl groups, differs from that obtained by WARBURG[19] and FISCHER AND DEILMANN[20], in which the formyl groups had also been reduced to methyl.

Table IV gives the absorption spectra of the dihydrocryptoporphyrin *a* ester and dihydrochlorocruoroporphyrin ester. The spectra of the 4-formyl and 2-formyl deuteroporphyrin esters are included for comparison.

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TABLE IV

ABSORPTION SPECTRA OF DIHYDROCRYPTOPORPHYRIN *a* ESTER AND DIHYDROCHLOROCRUOROPORPHYRIN ESTER COMPARED WITH THOSE OF MONOFORMYLDEUTEROPORPHYRIN ESTERS

Solvent : Chloroform.

		Bands *I*	*II*	*III*	*IV*
Dihydrochlorocruoroporphyrin	max (mμ)	641.5	580.5	558.5	518.5
	Ratio to band IV	0.18 :	1.21 :	1.85 :	1
Dihydrocryptoporphyrin *a*	max (mμ)	640.5	580	557.5	515.5
	Ratio to band IV	0.17 :	1.05 :	1.58 :	1
4-formyl deuteroporphyrin	max (mμ)	641	580	555	515
	Ratio to band IV	0.20 :	1.02 :	1.77 :	1
2-formyl deuteroporphyrin	max (mμ)	639.5	579	555	515
	Ratio to band IV	0.18 :	1.08 :	1.83 :	1

It is noted: (1) that the expected shift in band position towards shorter wave lengths on hydrogenation of the unsaturated side chains of cryptoporphyrin *a* and chlorocruoroporphyrin is obtained (cf. Tables I and IV), (2) that the ratio of bands III/IV and II/IV is increased in the dihydroporphyrins. In dihydrochlorocruoroporphyrin the III/IV ratio reaches that of the monoformyl deuteroporphyrins, as might be the result expected from the removal of the "anti-rhodofying" vinyl group in the neighbouring ring. The III/IV ratio of dihydrocryptoporphyrin *a* while also increased, remains considerably lower than that of the monoformyldeuteroporphyrin. Attempts to increase this ratio by repeated recrystallisation from chloroform–ether failed. This does not appear to be explained by assuming incomplete hydrogenation of cryptoporphyrin *a* since the shift of the absorption bands on hydrogenation was not smaller than that on hydrogenation of chlorocruoroporphyrin.

The origin of cryptoporphyrin a

Previous evidence adduced by the present author and reviewed by LEMBERG[6] (cf. also LEMBERG *et al.*[8], p. 442) had made it unlikely that cryptoporphyrin *a* can be derived from protohaemin as an isolation artifact. Although the protohaem from haemoglobin under certain conditions can be transformed into cryptoporphyrins *p*, these are quite different from cryptoporphyrin *a*, being ketonyl—not formyl—porphyrins and there is evidence that no cryptoporphyrins *p* are produced in the isolation of cryptoporphyrin *a* (PARKER, unpublished). That cryptoporphyrin *a* is not formed during isolation from protohaem was shown by the following expt. although there appeared to be at first some evidence to the contrary. Protohaemin formed from protoporphyrin (METHODS,) by re-introduction of iron, yielded small amounts (about 4%) of "apparent cryptoporphyrin *a*" according to spectrophotometric analysis (METHODS) on subsequent de-ironing, with or without previous standing of the haemin in acetone–HCl at room temperature. Paper chromatography (see METHODS), however, did not reveal the presence of any cryptoporphyrin *a* ester (R_F, 0.65); only protoporphyrin ester (R_F, 0.80) and 2 other porphyrin esters of R_F 0.0 and R_F, 0.27 (probably haematoporphyrin and hydroxyethyl-vinyl deuteroporphyrin esters) were found. Haematoporphyrin has indeed a ratio E_{555}/E_{538} far higher than that of

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protoporphyrin and about 15 % haematoporphyrin would account for 4 % "apparent cryptoporphyrin". These have probably been largely formed during the heating in glacial acetic acid during the introduction of iron into protoporphyrin, since they can also be obtained from crystalline haemin (directly prepared from blood) on heating in glacial acetic acid (PARKER, unpublished).

That cryptoporphyrin *a* is not formed from haem *a* is shown by the following observations. Haemin *a* was obtained by re-introduction of iron into porphyrin *a* (see METHODS) with ferrous acetate–dil. HCl. Paper chromatography of an esterified sample of the porphyrin had shown the absence of cryptoporphyrin *a* and protoporphyrin. Removal of iron was carried out by Method (1) under METHODS. The porphyrin thus obtained had no longer the same spectroscopic properties, in particular the ratio of bands III/IV was lowered from 2.01 to about 1.7. If the haemin *a* was kept for 24 h in acetone–HCl before removal of the iron, the ratio was further lowered to about 1.6. The formula given by LEMBERG AND PARKER[7] (p. 485) for the spectrophotometric estimation of mixtures of porphyrin *a* and cryptoporphyrin *a* indicated a cryptoporphyrin *a* content of 28 % (formed either during the introduction or the removal of iron), and an additional 12 % cryptoporphyrin *a* formed during the action of acetone–HCl on haemin *a* at room temperature. Paper chromatography of the ester of these products (see METHODS) showed three bands of R_F 0.72, 0.24 and 0.05, of which the first might have been cryptoporphyrin *a* ester (R_F, 0.65). It was, however, possible to separate this ester from other alteration products of porphyrin *a* and to show that it was not identical with cryptoporphyrin *a* ester. The ester of R_F 0.72 was obtained free from the other products of lower R_F by elution with a mixture of equal volumes of ether and light petroleum (40 to 60°) from a cellulose-powder column[16]. In Fig. 3 the spectra of this product from haemin *a*, of cryptoporphyrin *a*, and of porphyrin *a* are compared.

It is seen from an inspection of these curves (a) that the ratio of bands III/IV of the alteration product is higher (1.65) than that of cryptoporphyrin *a* (1.17), but lower than that of porphyrin *a* (> 2.0); (b) that the ratios $E_{580\,m\mu}/E_{513\,m\mu}$ of the alteration product is 1.15, similar to that of porphyrin *a*, but much higher than that of cryptoporphyrin *a* (0.65); (c) that the band II of the alteration product is

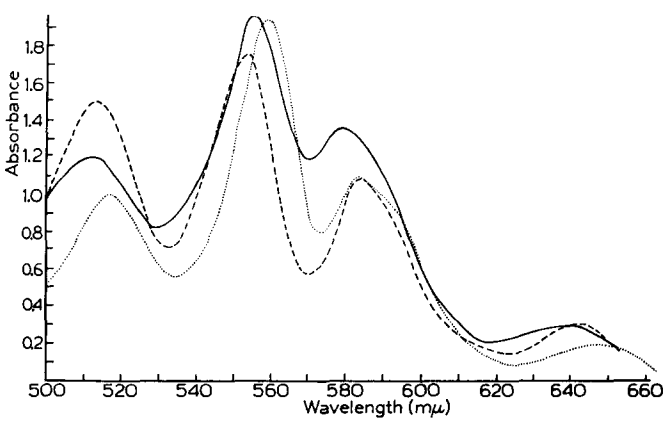

Fig. 3. Absorption curves of porphyrin *a* (·····) altered porphyrin *a* (———) and cryptoporphyrin *a* (– – – –) in acetone.

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found at 578 mμ, that of cryptoporphyrin *a* (and porphyrin *a*) at 583 mμ; (d) that the ratio $E_{578\,m\mu}/E_{610\,m\mu}$ is considerably higher for the alteration product than for cryptoporphyrin *a* or porphyrin *a*. These observations also exclude that the alteration products can be a mixture of cryptoporphyrin *a* with unaltered porphyrin *a*.

Variation of yield: The variation of yield of cryptoporphyrin *a* reported above is smaller than that observed in a previous paper[7]. No clear-cut evidence could be found for factors to which this variation could be ascribed, nor is it due to the presence of porphyrin *a* or altered porphyrin *a* in variable amounts in the 3% and 8% HCl extracts.

DISCUSSION

In the above experiments no evidence has been found to show that porphyrin *a* is formed from either protohaemin or haemin *a* under the conditions of its isolation from heart muscle.

The variation in the yield of cryptoporphyrin *a* from heart muscle has been previously considered as evidence for it being an artifact[8]. However, experimental difficulties of the isolation of cryptoporphyrin *a* from protoporphyrin are great. The amounts of cryptoporphyrin *a* in the first two HCl extracts cannot be determined with a great deal of accuracy owing to the presence of large amounts of protoporphyrin and the presence of some haematoporphyrin. Another source of error might have been the formation of cryptoporphyrin *p* from protohaem compounds of heart muscle, but no evidence for cryptoporphyrin *p* formation from adequately washed heart-muscle mince has been found.

The results of this paper indicate that cryptohaem *a* may be an independent constituent of heart muscle, probably in the form of a haemoprotein or cytochrome. Cryptohaem *a* is not a constituent of either cytochrome *a* or cytochrome a_3, since it occurs in far too small a concentration to account for either, whereas the yield of porphyrin *a* can account for both. CONNELLY, MORRISON AND STOTZ[24] have adduced evidence for the presence of cryptohaemin *a* among the haemins extracted from heart muscle.

So far no direct spectroscopic evidence for the existence of another cytochrome in addition to cytochromes *a* and a_3 has been found. If, as is probable, a haemoprotein with cryptohaemin *a* as prosthetic group exists, its small concentration makes it unlikely that it plays a major role as a cytochrome in the electron transport of the cell.

The results of this study show that cryptoporphyrin *a* is not identical with chlorocruoroporphyrin, though closely related to it. While NEGELEIN's spectroscopic findings[1] closely agree with ours, we cannot explain his analytic findings which give cryptoporphyrin *a* the analytical composition now claimed for chlorocruoroporphyrin. It is improbable, though not impossible, that pigeon-breast muscle contains chlorocruorohaem while ox heart contains cryptohaem *a*.

The amounts of pure cryptoporphyrin *a* ester obtained did not suffice for analysis, but nevertheless conclusions can be drawn as to its structure. Cryptoporphyrin *a* is, like chlorocruoroporphyrin, a dicarboxylic porphyrin with one formyl and one unsaturated side chain, in which the double bond is conjugated with the porphyrin ring. The low specific extinction of cryptoporphyrin *a* indicates that it differs from chlorocruoroporphyrin by having a large side chain (of the order of size of $C_{15}H_{31}$) which increases the molecular weight to 700–740. The great spectroscopic similarity

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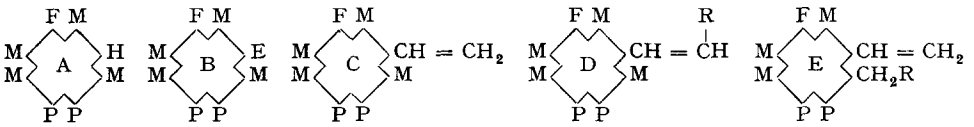
to chlorocruoroporphyrin indicates that the presence of this group, which may be called R, is the only difference between cryptoporphyrin *a* and chlorocruoroporphyrin. Moreover, the relative positions of the formyl and unsaturated side chain in both compounds are the same. In chlorocruoroporphyrin, the vinyl group is in position 4, in a pyrrole ring proximal to that bearing the formyl group in 2; this was established by FISCHER AND V. SEEMAN[21] and FISCHER AND DEILMANN[20] (Fig. 4C). The anti-rhodofying effect of the vinyl group decreases the ratio of the absorption band III to IV from 1.8 in monoformyldeuteroporphyrin (Fig. 4A) to 1.4. Hydrogenation of vinyl to ethyl abolishes the anti-rhodofying effect of vinyl, the resulting dihydrochlorocruoroporphyrin (Fig. 4B) having a ratio III to IV of 1.85, quite similar to that of monoformyl deuteroporphyrin. Cryptoporphyrin *a* has a III/IV ratio quite similar to that of chlorocruoroporphyrin, and the ratio is also increased by hydrogenation, although not to the same extent (1.32 to 1.58).



M = .CH_3 E = .$CH_2 \cdot CH_3$ P = .$CH_2 \cdot CH_2 \cdot CO_2H$ F = .CHO R = .$C_{12}H_{25}$ to .$C_{15}H_{31}$

Fig. 4. Relationship between cryptoporphyrin *a* and chlorocruoroporphyrin (using H. FISCHER's symbol for the porphyrin ring)

The difference of the ratio of absorption bands between dihydrochlorocruoroporphyrin and dihydrocryptoporphyrin *a* does not appear to throw any light on the position of the R group in the molecule, since no difference would be expected whether the grouping is CH_2—CH_2R or CH_2R (*cf.* Figs. 4E and 4D). The results of the resorcinol melt will distinguish between these two possibilities, since only formula 4D would yield deuteroporphyrin. Lack of material has prevented this experiment.

The spectroscopic differences between porphyrin *a* and chlorocruoroporphyrin are far greater than those between cryptoporphyrin *a* and chlorocruoroporphyrin, and it is clear that the relative position of the unsaturated side chain to the formyl group is different. On the other hand, both porphyrin *a* and cryptoporphyrin *a* contain a large alkyl group, and in the ether–pyridine HCl separation of RAWLINSON AND HALE[21], cryptohaemin follows haemin *a*, not protohaemin (see ref.[6]).

In view of the doubts which can now be entertained as to the formation of cryptoporphyrin *a* from haemin *a*, it would appear premature to discuss the relationship of cryptoporphyrin *a* and porphyrin *a* any further, although even if cryptoporphyrin *a* is not formed from haemin *a* in the isolation, the two haemins may still stand in close biogenetic relationship.

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CONCENTRATION OF PARATHYROID HORMONE ACTIVITY BY CHROMATOGRAPHY ON CARBOXYMETHYLCELLULOSE*

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SUMMARY

A method for the further purification of a fraction salted-out from a dilute hot hydrochloric acid extract of untreated ground bovine parathyroid glands is described. Chromatography on carboxymethylcellulose using a NaCl gradient rising to 1 *M* at pH 4.68 results in an active Ca-mobilizing fraction with a 6-fold increase in specific activity in moderate yield. This can be further increased 2- to 6-fold by recycling and eluting with a gradient rising to 0.5 *M* salt. The method is applicable to starting material containing either high or low amounts of activity.

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