Prosthetic Groups of the Cytochromes Present in *Corynebacterium diphtheriae* with Especial Reference to Cytochrome *a*

By W. A. RAWLINSON* (Wellcome Travelling Research Fellow, 1947–8) and J. H. HALE
Departments of Chemical Pathology and Bacteriology, University College Hospital Medical School, London

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As a preliminary to investigations on porphyrin synthesis by *Corynebacterium diphtheriae* with various isotopically labelled compounds, we deemed it essential to study the prosthetic group of the cytochromes of this organism. The present paper reports the results so far obtained.

With *C. diphtheriae*, controlled improvement of toxin production has led to a corresponding increase in the production of free porphyrin; accurate adjustment of iron concentration is essential for the production of optimum amounts of toxin, even when other factors such as pH, nutrients, etc. are optimal. Pappenheimer (1947*a*, *b*) has shown that there is a parallel rise and fall of toxin and porphyrin excreted into the medium during cultivation under different conditions. The porphyrin was identified tentatively by Campbell-Smith (1930) and Coulter & Stone (1931) and was characterized as coproporphyrin III by Gray & Holt (1947). A functional relationship between toxin and pigment was postulated by Pappenheimer (1947*a*) after he had obtained evidence to indicate that for every 4 atoms of iron added above the amount giving an optimum yield of toxin, there failed to appear in the culture fluid 4 mol. of porphyrin and 1 mol. of toxin. At such higher iron levels he found that cells contained greater amounts of a haemochromogen-like substance. *C. diphtheriae* contains large amounts of cytochrome *b* and smaller amounts of *c* and *a* (Fujita & Kodama, 1934). In the present work the prosthetic group of cytochrome *b* has been identified as protohaem no. 9 and the prosthetic group of cytochrome *a*, although it has not been identified with any structure of established constitution, has been investigated in some detail.

EXPERIMENTAL

Measurement of absorption spectra. The wave lengths of absorption maxima were measured with a Beck-Hartridge reversion spectrometer. In the case of intact cell suspensions, additional measurements were made by means of a Zeiss microscope eyepiece spectrometer.

For the measurement of optical densities, a Beckman photoelectric spectrophotometer was used. When dealing with solutions of pyridine haemochromogens, it was necessary to take the following precautions. The 1 cm. glass cell was filled completely with solution and the lid fitted without the introduction of air bubbles. The solid $Na_2S_2O_4$ used as reducing agent was dissolved by rotating the cell. On account of the variability of different batches of $Na_2S_2O_4$ the minimum amount required to effect complete reduction was found by trial. In order to prevent turbidity appreciable excess of $Na_2S_2O_4$ was avoided. Measurements at wave lengths shorter than 400 mμ. were not accurate as $Na_2S_2O_4$ shows significant absorption in this region.

Determination of iron in haems. The *o*-phenanthroline method of Drabkin (1941) was found to be satisfactory, but it was necessary to use twice the amount of ascorbic acid prescribed by him. With that modification and measuring optical densities at 510 mμ. (Beckman spectrophotometer) a straight-line relationship was found to obtain for solutions containing 0–100 μg. Fe/25 ml.

Cultivation of organisms. Two strains of *C. diphtheriae* were used: (*a*) Park Williams no. 8, substrain Toronto; (*b*) a variant of (*a*) supplied by Mr L. B. Holt of the Wright-Fleming Institute of Microbiology, London; the organisms were grown on the casein hydrolysate medium described by Holt (1948), the Fe concentration being either that found necessary for optimum toxin production (0·14μg./ml.) or 10 times that amount. In one instance a large batch (180 l.) was grown on beef papain digest medium.

In all cases the cells were harvested by centrifuging and washed 3 times with saline before further treatment.

RESULTS

Spectroscopic investigation of intact C. diphtheriae *cells grown at* Fe *level optimal for toxin production*

Aerated, thick suspensions of washed cells in phosphate buffer (pH 7·6) showed no definite absorption bands. Reduction with a small amount of $Na_2S_2O_4$ revealed bands at about *600*, 565 and *532* mμ., the first and last bands being of low intensity (figures given in italics indicate relatively weak absorption). The addition of pyridine, to give 25% (v/v) final concentration, brought about a shift of band positions to *590–580*, 557 and *527* mμ.

* Permanent address: Department of Biochemistry, University of Melbourne, Australia.

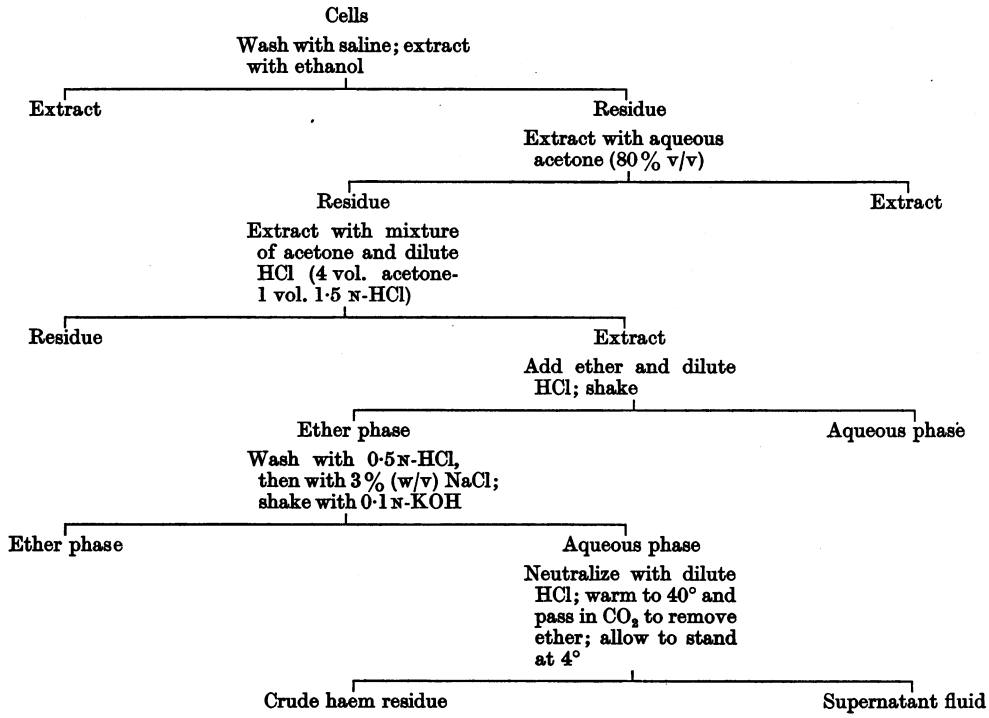


Fig. 1. Separation of intracellular haems from *C. diphtheriae*.

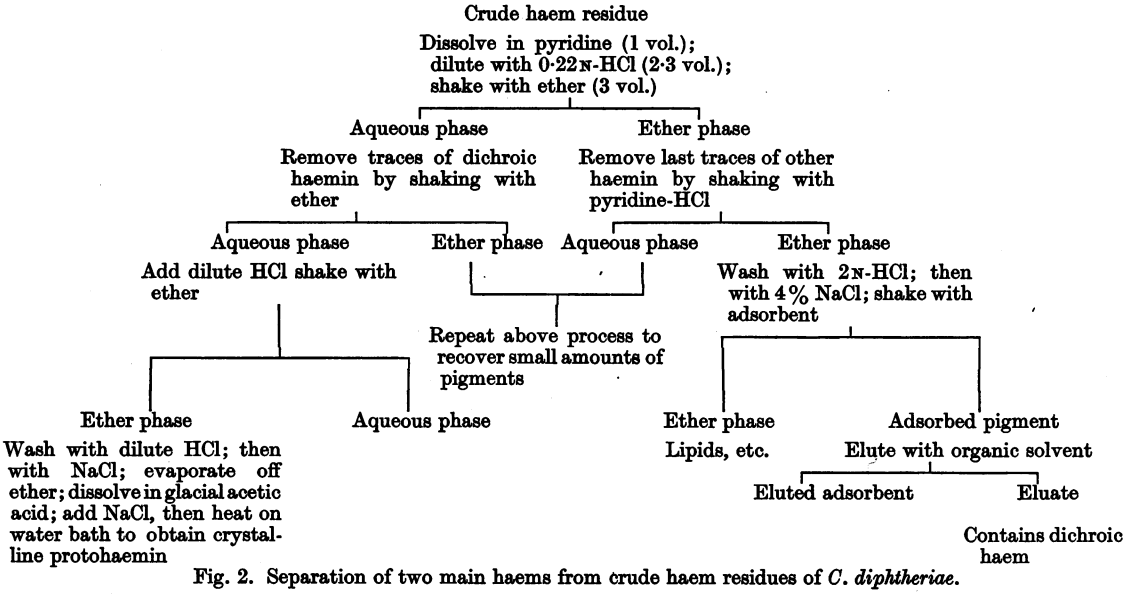
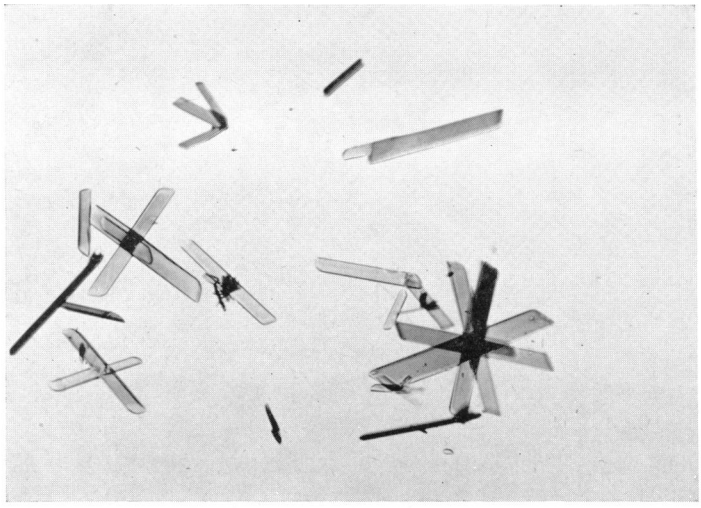


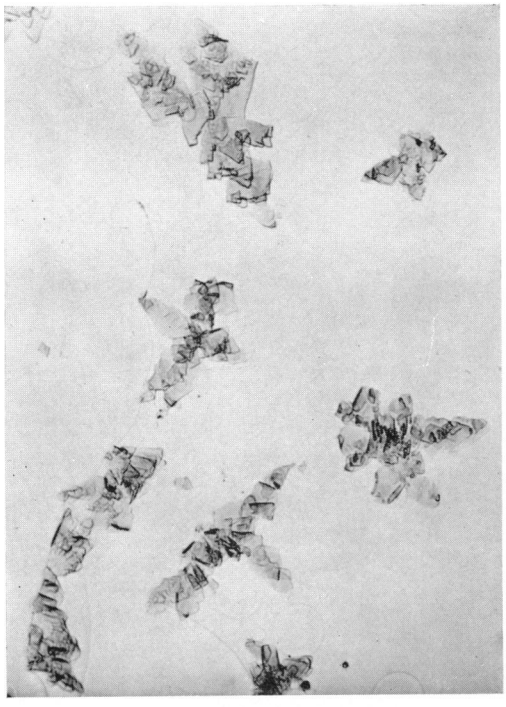
Fig. 2. Separation of two main haems from crude haem residues of *C. diphtheriae*.

Cells grown at higher iron levels inhibitory to toxin production

In respect of spectral pattern these cells were similar to those at a lower Fe level but the band intensities were at least five times as great. This enabled us to measure accurately the band positions which were (in mμ.): reduced cell suspension, 600; 568...554 (maximum at 563); reduced cell suspension, aerated (faint shading), 560...550; reduced



(*a*) Crystalline protohaemin from *C. diphtheriae* cells. × 100.



(*b*) Pyridine haemochromogen crystals from protohaemin of *C. diphtheriae* cells. × 100.

(*c*) Pyridine protohaemochromogen crystals. × 100.

cell suspension and addition of pyridine, 599...582 (maximum at 590); 564...549 (maximum at 556); 522 (diffuse).

This result indicated the presence of two principal intracellular haem components and we concluded that there was an increase in cytochromes *b* and *a* complex in cells grown at higher iron concentrations (cf. Pappenheimer, 1947*a*, *b*; Pappenheimer & Hendee, 1947).

Extraction and separation of intracellular haems from Corynebacterium diphtheriae

As attempts to extract the intact haemproteins were not successful, it was decided to extract the haem prosthetic groups by means of acid acetone. The flow sheet (Fig. 1) illustrates the process used.

Tests on the extracts confirmed the presence of haems exhibiting haemochromogen bands at 587, 556 and 522 mμ. The process outlined in Fig. 2 gave clean separation if attention was paid to the number of washings given to each phase. The removal of final traces of unwanted pigments was checked, after each washing, by haemochromogen tests on small samples.

The dichroic haem was either adsorbed on charcoal and eluted with pyridine, or it was adsorbed on alumina, in which case elution with warm glacial acetic acid was found to be more effective.

Table 1. *The absorption spectra of protohaemin from cells of* Corynebacterium diphtheriae *compared with authentic protohaemin no.* 9

(The two crystalline haemin samples were dissolved in acetone solution (0·3N with respect to HCl), transferred into ether, washed with 2N-HCl and then with water before transfer into 0·1N-KOH. The mixtures were diluted finally with 0·5 vol. of pyridine.)

	Protohaemin	
Solvent	*C. diphtheriae* (mμ.)	Authentic no. 9 (mμ.)
Acetone-HCl	642	642
	*589**	*590*
	546	547
	505	505
Ether (HCl washed)	638	638
	586	*586*
	544	544
	503	503
KOH (0·1N)	615	615
	575	576
KOH (0·1N) $+Na_2S_2O_4$	580	580
	543	544
KOH (0·1N) $+Na_2S_2O_4$ +pyridine	556	556
	524	523
	487	*486*

* Figures in italics indicate relatively weak absorption.

Characteristics of the haem passing into the aqueous phase

The pigment from the aqueous phase, obtained in the form of Teichmann crystals (Pl. 2*a*), gave with Takajama reagent crystals (Pl. 2*b*) identical with those of pyridine-protohaemochromogen (Pl. 2*c*). Table 1 records a comparison between this haemin and authentic crystalline protohaemin no. 9.

The quantitative spectral absorption curve (based on iron content, Fig. 3) further illustrates the identity of the haemin with protohaemin. The iron content of the crystalline product was 96% of that of protohaemin prepared from ox blood and after recrystallization this value increased to 97%.

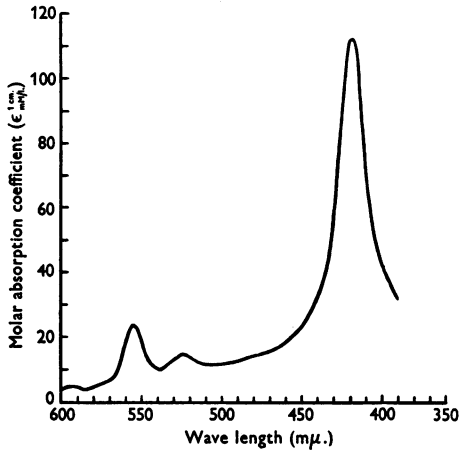


Fig. 3. Absorption spectrum of pyridine protohaemochromogen from *C. diphtheriae*.

Removal of iron. The $SnCl_2$-HCl method (Hamsik, 1931) was suitable for acetone solutions of the pigment, but for general purposes the ferrous acetate method of Warburg & Negelein (1932) was preferred. In Table 2 a comparison is made between

Table 2. *The absorption spectra of protoporphyrin derived from protohaemin of* Corynebacterium diphtheriae *cells compared with pure protoporphyrin no.* 9

	Protoporphyrin	
Solvent	*C. diphtheriae* (mμ.)	Pure no. 9 (mμ.)
Ether	632	632
	580	578
	537	537
	505	505
HCl (5 g. HCl/100 ml.)	601	600
	580	579
	556	555
$CHCl_3$ (from acidic solution)	605	604
	*585**	*584*
	559	559

* Figures in italics indicate relatively weak absorption.

the porphyrin obtained and authentic protoporphyrin no. 9.

Conversion of the haemin to mesoporphyrin. The hydriodic acid method of Fischer & Kögl (1924) was used. The mesoporphyrin was esterified with methanol saturated with hydrogen chloride, the ester purified in the usual way and crystallized four times from chloroform-methanol. A sample of protohaemin no. 9 was treated similarly. The mesoporphyrin methyl ester crystals obtained were identical in form and gave similar melting points (207° uncorr.). Spectrometric comparisons are given for the free porphyrins and methyl esters in Table 3.

Table 3. *The absorption spectra of mesoporphyrin derived from protohaemin of* Corynebacterium diphtheriae *cells compared with authentic mesoporphyrin no.* 9

	Mesoporphyrin	
	C. diphtheriae	Authentic no. 9
Solvent	(mμ.)	(mμ.)
Ether	623	623
	598	599
	578	580
	568	568
	527	527
	497	496
HCl (25 g. HCl/100 ml.)	594	593
	*573**	*574*
	550	550
$CHCl_3$† (from acidic solution)	595	595
	575	575
	551	552
	—	*527*
	496	490
$CHCl_3$† (washed with NH_3 soln., 12% diln. of conc.)	620	620
	595	*595*
	576	576
	567	567
	532	532
	500	500

* Figures in italics indicate relatively weak absorption.
† After conversion into dimethyl ester.

Characteristics of the haem from the ether phase

Solutions of this pigment were dichroic, concentrated solutions being red, while dilute solutions were yellow green. The pyridine-haemochromogen test on this haem showed a strong band at 587, a weak band at 553 and a very faint band at 525 mμ. (see Fig. 4). This indicated that the pigment contained more than one component. The 553 and 525 mμ. bands were observed to increase in intensity relative to the 587 mμ. band when solutions of this fraction were left standing. The absorption curve was therefore assumed to be the resultant of a main component with absorption maximum at 587 mμ., and a product derived from it with bands at 553, 525 and about 418 mμ. The addition of cysteine hydrochloride to a solution of the pyridine-haemochromogen mixture caused the slow disappearance of the 587 mμ. band and a simultaneous appearance of extra bands close to the 553 and 525 mμ. bands.

Removal of iron. The method of Warburg & Negelein (1932) was used to remove the iron from this fraction and an inspection with the reversion spectrometer clearly revealed the presence of two sets of porphyrin bands. These were (pyridine solution): I (*a*) 645, (*b*) 627; II, 593...581; III (*a*) 559, (*b*) 541; IV, 505 mμ. Order of intensity: III (*a*), II, IV, I (*a*), I (*b*), III (*b*). A partial separation of the two components was achieved by extraction from ether solutions by means of hydrochloric acid solutions of different concentration. Neither porphyrin was readily extractable by solvents less acidic than 3N-HCl.

Comparison of the a component from Corynebacterium diphtheriae *cells with that from heart muscle*

At this stage it was decided to reconsider the value of further investigations on the pigments in this ether fraction. Although partial separation of the two porphyrins was possible by differential acid extraction, the extra manipulations involved and the difficulties experienced in eliminating reactive substances carried over from the original extracts led us to attack the problem in a different manner. The justification for this decision will be discussed below, but for the sake of continuity a brief comment is needed. The pigment responsible for the 587 and 430 mμ. pyridine haemochromogen bands was assumed to be derived from the cytochrome *a* complex. It was therefore considered worth while to investigate another tissue (ox-heart muscle) known to be rich in this component.

Modifications of extraction and separation processes used in isolating the dichroic haem a from heart muscle. Ox hearts were minced after freeing them from fatty and ligamentous tissue and then given either a preliminary dehydration with ethanol or extracted directly with acetone. Either anhydrous or 80% (v/v) aqueous acetone was equally satisfactory. A mechanical press was used to remove fluid, and extractions were continued until yellow pigments were no longer apparent (normally three extractions). The intracellular haems were extracted as before, care being taken to compensate for acid bound to tissue material during the first extraction. We have used successfully for first extractions mixtures with final concentrations 0·3N with respect to HCl and ranging from 80 to 98% aqueous acetone. With subsequent extractions less HCl was required to maintain acidic conditions in the extraction fluid. The greater part of the haem pigments was extracted rapidly, prolonged treatment being necessary to recover further significant amounts. Extractions were made at 4° to minimize the risks of decomposition.

From extracts containing little water and HCl, the pigments were precipitated by addition of saturated sodium acetate solution. The crude haem residue obtained by washing the precipitate with acetone and then dilute acetic

acid was now ready for solution in pyridine and isolation. This technique was most useful in removing lipid matter. If the extracts would not yield precipitated haem, by such direct treatment, the pigments were transferred into ether by the process outlined in Fig. 2, transfer into dilute alkali being found unnecessary.

The aqueous pyridine phase contained at least one other haem compound in addition to protohaem, as haemochromogen tests showed two additional bands at wave lengths slightly lower than the α and β bands of protohaemochromogen. The dichroic haem was adsorbed by passing the washed ether phase through a column of alumina after which the alumina was washed well with fresh ether. By stirring the dry residue with warm glacial acetic acid (50–60°) the haem was eluted to give a dark brown solution. In this condition the pigment remained stable even after long standing at room temperature.

Characteristics of haem a *isolated from ox-heart muscle.* The results of spectroscopic investigations of this haem are summarized in Table 4.

Pyridine haemochromogen a. The quantitative absorption curve illustrated in Fig. 4 was obtained by dissolving a sample of the dichroic haem, containing 34·9 μg. Fe in 6·25 ml. pyridine (A.R.) and 12·5 ml. 0·1N-NaOH and adding $Na_2S_2O_4$. This solution was diluted to 25 ml. with distilled water and measurements were made with a Beckman spectrophotometer. For wave lengths below 474 mμ., 2·0 ml. stock solution was diluted to 7·0 ml. with control solution (25% pyridine, 0·05N-NaOH) and in this way maximum density values above 0·7 were avoided. This solution shows only a single band at 587, but when left standing for several hours in the presence of cysteine, was found to undergo change in the same way as the haem obtained from *C. diphtheriae* cells did. The 587 mμ. band faded and was replaced by two bands at 553 and 525 mμ.

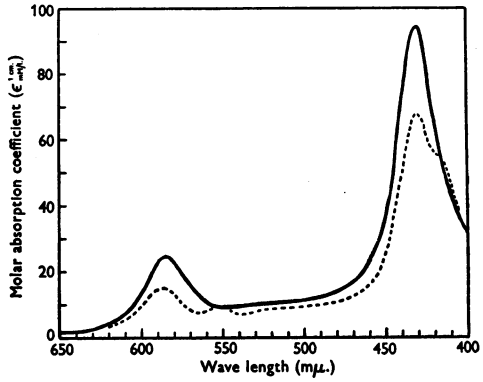


Fig. 4. Absorption spectra of pyridine haemochromogens. Heart haemochromogen *a* ——; *C. diphtheriae* haemochromogen *a* - - - -. Solvent, 3·11 M-pyridine in 0·05 N-NaOH.

Removal of iron. The method of Warburg & Negelein (1932) was used for the preparation of the porphyrin. In Table 5 are presented the absorption maxima and orders of intensities of the pigment in various solvents. Neutral organic solutions were coloured reddish when the porphyrin concentration was high and yellowish green in dilute solution. The porphyrin was insoluble in light petroleum (b.p. 40–60°).

From the data presented it was concluded that haem *a* isolated from ox-heart muscle was identical with that from *C. diphtheriae* cells, but, fortunately,

Table 4. *Absorption spectra of haem* a *derivatives*

Compound	Solution	Absorption bands I (mμ.)	II (mμ.)	Soret (mμ.)	Order of intensity
Haem *a*	KOH (0·17N)	635	577	—	I, II
Haem *a*	Alkali + $Na_2S_2O_4$	578	531	—	I, II
CO-haem *a*	Alkali + $Na_2S_2O_4$ + CO	591	542	—	I, II
Pyridine haemochromogen *a*	Alkali + pyridine + $Na_2S_2O_4$	587	—	430	—

Table 5. *Absorption bands of ox heart dichroic porphyrin* a*

Solvent	I (mμ.)	II (mμ.)	III (mμ.)	IV (mμ.)	E.A.† (mμ.)	Order of intensity
$CHCl_3$	648	590	561	520	450	III, II, IV, I
Benzene	647	591	561	519	451	III II, IV, I
Glacial acetic acid	641	591	560	520	454	III, II, IV, I
Ether	642	587	556	515	439	III, II, IV, I
Pyridine	648	589	559	527	444	III, II, IV, I
Pyridine-ether	647	589	558	517	455	III, II, IV, I
HCl (10 g./100 ml.)	618	563				II, I
KOH (0·1N)	582	565	519		447	II, III, I,

* From measurements with the Beck-Hartridge spectrometer.
† End absorption.

the former contained none of the secondary product seen in the material isolated from the micro-organisms.

Experiments to confirm the suitability of isolation procedures

In order to test the possibility that the dichroic pigment might have been an artifact derived from protohaem, similar processes of extraction and separation were performed on aqueous acetone solutions (acid, neutral and alkaline) of pure protohaemin. After these solutions had been allowed to stand for 2 days at room temperature, with frequent shaking in air, no haem component was detected with characteristics similar to the above dichroic pigment. Whole ox blood, when subjected to the processes, did not yield any pigment with preference for the ether phase.

Further experiments with haem a

The more readily obtainable product from ox-heart muscle was used in these investigations.

Effect of carbon monoxide on haem a. On bubbling CO through an alkaline solution of haem *a* reduced with $Na_2S_2O_4$, there was an immediate shift of the absorption spectrum to give bands at 591 and 541·5 mμ.

Effect of hydroxylamine on haem a. A solution of Na_2CO_3 was added to a pyridine solution of the haem to give a 20% (v/v) final concentration of pyridine. The addition of a few crystals of hydroxylamine hydrochloride followed a few minutes later by the addition of $Na_2S_2O_4$ resulted in the appearance of a haemochromogen spectrum with maxima at 570 and 533 mμ. (cf. haemochromogen spectrum of original haem 587 mμ.).

Further experiments on the porphyrin obtained from haem a

Effect of cysteine on porphyrin a. The addition of cysteine hydrochloride to a warm pyridine solution of the porphyrin, containing anhydrous Na_2CO_3, resulted in a shift of band positions to I, 634; II, 578; III, 546; IV, 508 mμ. (order of intensity: III, IV, II, I).

Effect of hydroxylamine on porphyrin a. The porphyrin was dissolved in pyridine and anhydrous Na_2CO_3 added. The addition of a few mg. of hydroxylamine hydrochloride was followed by an immediate shift of band positions, even at room temperature, and after this change no further shift was observed by heating at 100° for 1 hr. The mixture was diluted with water and shaken with ether. The pigmented ether phase was washed well with 1% (w/v) HCl and finally with water to remove traces of acid. The spectrum in washed ether was I, 639; II, 582; III, 547; IV, 509 mμ. End absorption (E.A.): 444 mμ. (order of intensity: III, IV, II, I). The addition of an equal volume of pyridine gave the following bands: I, 637; II, 582; III, 549; IV, 509 mμ.

In Fig. 5 this oxime reaction at 15° is demonstrated by measurements made in the Beckman spectrophotometer. The solutions used to obtain the curves were identical except for the addition to one of a small amount of hydroxylamine hydrochloride.

Copper complexes of porphyrin a. The copper complex was formed by adding a small amount of cupric acetate to a solution of the free porphyrin in glacial acetic acid and heating in a boiling water bath for 5 min. The absence of red fluorescence under ultraviolet radiation indicated completion of the reaction. In Table 6 are shown the positions of absorption band maxima in various solvents.

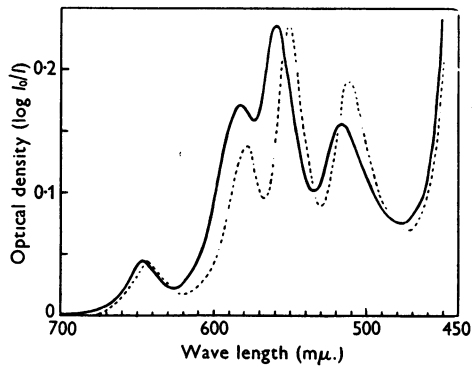


Fig. 5. Absorption spectra of heart porphyrin *a* derivatives. Heart porphyrin *a* ——, heart porphyrin *a* oxime - - - -. Solvent pyridine.

Table 6. *Absorption bands of copper complex of heart dichroic porphyrin* a

Solvent	I (mμ.)	II (mμ.)	III (mμ.)	Order of intensity
Glacial acetic acid	597	552	—	I, II
Pyridine	599	553	—	I, II
Ether	594	548	525	I, II, III
Pyridine-ether	597	552	530	I, II, III

The addition of hydroxylamine hydrochloride to a pyridine solution of the copper complex containing anhydrous Na_2CO_3 brought about a shift of band positions within a few minutes at room temperature, to I, 587; II, 542; III, *504* mμ. E.A.: 442 mμ. (order of intensity: I, II, III).

Esterification of porphyrin a. The porphyrin *a* was allowed to stand 2 days at 4° in methanol saturated with HCl; absorption bands I, 605; II, 563 mμ. (order of intensity: II, I). The pigment was taken into $CHCl_3$ after dilution with water. The $CHCl_3$ solution was extracted 4 times with 2 N-NH_4OH, 3 times with 10% (w/v) NaCl and then run through a dry filter paper. After evaporation to dryness and solution of the residue in pyridine-ether solution, bands were observed at 648, *630*, 589, 560, 510 mμ. The presence of the band at 630 mμ. and the general appearance of the band pattern indicated that a small amount of the pigment had undergone change. After extraction of this solution with 10% (w/v) HCl to remove pyridine and traces of modified porphyrin the absorption bands were measured again after washing with water and dilution with an equal volume of pyridine (646, 589, 558, 516 mμ.) cf. Table 4. The treatment of porphyrin *a* (dissolved in ether) with diazomethane did not appear to produce any modified pigment and so was considered preferable to the method described above for esterification.

DISCUSSION

The spectrometric tests made on intact cell suspensions of *C. diphtheriae* demonstrate the effect of iron concentration upon cytochrome content. The faint

absorption bands seen in washed cells grown at the lower iron levels agreed in wave length with the three main bands of cells grown at higher levels. It is concluded that, although the main types of haem constituents bound within the cells are independent of iron concentration, the quantity produced is decreased by low concentrations of iron and such inhibition is associated with an increased excretion of coproporphyrin III. A five- to ten-fold increase of intracellular haem components seems to take place when cells are grown at iron levels sufficient to reduce toxin and coproporphyrin production in the medium from optimal to insignificant amounts.

The absorption patterns and mean positions of wave lengths of maxima seen in reduced cell suspensions demonstrated the presence of cytochrome components. The intense band at 562 mμ. and the band of lower intensity at 531 mμ. resemble the α and β bands of cytochrome *b* except that they are shifted to values slightly lower than the *b* bands of some other tissues. Other micro-organisms show similarly low values. Small differences in band positions measured by direct vision spectroscopy, may not always be significant, and confirmatory specific functional tests are needed before they are attributed to new components.

The absence of cytochrome *c* was apparent by examination of the 562 mμ. band. The general appearance of this band with its edges at 568 and 554 mμ. makes it difficult to assume any contribution from the 550 mμ. α-band of cytochrome *c*. This was further confirmed by comparison of *C. diphtheriae* cells with other micro-organisms known to possess cytochrome *c*. The absorption band at 600 mμ. indicates the presence of the cytochrome *a* complex. Experiments similar to those used in general cytochrome studies allowed us to confirm the presence of cytochrome oxidase (Pappenheimer, 1947*b*).

The shift of absorption bands to shorter wave lengths on the addition of pyridine to reduced cell suspensions results from a displacement, by this base, of the normal protein moieties of the cytochrome. The 587 mμ. band represents the pyridine haemochromogen complex of the *a* component and the bands at 557 and 526 mμ. that of the prosthetic group of cytochrome *b*. The positions of the latter bands coincide with the α and β bands of pyridine protohaemochromogen. Pyridine coprohaemochromogen exhibits bands at 548 and 518 mμ., and as these cannot be demonstrated in 'high iron' cells the iron complex of coproporphyrin III must be absent. It was concluded from the above tests that at least two cytochrome components were present (viz. *a* and *b*) with dissimilar prosthetic groups. The identification of the major component as protohaem no. 9 is confirmed by all tests on the crystalline product isolated from the aqueous phase. Quantitative measurements on acid-acetone extracts, after conversion of the pigment into pyridine haemochromogen or protoporphyrin, gave values equivalent to 1·0–1·1 mg. protohaem/l. of culture. As the 587 mμ. pyridine haemochromogen band of component *a* (see p. 254) is known to possess a similar extinction value to protohaemochromogen, its low intensity compared with that of the 557 mμ. absorption band of the latter component in intact cells must mean that it represents a small fraction of the total intracellular haems.

The dichroic pigment in the ether phase (from *C. diphtheriae* cells) was composed of two haems. The following facts point to derivation from one original component: both haems showed preference for the ether phase, the porphyrins derived from them both possessed high acid numbers, and on standing in solution the component with haemochromogen bands at 553 and 525 mμ. increased at the expense of the predominant 587 mμ. component. The reactions with hydroxylamine and cysteine also supported this conclusion.

As the interpretations of data were complicated by the presence of a product derived from the original haem it was decided that further studies to ascertain the constitution of the pigment might be facilitated by preparation of material from a more convenient source than *C. diphtheriae* cells. The haem *a* from ox-heart muscle possessed spectroscopic characteristics similar to those of the haem from *C. diphtheriae* cells and (presumably due to smaller amounts of reactive impurities) was far more stable during its separation, and was therefore the material of choice. Lipid material accompanied the haem so closely during extraction that the possibility that some of it is an integral constituent cannot be excluded (cf. chlorophylls *a* and *b*).

In experiments with heart muscle our primary aim was to study the dichroic haem *a*. One pigment with haemochromogen α and β bands at wave lengths slightly shorter than protohaemochromogen was found in small amounts in the aqueous acid-acetone phase after extraction with ether. Further work is in progress to establish the nature and significance of this pigment. Although cytochrome *c* is not dissociated by treatment with acetone and hydrochloric acid it is not impossible that some pigment is dissolved. The great affinity of the *c* component for aqueous phases could then explain the presence of a haem derivative in the acid-acetone layer.

Although it had been concluded that the pigment responsible for the 587 and 430 mμ. absorption bands, in pyridine extracts of *C. diphtheriae* cells and ox-heart muscle, was the prosthetic group of the cytochrome *a* component it was necessary to prove that this haem had not arisen as a degradation product of protohaem. Attempts to produce degradation products of protohaem under the conditions applicable during extraction procedures failed (cf. Negelein,

1932*a*, *b*; Negelein, 1933; Roche & Benevent, 1936). The claim was made in the paper last mentioned that another derivative, obtained from heart muscle by a different process and possessing pyridine haemochromogen bands at 587, approx. 554, 530 and 425 mμ., was, in fact, the true haem *a* from cytochrome *a*. An inspection of the absorption curve of this pigment, however, leads us to suggest that the product of Roche & Benevent (1936) was a mixture composed of one component with a band at 587 mμ. and another component with bands at approx. 554 and 530 mμ., similar to the haemochromogen of the pigment found to accompany haem *a* in preparations from *C. diphtheriae* cells. For reasons stated earlier we consider this material (maxima 553, 525 mμ.) to have been derived from the haem *a* by combination with substances accompanying the latter during extraction (compare the action of cysteine on haem *a*). The stability of our haem *a* to acid offers another distinct contrast with the product described by these workers. The 'kryptoporphyrin' obtained by the transformation of protoporphyrin (Negelein, 1932*b*) possessed a lower acid number than the dichroic pigment isolated by us from *C. diphtheriae* cells and heart muscle.

The α-band position of the carbon monoxide-haem *a* complex (591 mμ.) is reminiscent of the position of one band of the light-sensitive carbon monoxide compound of Warburg's respiratory enzyme (cytochrome oxidase of Keilin). At the present time it is considered that cytochrome *a* and cytochrome oxidase are related at least in the nature of their prosthetic groups.

The quantitative absorption curve of pyridine haemochromogen *a* (Fig. 4) shows that it differs considerably from the normal type of haemochromogen. In general, α and β bands are observed in the visible region of the spectrum, and even in the case of chlorocruorin haemochromogen we find, in addition to an α band at 583 mμ., a second, weaker band at 542 mμ. The extinction coefficients of the band maxima at 587 and 430 mμ. of haemochromogen *a* approach those of the majority of haemochromogens, but the greater width of the former band makes it difficult to appreciate in direct vision spectroscopy that such is the fact.

The effect of cysteine on the pyridine haemochromogen *a* is similar to that reported for many haem derivatives (Fischer & Mittermair, 1941). Fischer & Mittermair concluded from experiments with derivatives of chlorophyll *b* that the shift was due to the presence of a reactive aldehyde group which with cysteine gave compounds of the mercaptal acetal type. The experiments we have reported with hydroxylamine and cysteine on both haem *a* and its porphyrin therefore indicate the presence of a carbonyl group in our material. There is the possibility that the carbonyl groups are ketonic, but the rapidity of the addition reactions suggests that labile aldehyde groups are involved.

The reaction of haem *a* with hydroxylamine results in the formation of an oxime, the pyridine haemochromogen bands of which are situated at 570 and 533 mμ. In the case of chlorocruorin haemochromogen (1:3:5:8-tetramethyl-2-formyl-4-vinylporphin-6:7-dipropionic acid iron complex), oxime formation shifts its 583 mμ. band to 557 mμ., i.e. identical with the haemochromogen of protoporphyrin no. 9, and, on this fact, it is assumed that the $-CH=NOH$ group exerts an effect similar to that of a $-CH=CH_2$ group (compare the haemochromogens of mesoporphyrin and protoporphyrin). The shift of band position shown by haemochromogen *a* on conversion to oxime is 17 mμ., a value which is similar to the shift (16·5 mμ.) shown by formylpyrroporphyrin no. 15, but much lower than in the case of chlorocruorin haemochromogen (26 mμ). This comparison indicates that haemin *a* must possess structural groups (other than such active carbonyl groups) either different in type or in their arrangement around the nucleus from those found in protohaemin or chlorocruorohaemin.

The method used for preparing porphyrin *a* appeared to involve no significant modification of substituent groups, as the re-introduction of iron produced the same type of haemochromogen spectrum as that of the original substance. We could not find any evidence that the reaction with hydroxylamine proceeds in two stages, and so it must be assumed that in porphyrin *a* there is only one labile aldehyde group/mol. of pigment. The curves of Fig. 5 illustrate the differences between the oxime and original porphyrin *a*. All bands are shifted towards the blue, bands I and III remain unchanged in extinction values, but bands II and IV reverse their relative intensities. The oxime shows the band relationships (III, IV, II, I) characteristic of the 'rhodo-type' of porphyrin.

SUMMARY

1. The cells of *Corynebacterium diphtheriae*, when grown at iron levels sufficient to inhibit the excretion of toxin and coproporphyrin, showed a five- to ten-fold increase of haem components over the level present when toxin and porphyrin excretion are maximal. The presence of cytochrome oxidase (cytochrome *a* complex) and cytochrome *b* were confirmed spectroscopically.

2. Methods for the separation of the two main haem prosthetic groups are described. Crystalline protohaemin, which is assumed to be derived at least in part from cytochrome *b*, and a dichroic haem, presumably from the cytochrome *a* complex, were obtained.

3. The dichroic haem was identified with the

haem of cytochrome *a* of ox-heart muscle and further studies on its characteristics were made on material from this more accessible source.

4. The general properties of this haem and its reactions with pyridine, cysteine, carbon monoxide and hydroxylamine were investigated.

5. The free porphyrin, like the haem from cytochrome *a*, reacted with cysteine and hydroxylamine.

6. The results of the tests described indicate that this haem possesses at least one aldehyde group.

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The Oxidation of Manganese by Plant Extracts in the Presence of Hydrogen Peroxide

By R. H. KENTEN and P. J. G. MANN
Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts

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Manganese is known to be an essential micronutrient of plants, although its function is unknown. It apparently plays a part in plant respiration; Lundegårdh (1939) found that the oxygen uptake of manganese-deficient wheat roots was raised by 155–470 % by the addition of 5×10^{-5} M manganese chloride. Such an effect might be brought about by the activation of certain enzyme systems, e.g. arginase (Waldschmidt-Leitz & Purr, 1931), phosphoglucomutase (Cori, Colowick & Cori, 1938), leucylpeptidase (Berger & Johnson, 1939) or by a system in which Mn^{++} undergoes alternate oxidation and reduction. It has been shown that soil micro-organisms can oxidize Mn^{++} (e.g. Beijerinck, 1913; Gerretsen, 1937; Leeper & Swaby, 1940; Mann & Quastel, 1946). No satisfactory evidence has been put forward to show that Mn^{++} is oxidized in higher plants and the present work was undertaken to investigate whether such oxidation does take place.

MATERIALS AND METHODS

Preparation of plant extracts. The roots were scrubbed free from soil and minced twice in a meat mincer. The mince was weighed and the juice squeezed out by hand through madapollam. Water to one-quarter of the original weight of the mince was added to the residue, which was then ground in a mortar with sand and squeezed through madapollam. This treatment was repeated twice. The extracts were combined and filtered through Whatman no. 1 filter paper, and stored in a refrigerator. Most of the work was done with horse-radish (*Cochlearia armoracia*); it was found that extracts of this root showed little loss of activity over a period of several weeks. In view of the possibility of contamination, however, fresh extracts were frequently made. Extracts of other roots were made and used on the same day. This method was originally adopted with horse-radish as benzidine-H_2O_2 tests showed satisfactory extraction of peroxidase.

Catalase preparation. The caps of the Basidiomycete, *Marasmius oreades*, were ground with sand, squeezed through madapollam and centrifuged at 3500 rev./min.