considered; preparations of kidney tissue rapidly form 5-hydroxytryptamine from added 5-hydroxytryptophan (Clark *et al.* 1954).

SUMMARY

1. The occurrence of indole bases in human urine has been studied by chromatography of urine on Zeo-Karb 226, elution of absorbed bases with acid ethanol and concentration to 0·002 of the volume of urine used. Indoles were investigated by paper chromatography and tests on guinea-pig ileum.

2. Urine extracts contained two indoles. Evidence strongly supporting the identity of one of these with 5-hydroxytryptamine and the other with tryptamine was obtained.

3. *N*-Methyl-5-hydroxytryptamine or bufotenin was not detected in the extracts.

4. By estimation of spot intensities on paper chromatograms recovery of 5-hydroxytryptamine, *N*-methyl-5-hydroxytryptamine, bufotenin and tryptamine added to urine was of the order of 70 %.

5. The excretion of 'urinary 5-hydroxytryptamine' in twelve normal adults ranged from 45 to 120 μg./24 hr.; similar values were found for 'urinary tryptamine' output (six subjects).

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The Prosthetic Group of Cytochrome a_2

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Cells of *Escherichia coli* and *Shigella dysenteria* which had been grown under aerobic conditions were shown by Yaoi & Tamiya (1928) to possess an absorption band which differed from those of previously described cytochromes in that it lay well within the red region of the spectrum. Keilin (1933) attributed this band to a component of the cytochrome system in these bacteria. Negelein & Gerischer (1934) and Fujita & Kodama (1934) independently published spectroscopic evidence that cytochrome a_2, as it had been designated, was autoxidizable and could combine with carbon monoxide and cyanide. Unlike other cytochromes that had been observed the oxidized form showed a band in the visible region of the spectrum, at 645 mμ. Fujita & Kodama (1934) also showed that this cytochrome was widely distributed amongst other bacteria, e.g. *Azotobacter chroococcum*, *Proteus vulgaris*, *Acetobacter pasteurianum*, *Eberthella typhosa* and *Salmonella paratyphi*. The spectroscopic evidence of the properties of cytochrome a_2 led to the assumption that in these organisms it had the function of a cytochrome oxidase. This view remained current until the recent work of Tissières (1952), Moss (1952) and Chance (1953) threw doubt on it.

No attempt seems to have been made to establish the nature of the prosthetic group of cytochrome a_2; though Negelein & Gerischer (1934) suggested that it might be related to the ferrophaeophorbides, and

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Lemberg & Wyndham (1937) considered that there were spectroscopic resemblances to biliviolin–iron complexes. From a study of the difference spectrum of the CO-compound of cytochrome a_2, Chance concluded that the spectrum of its prosthetic group would lack a distinctive Soret band, and considered this as evidence in favour of the assumption of Lemberg & Wyndham.

The probable importance of cytochrome a_2 as an electron carrier and especially its possible role as a terminal oxidase suggested the present attempt to isolate and characterize its prosthetic group.

Aerobacter aerogenes was used as the source of cytochrome a_2, but other organisms have also been examined. Both the haemin and the iron-free pigment, a new chlorin, have been partially purified, the latter to a much greater degree. Comparison of spectra, a study of the side chains and conversion into a porphyrin have led to the conclusion that chlorin a_2 is a dihydroporphyrin derived from either protoporphyrin or a similar porphyrin. A preliminary note covering an early part of this work has been published (Barrett & Lemberg, 1954).

METHODS

Bacteria. A strain of *Aerobacter aerogenes* supplied by Dr F. Moss of Sydney University was used at first, but was later replaced by a more vigorously growing strain isolated from soil in the grounds of this Institute. Both strains constantly gave rise to a mucoid variant which gave extremely viscid cultures on aeration. Consequently the organism was frequently plated out and cultures were made from dew-drop-type colonies. *Aero. aerogenes* was grown in a medium of the following composition: $(NH_4)_2HPO_4$, 64 g.; KH_2PO_4, 16 g.; NaCl, 10 g.; $MgSO_4,7H_2O$, 4 g.; sodium citrate, 18 g.; $FeSO_4,7H_2O$, 0·2 g.; peptone, 32 g.; sucrose, 200 g.; tap water to 15 l.; pH 6·8. Gas mixture ($O_2 + CO_2$, 95:5) was bubbled through a sintered-glass disk (1 in., grade 4), and provided optimum aeration and gentle stirring of the medium without excessive frothing. Cultures were incubated at 37° for 18 hr. in the early part of this work, but were later left for 72 hr. to give higher yields of cytochrome a_2 per unit weight of cell material. A cell mass equivalent to 15–20 g. in dry weight was normally obtained from 15 l. of culture.

Escherichia coli and *Proteus vulgaris*, *Bacillus subtilis*, *B. mycoides* and *Pseudomonas aeruginosa* were grown at 37° in a similar medium, glucose replacing sucrose and the peptone concentration being increased to 1%. Aeration was carried out as described above. Cells were harvested after 18 hr. *Azotobacter vinelandii* was grown at 25° in 10 l. amounts, in 16 l. bottles, in the medium of Burk & Lineweaver (1930). Vigorous aeration of the culture was maintained with compressed air. Cells were harvested at 72 hr. *Torulopsis utilis* was grown in a sucrose–mineral salt medium in 10 l. amounts. Cultures were vigorously aerated with $O_2 + CO_2$, and cells were harvested after incubation for 72 hr. at 25°.

Solvents. Peroxide-free ether was used. Acetone, A.R., was used as supplied for extraction of the cells, but for other steps in the preparative work it was stood over CaO overnight, filtered and distilled. All other organic solvents used, except kerosene, were distilled once if supplied as A.R. but twice if of lower purity.

Silica-gel columns. Silica gel (British Drug Houses Ltd., 100 mesh) was washed twice with 95% ethanol, twice with water, then dried at 22°. The silica gel (9 g.) was taken into a round-bottom flask and the air was replaced by nitrogen, the flask being continuously shaken. A methanol–water mixture (70:30, 4 ml.) was added and the contents of the flask were shaken under nitrogen. This procedure leaves the particles of silica gel still dispersed, which is essential for smoothly working columns. To the silica gel was added 60 ml. of light petroleum (b.p. 68°). A column 1·5 cm. × 20 cm. was used for the haemins from 20 g. dry weight of cells.

Hydrochloric acid concentrations. Because the term HCl number is widely used throughout the literature of porphyrin chemistry, the form % (w/v) of HCl is used instead of normality.

Paper chromatography. For analytical purposes the method of Chu, Green & Chu (1951) with slight modifications was used. The methyl esters of the porphyrins and chlorins were run on separate sheets in kerosene–chloroform (4·0:2·6) and kerosene–propanol (6:1). Trichloroethylene alone was also used. A 10 cm. migration of the solvent front was used throughout.

For preparative purposes sheets of Whatman no. 1 paper of 19–50 cm. width were used. The paper was washed in 95% ethanol, followed by 95% acetone, and dried in air. After development in one of the kerosene systems and marking of the bands that fluoresced under u.v. light, the paper was cut into horizontal strips and these were washed in light petroleum (b.p. below 40°), followed by drying *in vacuo* at 25°. The chlorin or porphyrin was then concentrated by placing one end of the strip in a few ml. of acetone in a test tube. The sharp band of pigment which developed at the top of the strip was cut off and the pigment eluted with acetone. When trichloroethylene was used washing with light petroleum was not necessary.

Spectrophotometry. Absorption spectra were measured with a Hilger Uvispek spectrophotometer. The wavelength scale was set with reference to the hydrogen lines at 656·3 and 486·1 mμ. with the hydrogen-lamp source as supplied with the instrument.

EXPERIMENTAL AND RESULTS

Preparation of haemin a_2

Nomenclature. Throughout this paper the nomenclature of Lemberg & Legge (1949) has been used.

Extraction of haemins from cells of Aero. aerogenes. The cells were dispersed in a Waring Blendor with sufficient water, acetone and HCl to give, for each 10 g. dry weight, 200 ml. of suspension containing 60 ml. of water, 140 ml. of acetone and 0·7 g. of HCl. Extraction was complete in 10–20 sec. Lower concentrations of acetone gave incomplete extraction of the haemins. The use of methanol instead of acetone, or prior extraction of the cells with neutral acetone or methanol, gave rise to low yields of haemin a_2.

The cell residue was separated from the acid–acetone solution by centrifuging. An equal volume of ether was added to the supernatant immediately on separation, and the ethereal solution of haemins obtained washed with 1% HCl until free from acetone. The ether solution contained much fat, and great care was necessary to avoid the

formation of emulsions. At the completion of this stage the combined haemin solutions were stood at −16°.

Spectroscopic observation of the brown ethereal solution of haemins showed a diffuse but strong band at 603 mμ., in addition to the bands of protohaemin at 635, 540 and 508 mμ. The intensity of this band relative to the 635 mμ. band of protohaemin was found to be of the same order as the intensity of the 630 mμ. band of reduced cytochrome a_2 relative to the 560 mμ. band of reduced cytochrome b_1. Fig. 1 shows the absorption spectrum of the haemins from cells with a cytochrome a_2 band of medium strength compared with the absorption spectrum of protohaemin. Ether solutions to be measured were washed with 5% HCl to ensure complete formation of the haemin. Extinctions at 635 and 603 mμ. were measured to follow the separation of haemin a_2 from protohaemin. The average batch of cells gave haemin extracts with a value of 1·2 for the ratio $E_{603\ m\mu.}/E_{635\ m\mu.}$. Exceptionally a ratio of 1·3 has been obtained. Cells with weak cytochrome a_2 band gave a ratio of 1·0.

Precipitation of phospholipids. The ether solution of haemins from 100 g. dry weight of cells contains approx. 14 g. of lipids, mainly phospholipids, the greater part of which was removed by acetone precipitation. The ether solution was first chilled at −16°, filtered to remove ice crystals, and the volume reduced to one-fiftieth by distillation *in vacuo*, nitrogen being passed through the capillary. Acetone was added in small amounts, the precipitated phospholipid being centrifuged off after each addition. When no more precipitation occurred the volume was reduced *in vacuo*, and any precipitate removed. The solution was cooled to −16°. To avoid adsorption of the haemins on the precipitating lipids the temperature was lowered by stages, the phospholipid being filtered off as it precipitated. Finally, all solvent was removed *in vacuo*, thus ensuring removal of residual water, and the oily residue was dissolved in dry acetone and stood at −16°.

Chromatography on silica gel. The acetone was removed *in vacuo* from the solution of haemins and the oily residue (approx. 1 g.) taken into a small volume of benzene. The protohaemin that precipitated was removed by centrifuging. By chilling at 5° overnight a second, smaller precipitate could be obtained. The haemins in benzene were applied to a silica-gel column (see Methods). Light petroleum (b.p. 68°) quickly eluted a yellow zone of lipids (fatty acids, phospholipid and a deep-yellow neutral lipid). This solvent was followed by light petroleum (b.p. 68°) which had been equilibrated with an equal volume of a mixture of methanol and water (70:30), then by wet benzene to which was added gradually increasing amounts of methanol. At a concentration of 0·5–1·0% of methanol, separation of a green zone of haemin began to develop at the head of the column, and the haemin was eluted. The methanol content of the benzene was further increased until all the haemin material had been removed from the column. All solvents were run through the column under nitrogen.

The green fraction coming off the columns showed a weak band at 603 mμ. and a strong band at 660 mμ. Washing of the fraction with 1% HCl caused a disappearance of the 660 mμ. band and a considerable increase in the intensity of the 603 mμ. band. The early benzene fractions accounted for 60% of the total absorption at 603 mμ. of the material applied to the column. The ratio $E_{603\ m\mu.}/E_{635\ m\mu.}$ was 2·88. The later benzene fractions of less-pure haemin a_2 were combined and put through a similar column, yielding haemin a_2 of the same $E_{603\ m\mu.}/E_{635\ m\mu.}$ ratio.

Preliminary work has shown that the crude haemins in light petroleum, without prior precipitation of phospholipids, may be applied to these improved silica columns, and developed in the same manner, except that wet ether is used in place of benzene. Haemin a_2, with a value of 3·18 for $E_{603\ m\mu.}/E_{635\ m\mu.}$, has been obtained readily in this way. Fig. 2 shows a spectral curve for the haemin in ether which had been washed with 5% HCl.

Countercurrent distribution and paper chromatography of haemins a_2. The green haemin obtained from the improved column was still oily on removal of solvent. Therefore,

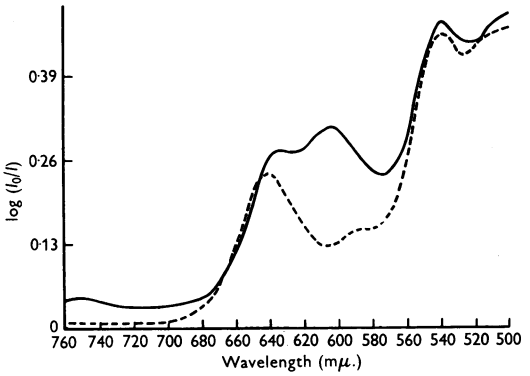

Fig. 1. Absorption spectrum of bacterial haemins, $E_{603\ m\mu.}/E_{635\ m\mu.} = 1{\cdot}13$, (—) and protohaemin (- - -). Solutions in ether washed with 5% (w/v) HCl.

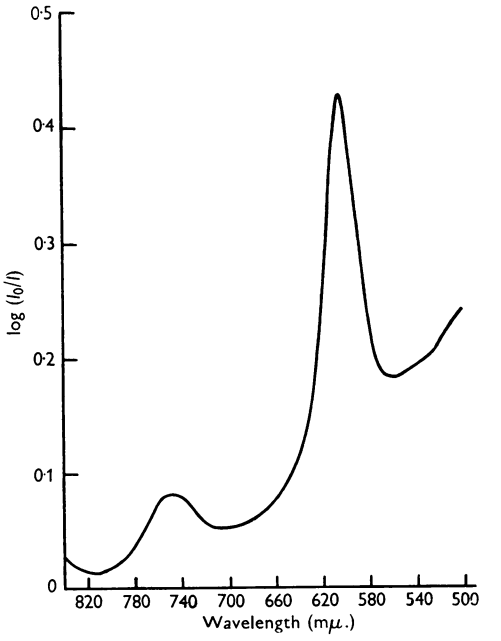

Fig. 2. Absorption spectrum of haemin a_2, $E_{603\ m\mu.}/E_{635\ m\mu.} = 3{\cdot}18$. Solvent, ether washed with 5% HCl.

attempts were made to remove further lipid by liquid–liquid extraction. The best solvent system was methanol–1% HCl–light petroleum (b.p. 68°) (70:30:100). A six-stage distribution was carried out according to the method of Bush & Densen (1948), six methanol and six light-petroleum fractions being obtained. A small amount of yellow fat moved rapidly with the light petroleum phase, the haemin staying in the first two tubes of the methanol phase. When benzene was used instead of light petroleum some of the haemin migrated with the fat. Haemin a_2, though still oily in appearance, could now be precipitated from acetone by means of light petroleum or benzene.

Attempts were made to obtain separation of haemin a_2 from an accompanying trace of protohaemin by means of paper chromatography. Of many solvent systems tried only benzene–butanol (10:1), with the ascending method of chromatography, gave a marked difference of the R_F value for haemin a_2 (R_F 0·6) and protohaemin (0·1). Haemin a_2 gave a slightly elongated spot, but no separation from the protohaemin impurity was obtained. The failure to obtain separation is attributed to presence of residual lipid.

Spectroscopic properties of haematin a_2 compounds

Haemin a_2 ($E_{603\,m\mu}/E_{635\,m\mu} = 2{\cdot}86$) for these experiments was prepared by silica-gel chromatography, followed by precipitation at $-70°$ of some of the protohaemin and lipid impurity from an acetone solution of the haemin.

The experiments were carried out in a Thunberg tube modified for spectrophotometry. Before the addition of $Na_2S_2O_4$ to obtain the reduced compounds, the tube was evacuated. Sufficient $Na_2S_2O_4$ was added as a molar solution in 0·2M phosphate buffer, pH 7·6, to ensure full development of the maxima. Excess was avoided as this led to rapid decay of the maxima of the haematin a_2 compounds. Maxima were measured in the spectrophotometer and with the Hartridge reversion spectroscope, in separate samples.

The pyridine haemochrome was formed by adding $Na_2S_2O_4$, contained in the side-arm of the Thunberg tube, to haemin a_2 in a mixture of pyridine and 0·2N-NaOH (1:2). The ferric compound gave a diffuse band with its maximum at 603–605 mμ.; reduction to the haemochrome gave a sharp peak at 613–615 mμ. This peak decayed fairly rapidly. Lower concentrations of NaOH (to 0·001N), and various proportions of pyridine to NaOH were tried, but the conditions as stated gave the maximum development of the 613–615 mμ. band and the slowest rate of decay.

When crude haemin preparations ($E_{603\,m\mu}/E_{635\,m\mu} = 1{\cdot}26$) were converted into the pyridine haemochrome, the intensity of the 613 mμ. band was much weaker than that to be expected from the haemin a_2 content of the extracts. Only a faint band, which disappeared very rapidly, could be seen at 613 mμ., though strong bands due to protohaemochrome (at 557 and 525 mμ.) were present and persisted.

Haemin a_2 in 0·001–0·2N-NaOH or in 0·2M phosphate buffer, pH 7·6, gave a wide absorption band with its maximum at 664 mμ. (alkaline haematin). Reduction gave a diffuse band at 618 mμ. (haem) as long as the solution remained alkaline.

The CO-haem compound was formed by adding $Na_2S_2O_4$ to the haemin in 0·2M-NaOH or 0·2M phosphate buffer, pH 7·6, the solution being saturated with coal gas beforehand. A strong band with its maxima at 618–620 mμ. appeared. Reduction to the haem before gassing did not give as stable a preparation.

The cyanide complex was formed by adding an excess of KCN to haemin a_2 in 0·2N-NaOH. The 664 mμ. peak of the alkaline haematin disappeared and was replaced by a broad band of absorption with its centre at 603–605 mμ. On reduction with $Na_2S_2O_4$ a strong band with its maximum at 618 mμ. was obtained. This ferrous cyanide compound was the most stable of the ferrous haem a_2 compounds.

Table 1 gives the position of the principal maxima of haematin a_2 compounds.

Chlorin from haemin a_2

Removal of iron from the haemins. The ferrous acetate method of Warburg & Negelein (1932) was used with modifications. First, it was found possible to remove the iron without heating the haemin solution, thus avoiding destruction of the chlorin. Secondly, after much experience it was apparent that the amount of HCl added to the haemin–ferrous acetate mixture was critical, even a small excess causing some destruction of the chlorin.

The iron may be removed from the haemins at any stage after taking them into ether from the acid aqueous–acetone extracts. For preparative purposes precipitation of the phospholipid was first carried out.

The combined haemins were taken into acetic acid to give a solution with $E^{508\,m\mu}_{1\,cm.} = 2{\cdot}0$. Carbon dioxide was bubbled through this solution at 20°. To each 8 ml. was added 1 ml. of a ferrous acetate solution prepared by heating under CO_2 2 mg. of Ferrum reductum (British Drug Houses Ltd.) per ml. of acetic acid. The hot ferrous acetate solution did not raise the temperature of the haemin solution

Table 1. *Haematin a_2 compounds*

	Wavelength (mμ.) of the maximum of absorption
Acid haematin (haemin)	603
Alkaline haematin	662–664
Haem	618
CO-haem	618–620
Pyridine haemochrome	613
Cyanide haemochrome	618

above 30°. After 30 sec., 0·1 ml. of conc. HCl was added. The emergence of the main band (630 mμ.) of chlorin a_2 hydrochloride and that of protoporphyrin hydrochloride was followed with a hand spectroscope. After 2–5 min. the acetic acid solution was tipped into ether over dilute sodium acetate. Acetic acid was largely neutralized with $NaHCO_3$, followed by thorough washing with water.

Spectroscopic examination of this ether solution showed, in addition to the bands of protoporphyrin at 633, 576, 537 and 503 mμ., a band at 653 mμ. (the principal band of chlorin a_2).

The ratios $E_{653\ m\mu.}/E_{633\ m\mu.}$ and $E_{653\ m\mu.}/E_{503\ m\mu.}$ have been used to follow the separation of chlorin a_2 from protoporphyrin and related porphyrins. Values for these ratios obtained after removal of the iron from haemin preparations of different $E_{603\ m\mu.}/E_{635\ m\mu.}$ ratios are given in Table 2. This shows that as the haemin a_2 content of these preparations increased there was an increase in the proportion of chlorin a_2 to protoporphyrin.

Table 2. *Correlation of spectrophotometric ratios for bacterial haemins and for the chlorin–porphyrin mixture obtained on removal of iron from the haemins, with the molar ratio of protoporphyrin to chlorin a_2*

Haemins in ether–HCl	Chlorin a_2–porphyrin mixture in ether		Molar ratio of protoporphyrin to chlorin a_2*
$E_{603\ m\mu.}/E_{635\ m\mu.}$	$E_{653\ m\mu.}/E_{633\ m\mu.}$	$E_{653\ m\mu.}/E_{503\ m\mu.}$	
0·85	0·81	0·34	10·4
1·00	—	—	7·1
1·05	1·23	0·49	6·4
1·09	—	—	5·9
1·14	1·33	0·53	5·3
1·26	—	—	3·6
1·28	1·82	0·81	3·3

* These values have been calculated from the extinctions at 653 and 576 mμ. (see text).

Chromatography on silica gel. Silica-gel columns (see Methods) of similar dimensions to those used for the haemins readily gave good yields of chlorin a_2 of purity greater than obtainable by any other means, including the classical method of fractionation between ether and HCl. After precipitation of phospholipid with acetone (see haemins) the chlorin was taken into benzene, and the precipitated porphyrin removed by centrifuging. The greenish brown supernatant was applied to a silica column. The sequence of solvents and procedure was as described for the haemins. Benzene which had been equilibrated with water, and to which was then added 1% of methanol, eluted 40% of the chlorin a_2 applied to the column. The value of $E_{653\ m\mu.}/E_{503\ m\mu.}$ for this fraction varied from 2·94 to 2·57. The subsequent fractions obtained by increasing the methanol content of the benzene accounted for 50–55% of the chlorin applied to the column. These fractions were recombined and run through a second column; a chlorin a_2 fraction with ratio $E_{635\ m\mu.}/E_{503\ m\mu.}$ of 2·82 was given. Rechromatographing the best fraction did not significantly raise the $E_{653\ m\mu.}/E_{503\ m\mu.}$ ratio, though a small amount of lipid could be removed.

Further purification of chlorin a_2. The best chlorin fractions from these columns were oily after removal of the solvent. Countercurrent distribution was therefore carried out as described for the haemins, except that ten stages were used. Some fat ran with the light-petroleum phase. Most of the chlorin was in the methanol fractions 1 and 2, and these two fractions were combined and taken into ether. The ratio $E_{653\ m\mu.}/E_{503\ m\mu.}$ was 3·0. The chlorin could now be completely extracted from ether with 10% HCl. The chlorin was returned to ether by partial neutralization of the acid extracts with $NaHCO_3$, and this solution exhaustively extracted with 1, 3 and 5% HCl. A small amount of porphyrin and a trace of chlorin were extracted by 1% HCl, and the 3 and 5% fractions contained equal amounts of chlorin a_2. The ratio $E_{653\ m\mu.}/E_{503\ m\mu.}$ for the 5% fraction in ether was 3·3. In ether the chlorin showed bands at I 653, II 598, III 573, IV 534 and V 503 mμ. The decreasing order of intensity was I, V, II, IV, III.

Table 3. *R_F values obtained on paper chromatography of the methyl ester of chlorin a_2 and related chlorins and porphyrins*

	Solvent system		
	Kerosene–propanol	Kerosene–chloroform	Trichloroethylene
Chlorin a_2*	0·55	0·32	0·77
Mesochlorin	0·88	0·89	0·96
Pyrrochlorin	0·96	0·97	0·96
Rhodochlorin	0·90	0·91	0·96
Dioxyprotoporphyrin†	0·34	0·00	0·00
Dioxymonovinylmonohydroxyethyldeuteroporphyrin‡	0·26	0·00	0·00
Protoporphyrin	0·75	0·80	0·96
Mesoporphyrin	0·82	0·82	0·96
Haematoporphyrin	0·30	0·05	0·05
Monovinylmonohydroxyethyldeuteroporphyrin	0·40	0·28	0·00
'626 mμ.' porphyrin impurity	0·40	0·28	0·00
Pyrroporphyrin	0·92	0·92	0·96
Rhodoporphyrin	0·77	0·82	0·96

* $E_{653\ m\mu.}/E_{553\ \mu m.} = 3·3$. † Prepared according to Fischer & Bock (1938).
‡ Prepared similarly from monovinylmonohydroxyethyldeuteroporphyrin.

Chromatography of the methyl esters of chlorin a_2 and porphyrin impurities. The methyl ester was prepared by esterification of chlorin a_2 with diazomethane. The spectra of chlorin a_2 and of its ester were identical.

Paper chromatography. Table 3 gives the R_F values obtained in three solvent systems for the methyl esters of chlorin a_2 and certain related substances and porphyrin impurities. Under u.v. light chlorin a_2 spots in all three systems displayed a brick-red fluorescence, whereas spots due to the porphyrin impurities were bright red.

Various preparations containing chlorin a_2 were chromatographed on a preparative scale. The best fraction from any system did not give a greater value than 2·5 for $E_{653\,m\mu.}/E_{503\,m\mu.}$. This low ratio was found to be due to admixture of a porphyrin of R_F 0·4 in the kerosene–propanol system. The absorption maxima of this porphyrin in ether were at 626, 576, 533 and 502 mμ.

Alumina chromatography. A mixture of chlorin a_2 and porphyrin, after precipitation of phospholipid and in some experiments further removal of lipid by chromatography on silica-gel columns, was esterified with diazomethane and an ether solution of the methyl esters applied to a column of alumina (British Drug Houses Ltd.) set up in ether. On eluting with ether, protoporphyrin moved rapidly down the column, followed slowly by a small amount of a porphyrin with absorption maxima at 626, 576, 534 and 502 mμ. On paper chromatography this porphyrin gave an R_F of 0·4 in the kerosene–propanol system and an R_F of 0·28 in the kerosene–chloroform system.

Chloroform and methanol eluted a small amount of haematoporphyrin (identified by its spectra and by paper chromatography) and a chlorin with a principal maximum of absorption at 648 mμ. The dark-green layer at the head of the column was then separated from the rest of the column and chlorin a_2 eluted from this with 70% aqueous acetone containing 1% (w/v) of HCl. In ether the value for $E_{653\,m\mu.}/E_{503\,m\mu.}$ for this fraction ranged from 2·0 to 2·5 in different experiments. The recovery of chlorin a_2 was of the order of 50%.

Other aluminas (Merck, Savory and Moore) and absorbents, e.g. magnesium oxide, magnesium carbonate, calcium oxide and calcium carbonate, were tried without success.

The '626 mμ.' porphyrin was also obtained from material precipitated with benzene and from certain fractions obtained on silica-gel chromatography of the benzene-soluble material. The amount from any preparation was always very small. It was not possible to crystallize this porphyrin, though protoporphyrin eluted from these columns crystallized readily.

Absorption spectra of chlorin a_2

A curve of the absorption in the visible region of the spectrum of an ether solution of chlorin a_2 is shown in Fig. 3. Chlorin a_2 preparations with a value of 3·3 for $E_{653\,m\mu.}/E_{503\,m\mu.}$ show practically no absorption in the 580–570 mμ. region. Very fatty fractions showed a shift of the two principal maxima of 1–2 mμ. towards longer wavelengths.

A number of the purest preparations of chlorin a_2 in ether solution were examined in the 420–200 mμ. region. These showed a Soret band with its maximum at 405 mμ., and two other main regions of absorption at 280–270 mμ. and 240–220 mμ. The purest fraction gave the ratios

$$E_{653\,m\mu.}:E_{503\,m\mu.}:E_{405\,m\mu.}:E_{280}:E_{230\,m\mu.} = 3{\cdot}3:1:12:3{\cdot}0:5{\cdot}0.$$

Some of the absorption in the u.v. region appears to be due to lipid impurity. A number of lipid fractions which had been separated by chromatography and countercurrent distribution from chlorin a_2 showed broad bands in the 280–200 mμ. region.

Chlorin a_2 in dilute HCl showed a strong band at 630 mμ. Higher concentrations exhibited a shading of the band towards the red region of the spectrum. In 20% HCl the maximum was at 647 mμ. Chlorin a_2 in 10% HCl gave a value of 0·71 for $E_{647\,m\mu.}/E_{630\,m\mu.}$ initially, but this ratio had risen to 1·1 after 24 hr. Fig. 4 shows spectral curves for chlorin a_2 in 8% and 16·5% HCl. The concentration of the chlorin is the same in both, and measurements were made immediately after preparation of the solutions. Dilution of the 16·5% HCl

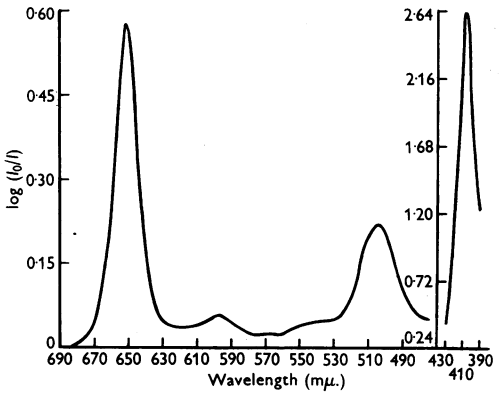

Fig. 3. Absorption spectrum of chlorin a_2, $E_{653\,m\mu.}/E_{503\,m\mu.} = 2{\cdot}63$. Solvent, ether.

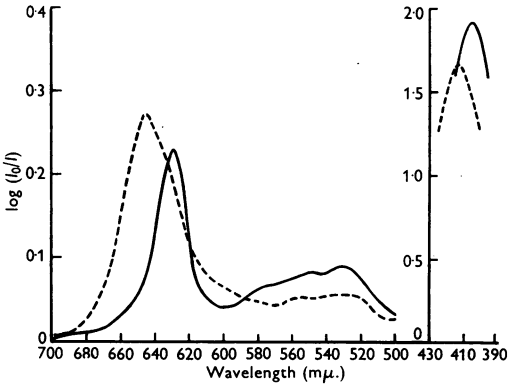

Fig. 4. Absorption spectra of equal concentrations of chlorin a_2 in 8% HCl (—) and in 16·5% HCl (- - -).

solution with water to give an 8% HCl concentration gave a spectral curve identical with that of chlorin a_2 in 8% HCl.

Metal complexes of chlorin a_2

Haemin. Iron was introduced into chlorin a_2 by the ferrous acetate method (Fischer & Orth, 1940). Quantitative conversion of chlorin a_2 into haemin a_2 could not be obtained. Prolonged heating of the chlorin with ferrous acetate and NaCl in acetic acid caused destruction of the chlorin and of the haemin formed. With short periods of heating or at low temperatures the iron was not introduced into the chlorin.

The haemin was converted into the acid haematin and into the pyridine haemochrome. The positions of the maxima of these compounds were the same as for those formed from the original haemin a_2.

Copper and zinc complex. The Cu–chlorin and Zn–chlorin complexes were formed by the methods of Fischer (Fischer & Orth, 1940). The positions of the maxima of absorption for the Cu complex were I 613, II 562, III 526 and IV 401 mμ.; those of the Zn complex I 615, II 564, III 529 and IV 408 mμ. The decreasing order of intensity was IV, I, II, III in both cases.

Evidence for the nature of the side chains of haemin a_2 and chlorin a_2

Effect of hydroxylamine on haemin a_2 and chlorin a_2. To a solution of the haemin in pyridine, excess of a mixture of equivalent amounts of hydroxylamine hydrochloride and Na_2SO_3 was added, and the mixture was stood for 2 hr. at 22° or gently refluxed for 5 min. After cooling, an equal volume of 0·1 N-NaOH was added to the pyridine solution, followed by a small amount of $Na_2S_2O_4$. No shift of the principal maxima of absorption of the pyridine haemochrome was observed. Chlorin a_2 was similarly heated with hydroxylamine in pyridine, followed by cooling and filtering. No shift of the absorption maxima of chlorin a_2 was found.

The presence of formyl or carbonyl groups in haem a_2 is thus excluded.

Table 4. *Effect of diazoacetic ester (DAE) on the position of the maxima of absorption in the red region of the spectrum*

Compound	Wavelength (mμ.) of the maximum of absorption: Before DAE treatment	After DAE treatment
Chlorin a_2	652	646
Phaeophorbide *a*	667	660
Dioxyprotoporphyrin	668	662
Protoporphyrin	633	626
Monovinylmonohydroxyethyldeuteroporphyrin	626	624
'626 mμ.' porphyrin from chlorin a_2	626	626
'626 mμ.' porphyrin from protoporphyrin	626	626

Effect of diazoacetic ester on the absorption spectrum of chlorin a_2. An excess of diazoacetic ester was added to chlorin a_2 in a small tube. The tube was flushed with nitrogen, stoppered and left in the dark for 48 hr. Heating at 70° for 18 hr. as carried out by Fischer (see Fischer & Orth, 1940) gave similar results, but this caused considerable destruction of the chlorin. Ether was added to the tube and the chlorin extracted with 20% HCl, taken back into ether and the positions of the maxima were determined. Protoporphyrin (two vinyl groups), phaeophorbide *a* (one vinyl group) and dioxyprotoporphyrin (two vinyl groups) were similarly treated. All these compounds showed the same degree of shift of the principal maxima of absorption after reacting with diazoacetic ester (Table 4). The presence of at least one vinyl group in chlorin a_2 is thus demonstrated.

Paper chromatography of the free chlorin. Chlorin a_2 was chromatographed according to the method of Nicholas & Rimington (1949), in lutidine–water, in an attempt to determine the number of free carboxylic acid side chains. Chlorin a_2 ($E_{653\,m\mu.}/E_{503\,m\mu.}=3{\cdot}1$) ran with R_F 0·87 (pyrrochlorin and pyrroporphyrin, R_F 0·95; protoporphyrin and rhodochlorin, R_F 0·8; coproporphyrin, R_F 0·50). The R_F value obtained for chlorin a_2 indicates that it probably has two carboxylic acid side chains (see Discussion).

Effect of acid and alkali on chlorin a_2. Because of the possibility of an esterified carboxylic acid side chain, chlorin a_2 was left to stand in 20% HCl or in NaOH overnight. No change in the R_F values was found. Esterification of a carboxylic acid side chain can thus be ruled out. Some destruction of the chlorin occurred in the alkali. Similar results were obtained with 1% methanolic potassium hydroxide. Heating of the chlorin in all these solvents resulted in its total disappearance.

Conversion of chlorin a_2 into porphyrins

As the spectrum of chlorin a_2 did not correspond to that of any known chlorin, further information was sought as to the nature of its side chains by attempting conversion into the porphyrin. Two methods were investigated, both of which had been used extensively by Fischer (see Fischer & Orth, 1940) during his studies on the structure of chlorophyll.

Catalytic hydrogenation and reoxidation. Chlorin a_2, in approx. 100 μg. amounts estimated spectrophotometrically (see below), was hydrogenated at 22° and atmospheric pressure, with palladium black as catalyst. The catalyst was removed from the solvent by centrifuging and the supernatant shaken in air for several hours. In some experiments the solution was left to stand, as a shallow layer, in the dark for several days. The products of the reaction were taken into ether and the spectra determined. Protoporphyrin, phaeophorbide *a* and dioxyprotoporphyrin were similarly treated.

When the reaction was carried out in acetone, chlorin a_2 gave a small yield of a porphyrin and a still smaller yield of a chlorin. The porphyrin was extracted with 3% HCl and returned to ether. The spectrum of this porphyrin was of the aetiotype (the four maxima of absorption increasing in strength from the first in the red to the fourth in the blue) and the maxima were at 623, 568, 526 and 496 mμ. On paper chromatography in the kerosene–propanol system the methyl ester of the porphyrin ran with R_F 0·8 with slight

streaking (mesoporphyrin, R_F 0·77; pyrroporphyrin, R_F 0·88; protoporphyrin, R_F 0·7). The chlorin had a principal maximum of absorption at 646 mμ. and ran with R_F 0·43 (chlorin a_2, R_F 0·60) in the same system. Similar products were obtained when ethanol was used as a solvent, the amount of 646 mμ. chlorin being greater. No porphyrin and only a trace of the 646 mμ. chlorin was obtained when acetic acid was used, no chlorin a_2 remaining.

Protoporphyrin was converted into mesoporphyrin and phaeophorbide *a* to phaeoporphyrin a_5 in all solvents.

In acetone or ethanol, hydrogenation converted dioxyprotoporphyrin into the leuco compound; oxidation of this yielded mesoporphyrin. When dioxyprotoporphyrin was hydrogenated in acetic acid the chlorin-like acid spectrum rapidly changed directly to that of mesoporphyrin hydrochloride. In all solvents protoporphyrin and dioxyprotoporphyrin yielded small amounts of a chlorin which in ether had a principal maximum of absorption at 646 mμ.

Conversion of chlorin a_2 into porphyrin with hydriodic acid. Treatment of chlorin a_2 with HI according to the method of Fischer (see Fischer & Orth, 1940), which involved heating, caused total loss of the chlorin, no porphyrin being obtained. By carrying out the reaction at 18–20°, however, in the presence of a great excess of HI, porphyrins could be obtained from chlorin a_2 without excessive loss of material. To approx. 200 μg. of the chlorin in 0·5 ml. of acetic acid in an atmosphere of CO_2, 0·1 ml. of HI (sp.gr. 1·94) was added, the tube stoppered and placed in the dark for 72 hr. Initially a strong band could be seen at 630–650 mμ. but this slowly disappeared, being replaced by a band at 560 mμ. After 72 hr. there was no further increase in strength of this band. The porphyrin was taken into ether over dilute sodium acetate, and partially neutralized with $NaHCO_3$, the free iodine was removed by washing with 1% (w/v) sodium thiosulphate and the ether washed thoroughly with water. By this method an ether solution with absorption maxima at 646, 626, 575, 534, 502 and 402 mμ. was obtained (from chlorin a_2 of $E_{653\,m\mu.}/E_{503\,m\mu.} = 2·95$). The maximum at 646 mμ. was caused by the presence of a chlorin of *meso*-type derived by reduction of the vinyl group of chlorin a_2. The other absorption bands indicated that a porphyrin of aetio-type spectrum had been formed.

A major portion of the ether solution was methylated with diazomethane. Paper chromatography, in kerosene–propanol, of the porphyrin esters gave a major spot of R_F 0·41, a secondary spot of R_F 0·26 and a faint spot of R_F 0·75 (haematoporphyrin ester, R_F 0·26; protoporphyrin ester, R_F 0·75). This mixture of porphyrin esters was chromatographed on an alumina column. Elution with ether gave a rapid-moving (*a*) and a slow-moving (*b*) fraction. A third fraction (*c*) could be eluted with methanol. These all showed aetio-type spectra. The amount of porphyrin in each fraction was estimated by measuring the absorption at 501–503 mμ. Fraction (*b*) contained 60%, fractions (*a*) and (*c*) 20% each, of the porphyrin eluted from the column. On paper chromatography in the kerosene–propanol system the major fraction (*b*) gave a strong spot at R_F 0·40 and also a trace of haematoporphyrin ester (R_F 0·2) and a trace of protoporphyrin ester (R_F 0·7). Fraction (*a*) gave a spot with R_F 0·7 and a secondary spot of R_F 0·27, and fraction (*c*) gave a spot of R_F 0·27 with a secondary spot of R_F 0·41. Fraction (*b*) was chromatographed on alumina. The porphyrin ester eluted with ether from this column, when run on paper chromatograms in kerosene–propanol moved with R_F 0·41, in kerosene–chloroform with R_F 0·28. No spots due to protoporphyrin or haematoporphyrin were seen. Fig. 5 shows an absorption curve obtained for this porphyrin. This porphyrin was still oily and could not be crystallized.

The sample that had not been treated with diazomethane was extracted with 0·1, 0·3, 1·0, 2·0, 5 and 10% HCl successively. The fractions were returned to ether and the absorption at the Soret maximum was read.

The porphyrins were then converted into the ester form and run on paper in the kerosene–propanol system. Table 5 summarizes the data obtained from this fractionation.

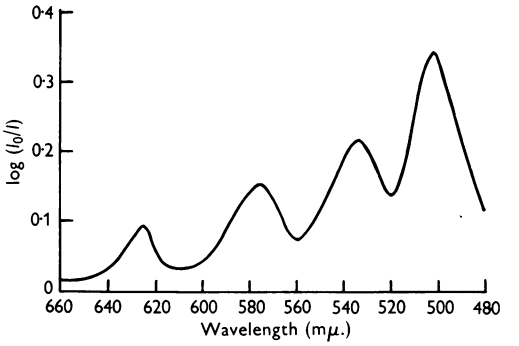

Fig. 5. Absorption spectrum of the principal porphyrin obtained on treating chlorin a_2 with hydriodic acid. Solvent, ether.

Table 5. *Hydrochloric acid fractionation of porphyrins obtained on treating chlorin a_2 with hydriodic acid*

HCl concn.	Wavelength of the Soret maximum (in ether)	Percentage of the total porphyrin based on the extinction of the Soret band	R_F values and relative intensities (>, =) of spots obtained on paper chromatography in kerosene–propanol of the methyl esters*
0·1	397	15	0·28 > 0·40
0·3	399	18	0·28 = 0·40
1·0	403	37	0·40 > 0·28
2·0	405	7	0·40 > 0·28
5 and 10†	—	—	—
Residue	405	22	0·76 = 0·40 = 0·28
		Total 97	

* Protoporphyrin, 0·76; monovinylmonohydroxyethyldeuteroporphyrin, 0·40; haematoporphyrin, 0·28 (methyl esters).
† These fractions were too small for analysis.

Comparison of the action of HI *on chlorin* a_2 *and other compounds.* At 18–20° it was necessary to use a molar ratio of HI to chlorin a_2 of 140:1. Dioxyprotoporphyrin at this level of HI was converted into protoporphyrin in a few seconds, and phaeophorbide *a* required a few hours for complete conversion into phaeoporphyrin a_5. When the molar ratio was reduced to 4:1, 18 hr. was required for completion of the reaction for both dioxyprotoporphyrin and phaeophorbide *a*. Chlorin a_2 at this low molar ratio gave no detectable porphyrin even when left for several days, but some formation of the 646 mμ. chlorin occurred.

Because of the similarity of the principal porphyrin obtained from the action of HI on chlorin a_2 to protoporphyrin and haematoporphyrin, a study of the action of HI on protoporphyrin at low temperatures was made at a molar ratio of HI to porphyrin of 140:1. After 48 hr. at 18–20° a porphyrin with an aetiotype spectrum and maxima at 626, 574, 533, 502 mμ. (R_F 0·40 in kerosene–propanol, R_F 0·28 in kerosene–chloroform) was obtained together with unaltered protoporphyrin and some haematoporphyrin. This study will be reported in full elsewhere.

The spectra obtained for the '626 mμ.' porphyrin from chlorin a_2 and the behaviour on chromatography of the methyl ester suggested that this porphyrin was either monovinylmonohydroxyethyl- or monoethylmonohydroxyethyl-deuteroporphyrin. Dr Granick kindly supplied a sample of monovinylmonohydroxyethyldeuteroporphyrin, obtained from a mutant of *Chlorella*. The maxima of this porphyrin in ether were identical in position with those of the chlorin a_2–porphyrin and of the '626 mμ.' porphyrin derived from protoporphyrin. On paper chromatography of the ester in kerosene–propanol an R_F of 0·40 was obtained, in kerosene–chloroform R_F was 0·28.

Diazoacetic ester (DAE) experiments. The '626 mμ.' porphyrin from chlorin a_2, monovinylmonohydroxyethyldeuteroporphyrin and the '626 mμ.' porphyrin from protoporphyrin were treated with diazoacetic ester for 24 hr. at 70° under nitrogen. In ether the product from monovinylmonohydroxyethyldeuteroporphyrin showed a small shift of the absorption band in the red end of the spectrum towards shorter wavelengths (Table 4). The other two porphyrins showed no detectable shift.

The diazoacetic ester complexes of chlorin a_2 and dioxyprotoporphyrin and phaeophorbide *a* were treated with hydriodic acid at molar ratio 140:1. Chlorin a_2 gave a porphyrin with maxima at 626, 574, 533, 501 mμ.; phaeophorbide *a* yielded a porphyrin with a spectrum corresponding to the DAE compound of phaeoporphyrin a_5, and dioxyprotoporphyrin gave the DAE compound of protoporphyrin.

Attempted oxidation of the copper complex. Fischer & Herrle (1937) were able to convert a number of chlorins into the corresponding vinyl porphyrin by oxidation of the copper complex. The copper complex of chlorin a_2 was shaken in acetic acid for several hours at 40°. In some experiments oxygen was bubbled through the solution. No formation of porphyrin had occurred after several hours. The copper complexes of dioxyprotoporphyrin and phaeophorbide *a*, similarly treated, also remained unchanged.

Concentration of haemin a_2 *and protohaemin in the bacteria*

Since it has not been possible to obtain crystalline material, the specific extinction of chlorin a_2 cannot be determined. However, an approximation may be made by utilizing the data published by Stern and his co-workers (Stern & Wenderlein, 1935, 1936; Stern & Deželić, 1937; Stern & Molvig, 1937; Stern & Pruckner, 1937), who determined the molar extinction coefficients (ϵ) of 29 chlorins and related pigments. For the principal maximum of absorption of these, ϵ ranges from 45 000 to 63 000. Of these compounds pyrrochlorin appears to be the most closely related to chlorin a_2 (see Discussion). Pyrrochlorin has a value of 45 000 for $\epsilon_{652\ m\mu.}$, and this has been assumed for the principal maximum of absorption of chlorin a_2. Further, it has been assumed (see Discussion) that crystalline chlorin a_2 would be protochlorin, and the value of 566 has therefore been used for the molecular weight throughout the following calculations.

In the region of maximum II (580–570 mμ.) of the spectrum of protoporphyrin and related porphyrins in neutral solvents, chlorin a_2 preparations of $E_{653\ m\mu.}/E_{503\ m\mu.} = 3{\cdot}3$ exhibit very little absorption ($E_{653\ m\mu.}/E_{576\ m\mu.} = 50$). In the initial crude preparations all absorption at 576 mμ. is taken as being due to protoporphyrin, $\epsilon_{576\ m\mu.} = 6450$. The absorption of protoporphyrin at 653 mμ. is 0·04 times that at 576 mμ.; thus $E_{653\ m\mu.} - 0{\cdot}04E_{576\ m\mu.}$ will give the absorption at 653 mμ. due to chlorin a_2. If E is measured in a 1 cm. layer, the concentration of chlorin a_2 will be $12{\cdot}6\ (E_{653\ m\mu.} - 0{\cdot}04E_{576\ m\mu.})$ μg./ml., and that of protoporphyrin $(87{\cdot}4E_{576\ m\mu.})$ μg./ml. Quantitative determination of the bacterial haemins was carried out by taking the haemins extracted from the bacteria into acetic acid, without prior separation from lipids, and removing the iron from the haemins as previously described. Measurements were made in ether at 653, 633, 576 and 503 mμ.

On the basis of the molecular weight assumed for chlorin a_2, *Aero. aerogenes* contained 1·4–4·3 mg. of haemin a_2 and 16–24 mg. of protohaemin/100 g. dry weight of cells. The amounts of the haemins obtained depended on the degree of aeration and length of incubation of the culture. Table 2 shows the relation between the ratio $E_{603\ m\mu.}/E_{635\ m\mu.}$ and the molar ratio of protoporphyrin to chlorin a_2 obtained on removal of iron from the haemins, as well as other spectrophotometric ratios (see above) which depend on this ratio. It thus appears possible to estimate roughly the haem a_2/protohaem ratio from the $E_{603\ m\mu.}/E_{635\ m\mu.}$ ratio of the acid haematins.

Haemin a_2 *and chlorin* a_2 *from other organisms*

Az. vinelandii cells harvested at 48 hr. showed a band due to cytochrome a_2 as well as the α-bands of cytochromes a_1, b_1 and *c*. The cells were extracted as described for *Aero. aerogenes*. The haematins in ether washed with 5 % HCl gave a value of 0·96 for $E_{603\ m\mu.}/E_{635\ m\mu.}$. This is equivalent to a protohaem/haem a_2 ratio of approx. 8. Haemin a_2 was separated from the mixture of haemins by chromatography on

silica gel. From this chlorin a_2 was obtained, identified by its spectrum in neutral and acid solvents and by paper chromatography of the methyl ester.

Esch. coli and *P. vulgaris* cells harvested at 18 hr. showed a band due to cytochrome a_2. On fractionation of the haemins, haemin a_2, and from this chlorin a_2, were obtained. In *Bact. subtilis*, *Bact. mycoides* and *Ps. pyocyaneus*, the cytochrome a_2 band was very weak, but small amounts of haemin a_2 could be obtained on fractionation of the haemins. No cytochrome a_2 could be seen in *Torulopsis utilis* cells. No band could be detected at 603 mμ. on examination of the acid haematins, nor was any chlorin a_2 found on removal of iron from the haemins.

Anaerobic cultures. Aero. aerogenes, *Esch. coli* and *P. vulgaris* when grown anaerobically showed no cytochromes. A trace of protohaemin but no haemin a_2 was extracted from these bacteria (30 g. dry weight of cells). A trace of coproporphyrin was found on extracting the culture with ether at pH 3·2.

Free chlorin and porphyrin present in bacterial cells. Small amounts of *a* chlorin, maxima of absorption at 668 and 500 mμ., and protoporphyrin were found in the 5% HCl washings of the ether solution of crude haemins. If the haemins were not extracted with HCl, the chlorin was found to run with the yellow-lipid fraction on silica-gel chromatography of the haemins. Extraction of the cells with neutral acetone yielded small amounts of the chlorin and porphyrin, and none could be found in the HCl washings of the haemins subsequently extracted from the cells with acid–acetone.

The amount of this free chlorin and porphyrin was always small, but was greater in cells from cultures that had been incubated for several days or from cultures where aeration had been too vigorous, resulting in poor yields of cytochrome a_2.

Haemin a in Aero. aerogenes. The presence of haem *a* in this organism was demonstrated by partitioning crude haemin preparations between ether and pyridine–HCl buffer (see Rawlinson & Hale, 1949). Haemin *a* remained in the ether phase and haemin a_2 went into the aqueous phase together with protohaemin. Haemin *a* was identified by its conversion into the pyridine haemochrome and into the porphyrin.

DISCUSSION

A green haemin which has been shown to be the iron complex of a hitherto undescribed chlorin has been isolated from various bacteria, all of which contained cytochrome a_2. The yield of this haemin was related to the intensity of the 630 mμ. band of the reduced cytochrome, and the haemin was never present in extracts from cells which did not show the band of cytochrome a_2. Further, apart from protohaemin from cytochrome b_1, and a small amount of haem *a* from cells which showed a strong cytochrome a_1 band, no other haemins were found. The green haemin could only be extracted from the bacterial cells by solvents containing acid, and this is evidence for the assumption that the haemin is linked to a protein *in situ*. It is concluded that this haemin is the prosthetic group of cytochrome a_2 and it is designated haemin a_2.

The position of the principal maximum of absorption of the pyridine haemochrome lies 17 mμ. towards the blue end of the spectrum as compared with the absorption band of reduced cytochrome a_2 (630 mμ.), that of the ferrous cyanide compound of haemin a_2 18 mμ. as compared with the position of the absorption band of the reduced cyanide compound of cytochrome a_2 (636 mμ.). The shift found for the pyridine haemochrome is similar in magnitude to that found for pyridine haemochrome *a* (587 mμ.) relative to the α band of reduced cytochrome *a* (604 mμ.), but is greater than that for pyridine protohaemochrome (557 and 527 mμ.) relative to the bands of cytochrome *b* (566 and 528 mμ.), and pyridine mesohaemochrome (547 and 528 mμ.) relative to reduced cytochrome *c* (550 and 521 mμ.). It is well established (see Lemberg & Legge, 1949) that the position of the absorption bands of the compound formed by a haem with a protein varies considerably according to the particular protein used. The small shift found for the latter two cases does not therefore invalidate the assumption that the degree of shift observed for pyridine haemochrome a_2, and for the cyanide compound of haemin a_2, can be ascribed to the replacement of the apoprotein of cytochrome a_2 by a different nitrogenous base rather than to a degradation of the prosthetic group.

Though it has been possible to obtain considerable purification of haemin a_2, protohaemin and lipid impurities are still present in the best preparations. Of several methods of purification, chromatography on a silica gel, which has been treated with aqueous methanol to modify its adsorptive properties, has provided the purest haemin.

By chromatography of the free chlorin on silica-gel columns followed by countercurrent distribution between organic solvents and hydrochloric acid fractionation it has been possible to obtain chlorin a_2 with only a trace of remaining porphyrin impurity. Despite these methods and even after precipitation from benzene the chlorin is still oily in nature. Like the haemin, chlorin a_2 is lipophilic and tends to fractionate according to the partition properties of certain accompanying free lipids. By spectrophotometric analysis and further fractionation of the eluted material it has been shown that chlorin a_2 in the presence of lipids can associate with porphyrins to form a complex which behaves as a single

entity on paper chromatography of the methyl esters. Thus it cannot be assumed that a spot of given R_F value represents pure chlorin a_2 where lipids are present.

Since reintroduction of iron into the chlorin obtained from haemin a_2 restores the spectrum of the haemin and of its compounds, it may be taken that no alteration to the nucleus or to the side chains has occurred during removal of the iron.

Of the spectra of the chlorins and related pigments given by Fischer & Orth (1940) only those of mesochlorin (2:4-diethyl-1:3:5:8-tetramethylchlorin-6:7-dipropionic acid) and pyrrochlorin (4-ethyl-1:3:5:8-tetramethyl-2-vinylchlorin-7-propionic acid), Fig. 6, approach that of chlorin a_2 in respect of the position of the absorption bands (Table 6). The porphyrin obtained by catalytic hydrogenation and reoxidation of chlorin a_2 closely resembles mesoporphyrin; neither the slight difference from mesoporphyrin in the position of the maxima, nor in the R_F value of the ester, would appear to prove a difference.

Pyrrochlorin ester runs with a much higher R_F value than chlorin a_2 on paper chromatography, and pyrroporphyrin ester gave a significantly higher R_F value than did the methyl ester of the porphyrin obtained from chlorin a_2 by catalytic hydrogenation. Thus it is certain that chlorin a_2 is not pyrrochlorin.

The porphyrins obtained by catalytic hydrogenation and by hydriodic acid treatment of chlorin a_2 are evidence also that this chlorin is not a rhodochlorin (CO_2H at position 6 of the tetrapyrrole ring). Further, rhodochlorin ester runs with a much higher R_F value than does chlorin a_2 on paper chromatography.

The absorption band of the haemin of mesochlorin is at 600 mμ. (haemin a_2, 603 mμ.), and that of the copper complex of mesochlorin at 613 mμ. (copper complex of chlorin a_2, 613 mμ.). This is further evidence that chlorin a_2 is closely related to mesochlorin.

The question arises whether chlorin a_2 is a true chlorin, with two extra hydrogens on one of the pyrrole rings, or a 'dihydroxychlorin', with two hydroxyl groups in this position. The 'dihydroxychlorins' prepared by Fischer (see Fischer & Orth, 1940) have a principal maximum of absorption which is removed by approximately 10 mμ. further into the red end of the spectrum, as compared with that of the corresponding true chlorins. It is unlikely that chlorin a_2 is a compound of this type since the position of the principal maximum of absorption of chlorin a_2 is only 9 mμ. further towards the red than that of mesochlorin, and this shift can be accounted for by the presence in chlorin a_2 of a vinyl group (see below). Further, the behaviour of the methyl ester of chlorin a_2 on paper

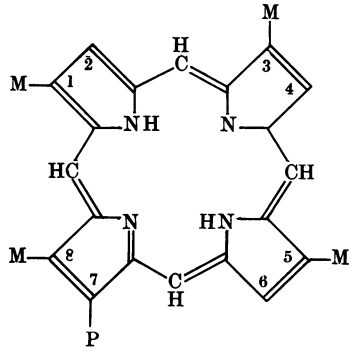

Fig. 6. Possible structural formulae for chlorin a_2 and those for certain related chlorins and porphyrins.

	Substituents at positions		
	2	4	6
Chlorin series*			
Mesochlorin	E	E	P
Pyrrochlorin	V	E	H
Rhodochlorin	V	E	CB
Chlorin a_2	V	HE	P†
	HE	V	
	V	E	
	E	V	
	V	V	
Porphyrin series			
Protoporphyrin	V	V	P
Mesoporphyrin	E	E	
Haematoporphyrin	HE	HE	
Monovinylmonohydroxyethyldeuteroporphyrin	V	HE	
	or	or	
	HE	V	
'626 mμ.' porphyrin derived from chlorin a_2	E	HE	
	or	or	
	HE	E	

Abbreviations: M, methyl; E, ethyl; V, vinyl; HE, hydroxyethyl; H, hydrogen; CB, carboxylic acid; P, propionic acid.

* These contain two extra hydrogens, conventionally placed at positions 7 and 8 of the tetrapyrrole ring.

† Or relatively non-polar carboxylic acid.

Table 6. *Position of the maxima of absorption of certain chlorins*

Compound	Wavelength (mμ.) of the maxima of absorption I	II	III	IV	Solvent
Chlorin a_2	653	598	534	503	Dioxan
Pyrrochlorin*	652	597	523	491	Dioxan
Mesochlorin*	644	591	522	490	Pyridine–ether
Mesopyrrochlorin*	642	589	518	487	Dioxan

* From Fischer & Orth (1940).

chromatography, and the difficulty with which this chlorin reacts with hydriodic acid, argue against the presence of hydroxyl groups at the β-positions of the pyrrole rings.

Substituents at the methine bridges of the tetrapyrrole ring may be ruled out from a consideration of the porphyrins obtained by catalytic hydrogenation and by hydriodic acid treatment of chlorin a_2. Neither of the methods used would remove a side chain, except a hydroxyl group, from this position. The presence of a hydroxyl group is unlikely, as both dioxyprotoporphyrin (see Fischer & Bock, 1938) and dioxymonovinylmonohydroxyethyldeuteroporphyrin have their principal maxima of absorption well within the red region of the spectrum at 669 and 661 mμ. respectively.

It has been shown that chlorin a_2 does not possess a carbonyl or formyl group, but that at least one vinyl group is present. The residual lipid impurities, which from their ultraviolet spectrum appear to be highly unsaturated, have prevented a determination of the number of vinyl groups by quantitative hydrogenation. Fischer (see Fischer & Orth, 1940) prepared the diazoacetic ester complex of a number of chlorophyll derivatives containing a vinyl group. The shift to the blue of the principal maximum of absorption of the diazoacetic ester compound ranges from 3 to 8 mμ. These values are for chlorins with single vinyl groups, no divinylchlorins having yet been synthesized.

A comparison of the position of the principal maximum of absorption of various vinylchlorins and chlorin-like compounds with that of their meso form, i.e. ethyl instead of vinyl group, shows a shift of 10–13 mμ. to the blue on saturation of the vinyl group. On catalytic hydrogenation of highly purified chlorin a_2 a new chlorin with its principal maximum of absorption at 645–646 mμ. has been obtained as one of the products. The shift to the blue is only 8 mμ. Since this chlorin could have arisen only from chlorin a_2 it can be assumed that it is a meso form of chlorin a_2. The combined evidence of the spectral shift obtained on formation of the diazoacetic ester compound and on hydrogenating chlorin a_2 favours the assumption that only one vinyl group is present in chlorin a_2.

The spectrum and chromatographic behaviour of the principal porphyrin obtained on treatment of chlorin a_2 with hydriodic acid is very similar to, if not identical with, that of monovinylmonohydroxyethyldeuteroporphyrin isolated by Dr Granick from a mutant of *Chlorella*. The failure to demonstrate the presence of a vinyl group in the former porphyrin suggests, however, that it is ethylhydroxyethyldeuteroporphyrin. A similar porphyrin was obtained by the action of hydriodic acid on protoporphyrin under the same conditions. It is evident that formation of a hydroxyethylporphyrin precedes the formation of haematoporphyrin under the specific conditions of these experiments, and this suggests that the hydroxyethyl group is not present in chlorin a_2 itself.

To summarize, it can be concluded that chlorin a_2 is a true chlorin corresponding to protoporphyrin or a porphyrin with only one vinyl group such as monovinylmonoethyldeuteroporphyrin or, less likely, monovinylmonohydroxyethyldeuteroporphyrin. Either isomerism or even replacement of some alkyl groups by others is not excluded.

The R_F value obtained for free chlorin a_2 on paper chromatography in the lutidine–water system is midway between that for a monocarboxylic and that for a dicarboxylic chlorin. It is not known whether in this system lipid impurities could modify R_F values to such an extent. Treatment of chlorin a_2 with acid and alkali did not alter the R_F values obtained on paper chromatography or result in the further separation of lipid. This is evidence against the presence of an esterified side chain. It is possible that one of the side chains of chlorin a_2 is a relatively non-polar carboxylic acid.

The ability of the methyl ester of chlorin a_2 to run on paper chromatograms in kerosene–propanol, kerosene–chloroform and trichloroethylene solvent systems contrasts with its strong adsorption on alumina or magnesium oxide columns, from which the chlorin can be removed only by acid–acetone. The methyl ester of dioxyprotoporphyrin (with two hydroxyl groups on the methine bridges), monovinylmonohydroxyethyldeuteroporphyrin (with one hydroxyethyl side chain) and haematoporphyrin (with two hydroxyethyl side chains), all of which have lower R_F values than chlorin a_2 methyl ester on paper chromatography in these systems, may be readily eluted from alumina with ether or chloroform. The presence of hydroxyl groups in the chlorin would not, therefore, alone account for its behaviour on alumina columns. Mesochlorin was found to run on these columns, so that the reduction of one of the rings of the tetrapyrrole nucleus would not seem sufficient to explain the strong adsorption. It is possible that the adsorption is due to a lipid side chain or to a free lipid with which chlorin a_2 has formed a complex. Indeed the fraction eluted with acid–acetone is fatty, and some lipid can be separated from the chlorin on subsequent partition between ether and hydrochloric acid.

When partition of the haemins between ester and pyridine–hydrochloric acid buffer was carried out, haemin a_2 accompanied protohaemin into the aqueous phase, which contrasts with the behaviour of haemin *a*. Further, when, in the present work, the method of Kiese & Kurz (1954), by which haemin *a* may be separated from protohaemin, was applied to the haemins from *Aero. aerogenes*, no separation of

haemin a_2 from protohaemin was effected. These partition properties of haemin a_2 suggest that it is unlikely that this haem possesses a long-chain alkyl group such as has been demonstrated to be present in haem *a* (Warburg & Gewitz, 1953), but the presence of a side chain which may give rise to the formation of complexes between haem a_2, or chlorin a_2, and certain lipids, is not excluded.

The principal porphyrin obtained after hydriodic acid treatment of chlorin a_2 closely resembles a porphyrin which has been found in very small amounts in some preparations of chlorin a_2. The possible identity of those two porphyrins raises the question whether the latter porphyrin is an artifact produced by hydroxylation of one of the vinyl groups of protoporphyrin or whether it arises through removal from chlorin a_2 of the two extra hydrogens. An attractive hypothesis is that the presence of unsaturated lipids is responsible for the dehydrogenation, a fatty acid peroxide being the active agent.

For the purpose of determining approximately the amount of haem a_2 in bacterial cells it has been assumed that the molar extinction of chlorin a_2 would be similar to that of other true chlorins. The molar ratio of chlorin a_2 to protoporphyrin obtained on removal of iron from crude haemin preparations was found to vary with the intensity of the 630 mμ. band of reduced cytochrome a_2 relative to that of the α band of reduced cytochrome b_1 of the bacterial cells. On the basis of the ratios obtained, cells with the strongest cytochrome a_2 band have a ratio of cytochrome a_2 to cytochrome b_1 of 1:4, and those with a weak cytochrome a_2 band a ratio of 1:10. The best synthesis of cytochrome a_2 has been found to occur under conditions of moderate aeration. This has also been found by Moss (in preparation), who has been able to measure accurately the oxygen tension of the medium during growth of the bacteria.

Because of the structural similarity of mesochlorin to chlorin a_2 it should be possible to use the extinction coefficients of the haemin of mesochlorin for estimations of haemin a_2 with the haemin spectra directly. This would avoid the difficulties associated with removal of iron from the haemins.

SUMMARY

1. A green haemin has been obtained from *Aerobacter aerogenes* and several other bacteria, all of which contained cytochrome a_2. The yield of this haemin was related to the intensity of the 630 mμ. band of cytochrome a_2.

2. This haemin was partially purified, but its lipophilic nature and the similarity of its side chains to those of protohaemin made complete separation from lipids or protohaemin difficult.

3. The spectroscopic properties of this haemin and of its ferrous, carbon monoxide, cyanide and pyridine compounds have been examined. These are consistent with the view that this haemin is the unaltered prosthetic group of cytochrome a_2.

4. Removal of iron from haemin a_2 yielded a new chlorin. This was highly purified, but still remained oily in nature.

5. The chlorin in ether has a principal maximum of absorption at 653 mμ., and three lesser maxima at 598, 573 and 503 mμ. The Soret band is at 405 mμ. In 8% hydrochloric acid the chlorin has its principal maximum at 630 mμ., but this is shifted to 647 mμ. in 16% hydrochloric acid.

6. Paper chromatography of chlorin a_2 methyl ester was carried out in various solvent systems and its behaviour compared with other chlorin-like pigments and with certain porphyrins.

7. Chlorin a_2 was shown to possess at least one vinyl side chain. The presence of a formyl or carbonyl group could not be demonstrated.

8. The chlorin has been converted into a porphyrin by (*a*) catalytic hydrogenation and reoxidation, (*b*) the action of hydriodic acid. The principal porphyrin obtained by the action of hydriodic acid was similar to, but not identical with, monovinylmonohydroxyethyldeuteroporphyrin. The porphyrin obtained by catalytic hydrogenation was similar to, if not identical with, mesoporphyrin.

9. The experimental evidence is discussed, and it is concluded that chlorin a_2 is a chlorin corresponding to protoporphyrin or to a closely related vinylporphyrin possessing a hydroxyethyl or ethyl side chain.

10. A molar extinction for chlorin a_2 based on its similarity to certain other chlorins was assumed. From this value the molar ratio of haemin a_2 to protohaemin has been calculated for the haemins extracted from bacteria of different cytochrome a_2 and b_1 content.

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Non-Enzymic Browning: the Reaction between D-Glucose and Glycine in the 'Dry' State

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The interaction between amino acids or protein and reducing sugars has been shown to be the cause of much of the browning that occurs during the manufacture and storage of dried foods (cf. reviews by Stadtman, 1948; Danehy & Pigman, 1951) and consequently much research has been done on the reaction. Many of the investigators, however, have conducted the experiments in aqueous solution at elevated temperatures, that is, they have used experimental conditions similar to those of Maillard (1912). Under these conditions a large number of compounds are formed (Chichester, Stadtman & Mackinney, 1952) and it becomes difficult to decide which are intermediates in the browning and which are products of side reactions. Also these conditions for the reaction are very different from those found in dried foods. It has been shown, however, (Gottschalk & Partridge, 1950) that mixtures of amino acids and simple sugars under 'dry' conditions, i.e. at low moisture content and at lower temperatures, turn brown and yield chromatographically relatively simple mixtures. These mixtures contain new compounds with the colour reactions of both an amino compound and, more weakly, of a sugar. On their evidence Gottschalk & Partridge (1950) suggested structure (III) for the compounds. Other workers (cf. Hodge, 1953) consider such intermediates to be 1-amino-1-deoxy-2-ketoses formed by an Amadori rearrangement of an initially formed *N*-substituted glycosylamine.

The present investigation is concerned with the interaction between a simple sugar (D-glucose) and an amino acid (glycine) at pH 6·7 in an atmosphere of 70% relative humidity at 37°, conditions which approximate to those which might be found during the tropical storage of foodstuffs.

EXPERIMENTAL

Chromatography. In the following experiments, sheet-paper partition chromatography was done by the descending method (Partridge, 1948) on Whatman no. 1 filter paper. R_F values were given with *n*-butanol–ethanol–water (40:11:19, by vol.) as the mobile phase. The chromatograms were run for about 15 hr., dried and then sprayed with one of the following reagents: (*a*) ammoniacal silver nitrate (Partridge, 1948); (*b*) aniline hydrogen phthalate (Partridge, 1949); (*c*) resorcinol–HCl in butanol (Forsyth, 1948) and modifications; (*d*) 0·2% ninhydrin in wet butanol; (*e*) the Elson & Morgan reagent for glycosylamines as modified for use as a spray (Partridge, 1948); (*f*) alkaline ferricyanide solution followed 5 min. later by ferric reagent (Borsook, Abrams & Lowy, 1955). If sprays (*a*)–(*d*) were used the papers were then heated.

5-Hydroxymethylfurfuraldehyde. This compound was prepared according to Haworth & Jones (1944).

Examination of the reaction between D-glucose and glycine

Equal volumes of M D-glucose and 0·25 M glycine were mixed and brought to pH 6·7 with HCl and NaOH. Portions (2 ml.) were transferred to specimen tubes, dried at 20° in a vacuum desiccator, equilibrated to 70% relative humidity, and then, still in the same atmosphere, held at 37°. At intervals two samples were removed, one for chromatographic and one for spectroscopic examination.

Chromatographic examination. The first sample, which was taken after 2 days of heating, had an amber colour when dissolved in water (2 ml.). In samples taken at longer time intervals the colour was progressively darker until at 8 weeks the solution was almost black. Chromatography revealed the presence of only one additional compound (R_F 0·11) apart from a brown material which did not move from the base-line. The quantity of the compound having R_F 0·11 was so small after 2 days that it was barely detectable on paper chromatograms, but by 6 days it was present