441. *Chlorophyll and Related Compounds. Part IV.* The Position of the Extra Hydrogens in Chlorophyll. The Oxidation of Pyrophœophorbide-a.*

By G. E. Ficken, R. B. Johns, and R. P. Linstead.

From the oxidation products of pyrophæophorbide *a*, an optically active acid imide has been obtained which yields a benzylamine salt indistinguishable in infrared spectrum from the salt of synthetic racemic *trans*-dihydrohæmatinic imide, the preparation of which is described in the following paper. The natural dihydroimide has been converted into racemic *transoid*-dihydrohæmatinic acid identical with synthetic material.

A number of other oxidation products have been either completely or partly separated. Of these the most important is an acid believed to belong to the 2 : 5-dicarboxypyrrole series. This is a new feature in a porphyrin degradation.

The main conclusions are that Fischer's 1940 formulæ for the phorbides and chlorophyll-*a* with the extra hydrogen atoms in ring IV are proved to be correct in this respect; and that the extra hydrogen atoms are in the *trans*-positions.

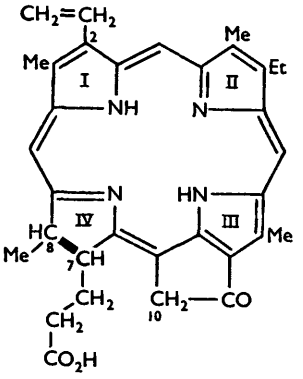
The implications of these results on the structures of chlorophyll-*b* and bacteriochlorophyll are briefly indicated.

In earlier Parts of this series,[1] evidence has been presented which confirms the representation of chlorophyll and its green derivatives as dihydrides of porphyrins. The present paper deals with the *position* which the two " extra " hydrogen atoms occupy.

* Part III, *J.*, 1956, 1655.

[1] Parts I—III : Eisner and Linstead, *J.*, 1955, 3742, 3749; 1956, 1655.

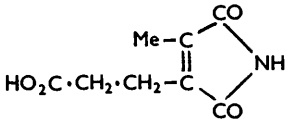
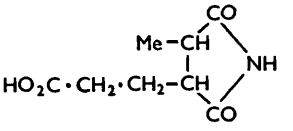
The usual formulæ for chlorophyll-*a* and its near derivatives are those put forward by Hans Fischer in 1940, in which the extra hydrogen atoms are placed on adjacent carbon atoms, $C_{(7)}$ and $C_{(8)}$, of ring IV. This is exemplified in formula (I) for pyrophæophorbide-*a*.



(I) One canonical form is shown; the extra hydrogen atoms are picked out by the thick line. The structure differs from that of chlorophyll-*a* only in three minor respects: the absence of a methoxycarbonyl group at $C_{(10)}$, the removal of the phytyl group from the propionic acid side chain at $C_{(7)}$, and the replacement of the central magnesium by hydrogen.

The evidence is briefly as follows: the retention of the continuous conjugation of the macrocycle was in harmony with the intense light absorption at high wavelengths; secondly, the structure explained the facts that chlorophyll itself was optically active (Stoll and Wiedemann [2]) and that the activity persisted in derived compounds, such as pyrophæophorbide-*a* (I), which contained no other asymmetric centres (Fischer and Stern [3]); thirdly, it was strongly indicated by Fischer's oxidation experiments.

Fischer and Breitner [4] found that ethylmethylmaleimide was always obtained by the chromic acid oxidation of green derivatives of chlorophyll and that a doubled yield of this imide was obtained if the 2-vinyl group was hydrogenated before oxidation. This indicated that rings I and II did not carry the extra hydrogen atoms. Fischer and Stern [5] first placed them on ring III but they were later assigned to $C_{(7)}$ and $C_{(8)}$ of ring IV on the following grounds (Fischer and Wenderoth [6]): (i) It was reported that citraconimide was isolated on oxidation of phyllochlorin, which would mean that ring III must be free from extra hydrogen: the experimental evidence cited, however, was hardly satisfactory. (ii) Hæmatinic imide (II) was never isolated from the oxidation of green derivatives of chlorophyll, which implies that ring IV is not at the ordinary porphyrin level of hydrogenation. This is important, if negative, evidence, for this imide is a regular product from the oxidation of many porphyrins of natural origin. (iii) In place of hæmatinic imide there appears an oily acidic imide which is optically active. Fischer and Wenderoth were unable to obtain this oxidation product solid or to characterise it other than by the formation of a disilver derivative, which gave a correct analysis for silver. They ascribed to it the structure of a dihydrohæmatinic imide (III). The persistence of the optical activity in the fission

(II)  (III) 

product was very important evidence in support of Fischer's formulation. However, the experimental identification was clearly incomplete, more particularly as neither of the two isomeric forms of the imide (III) had been synthesised in the pure state. The matter has rested in this unsatisfactory state for fifteen years.

[2] Stoll and Wiedmann, *Naturwiss.*, 1932, **20**, 706; *Helv. Chim. Acta*, 1933, **16**, 183, 307.
[3] Fischer and Stern, *Annalen*, 1935, **519**, 58; **520**, 88.
[4] Fischer and Breitner, *ibid.*, 1936, **522**, 151.
[5] Fischer and Stern, *ibid.*, 1935, **520**, 88.
[6] Fischer and Wenderoth, *ibid.*, 1939, **537**, 170; 1940, **545**, 140.

Our work is divided into two parts—the synthesis of the reference compounds, which is dealt with in the following paper,[7] and a reinvestigation of the degradation, which is now described. A preliminary account has already appeared.[8]

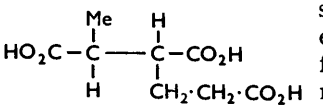
Oxidation of Pyrophæophorbide-a.—We have confined our oxidations to pyrophæophorbide-*a* (I). This is obtained from chlorophyll in two relatively simple series of operations. Hydrogenation of the vinyl group at $C_{(2)}$ would doubtless have simplified the separation of the fission products, as gross degradation of ring I would have been avoided; but we refrained from this step as it would have introduced a slight ambiguity as to the identity of the " natural " extra hydrogen atoms. A commercially available mixture of phæophytins (as I, with phytyl on the $C_{(7)}$-side chain, $-CO_2Me$ at $C_{(10)}$) was a convenient source of material in sufficient quantity. This was subjected to acid fractionation, following Fischer, Broich, Breitner, and Nüssler,[9] a process which removed impurities, separated the *a* and the *b* component, hydrolysed the phytyl group, and largely removed the 10-methoxycarbonyl group by ketonic fission. The process of conversion of phæophorbide-*a* into pyrophæophorbide-*a* was completed by boiling with pyridine. The identity of the pyrophæophorbide-*a* was established by conversion into the methyl ester, spectroscopically identical with that of Fischer, and into the oxime.

Oxidations of pyrophæophorbide were carried out by means of chromic acid in dilute sulphuric acid following the method introduced by Küster, used by Willstätter and by Fischer, and recently studied in considerable detail (for the oxidation of hæmin) by Muir and Neuberger.[10] Some variations in the experimental procedure were examined, in particular the scale and the temperature of the initial reaction, which was varied between $-30°$ and $-10°$. The method described in the Experimental section was standardised as providing fairly reproducible quantities of dihydrohæmatinic imide.

The reaction products constitute a most complex mixture. We have identified, or partly identified, ten products in it and doubtless there are many more. A very wide range of experimental techniques has been examined for the separation, of which the most promising were partition chromatography, solvent extraction at different acidities, and purification through benzylamine salts. The two best procedures were partition chromatography on silica gel, or alternatively a separation into neutral, weakly acid, and strongly acid fractions by successive solvent extractions, first from alkaline solution, then at pH 4, and finally at pH 2. The Experimental section describes details of these methods of separation, and also of some other procedures which readily enabled individual products to be isolated. For the control of isolation procedures, paper chromatography has been used, imide spots being detected by Reindel and Hopper's method [11] (cf. Rydon and Smith [12]). The separation and control procedures were standardised against known mixtures of the key compounds. In agreement with Fischer, the neutral product readily yielded ethylmethylmaleimide as the only detectable product. The " weakly acid " product, isolated by extraction with ethyl acetate at pH 4, yielded an optically active oil which failed to solidify under all the usual stimuli and could not be separated into solid components by chromatography. On treatment with benzylamine it readily gave a crystalline optically active product which gave analytical figures corresponding to the monobenzylamine salt of dihydrohæmatinic imide. The infrared spectrum of the benzylamine salt was identical with that of the benzylamine salt of racemic *trans*-dihydrohæmatinic imide.[7] The identity (apart from the optical activity) was confirmed by m. p. and mixed m. p. determinations. Regeneration of the acid imide from the salt gave an active colourless oil which again failed to solidify. Judged from the optical activity, the purity of the imide was not substantially affected by conversion into the salt and regeneration. Our yield of this optically pure material was considerably higher than that reported by Fischer. As the synthetic racemic *trans*-imide melts at 85°, the obstinate refusal to crystallise of the lævorotatory material

[7] Ficken, Johns, and Linstead, following paper.
[8] Linstead, Eisner, Ficken, and Johns, Chem. Soc. Special Publ. No. 3, 1955.
[9] Fischer, Broich, Breitner, and Nüssler, *Annalen*, 1932, **498**, 241.
[10] Muir and Neuberger, *Biochem. J.*, 1949, **45**, 163; 1950, **47**, 97.
[11] Reindel and Hopper, *Chem. Ber.*, 1954, **87**, 1103.
[12] Rydon and Smith, *Nature*, 1952, **169**, 922.

from natural sources (both ours and Fischer's) is worthy of note. It may well be due to slight contamination by a similar material, for our synthetic imide often crystallised with great reluctance. After various trial experiments, the identification of the natural imide was completed by the following procedure. The imide, regenerated from the benzylamine salt, was completely hydrolysed to the triacid which was esterified with diazomethane. The trimethyl ester was refluxed with sodium methoxide in methanol (to permit racemisation through tautomeric change involving the asymmetric centres), and the product was hydrolysed. The triacid was purified through the anhydride,[7] hydration of which gave crystalline optically inactive *transoid*-dihydrohæmatinic acid (V).* This was identified with synthetic material by its m. p., mixed m. p., infrared spectrum, and analysis.



(V)

Before this result is discussed, the rest of the data on the oxidation will be summarised. The product from which the neutral and the weakly acid components had been removed was acidified to pH 2 and continuously extracted with ether. The strongly acid components so isolated were separated by partition chromatography on silica gel, assisted by paper chromatography and separation as benzylamine salts. In this way there were separated: (i) oxalic acid; (ii) succinic acid; (iii) a pyrrole acid; (iv) traces of *transoid*-dihydrohæmatinic acid; (v) traces of *trans*-dihydrohæmatinic imide; (vi) at least two other unidentified acids, present only in very small amount, and isolated as benzylamine salts. Data for these compounds are in the Experimental section.

The succinic acid clearly came from fission of the side chain at $C_{(7)}$. The *transoid*-dihydrohæmatinic acid, which was identified by paper chromatography, must be an artefact formed by hydrolysis of the imide during the prolonged extractions in acid media. The most interesting and novel material is the pyrrole acid which was present, albeit in very small amount, in the first fractions from the silica-gel columns. This material was a solid, decomposing at 202°, gave a strong Ehrlich reaction, and showed selective ultraviolet absorption, with maxima at 279 and 222 mμ. On treatment with diazomethane it yielded an oily ester which could not be completely purified but clearly resembled in ultraviolet and infrared spectra dimethyl 3-ethyl-4-methylpyrrole-2 : 5-dicarboxylate. We therefore think it highly probable that the acid is 3-ethyl-4-methylpyrroledicarboxylic acid. However, the intensities of the ultraviolet absorption show that the product was not pure and lack of material has prevented a more certain identification. The evidence for the structural type, however, is strong and of some importance, because the isolation of a pyrrole-2 : 5-dicarboxylic acid has, to our knowledge, not yet been recorded in oxidations of porphyrins or chlorins. Muir and Neuberger [10] suggested that some dipyrrolic material may be present in the fragments from the oxidation of *meso*porphyrin. Pyrrole diacids might be formed from the oxidation of ring I, II, or III in appropriate canonical forms of the pyrophæophorbide molecule (I). However, no significant degradation products have ever been isolated with certainty from rings of type (I) or (III) in this molecule and it may well be that the vinyl group (on ring I) or the keto-group (on ring III) provides a point of weakness which leads to entry into the pyrrole ring and its disruption. Any pyrrolic material which survived oxidation would, therefore, be expected to come from ring II, and would be 3-ethyl-4-methylpyrroledicarboxylic acid as seems, in fact, to be the case.

The main significance of the isolation of a pyrrole-2 : 5-dicarboxylic acid is that it would provide for the first time positive analytical evidence that the pyrrole rings in porphyrins and chlorins are linked through carbon links in the α-positions.

Two other features of the oxidations should be mentioned. An appreciable amount of colourless solid was isolated during separation of the acids. This was insoluble in the usual solvents and clearly complex in nature. Although not identical with starting material it had some structural similarity to it, as it yielded dihydrohæmatinic and succinic acid on renewed oxidation. The oxidation products also contained very small quantities of organic material volatile in steam. This material shows carbonyl reactivity and preliminary examination suggests that it may be a saturated aldehyde.

* For nomenclature see the following paper.

The "Extra" Hydrogen Atoms of Chlorophyll.—The evidence reviewed above leaves no doubt than an optically active imide is obtained by the oxidation of pyrophæophorbide-*a*; that this is a dihydrohæmatinic imide and that it belongs to the stereoisomeric series of which the parent acid and imide (in their racemic forms) melt at 143° and 85° respectively (see following paper). The configuration of the imide is not known with certainty but it is very probable that in it the two hydrogen atoms are on opposite sides of the imide ring, as in DL(*trans*)-dimethylsuccinimide (Linstead and Whalley [13]). From these results it is regarded as proved that pyrophæophorbide-*a* has extra hydrogen atoms on $C_{(7)}$ and $C_{(8)}$ in ring IV, as shown in formula (I), and in accordance with the Fischer formulæ of 1940 for chlorophyll and the phorbides in general. There is every reason to believe that this arrangement is generally true throughout the chlorophyll-*a* series. In view of the known close relation [14] between chlorophylls *a* and *b* the extra hydrogen atoms in the *b* series must be in the same positions. Moreover, the evidence can be carried forward to the structure of bacteriochlorophyll. This substance is generally believed to contain four extra hydrogen atoms, although the evidence is incomplete. As it has been converted, by processes involving partial dehydrogenation, into phæophorbide-*a* (Fischer, Mittenzwei, and Hever [15]), two of the extra hydrogen atoms of this compound must also be at $C_{(7)}$ and $C_{(8)}$.

So far, therefore, the new results confirm the Fischer formulation of ring IV. They also provide a basis for an interpretation of the stereochemistry of the chlorophyll molecule. If the key imide (III) obtained by the oxidation of phæophorbide-*a* has, as we believe, the *trans*-structure then the same must be true for the parent phorbide. There are many reasons why this stereochemical connection can be postulated with confidence. First, in the analogous oxidative fission of the hydride of octamethyltetrazaporphin the very labile *cis*-dimethylsuccinimide can be isolated from the products (Linstead and Whalley [16]) and we have now found that *cis*-dihydrohæmatinimide is not inverted under the conditions of oxidation. If therefore the macrocycle has a *cis*-configuration it will appear in the fission product. There is also more direct and conclusive evidence: If an inversion occurred during the total series of operations, it would be accompanied by racemisation. None occurs. It also appears in the highest degree probable that the configuration existing in pyrophæophorbide is also that present in chlorophyll.

In summary, therefore, there are reasonable grounds for assigning a *trans*-dihydro-structure to the chlorophylls and to the natural chlorins and phorbides derived from them.

Experimental

Microanalyses are by Mr. F. H. Oliver, and infrared measurements by Mr. R. L. Erskine of this Department.

Preparation of Pyrophæophorbide-a from Commercial Phæophytin.—A solution of crude phæophytin (Chlorophyll C C; the Allen Chlorophyll Co.) (402 g.) in ether (6 l.) was poured with vigorous stirring into 38% hydrochloric acid (13·6 l.). After 45 minutes' stirring the solution was diluted with water (4 l.) and extracted continuously with ether until no more pigment was extracted. This removed most of the material other than chlorophyll products. The aqueous solution was diluted with water (4·0 l.) and continuously extracted with ether. This extract on evaporation gave only 2·25 g. of mixed phorbide. This was mainly of the *a*-series although from Fischer's work [9] it was expected that *b*-component would separate almost pure at this point. The aqueous solution was further diluted with water (4·3 l.). Ether-extraction yielded only 0·21 g. of pigment. Finally the solution was diluted with 20·5 l. of water and exhaustively extracted with ether. The extract was evaporated to small volume, and the pigment filtered off. The yield at this stage was 10·5 g. and the pigment was pyrophæophorbide contaminated with a little phæophorbide. It was shown spectroscopically to belong entirely to the *a*-series.

[13] Linstead and Whalley, *J.*, 1954, 3722.
[14] Fischer and Gibian, *Annalen*, 1942, **522**, 153; for summary of earlier work see Linstead, *Ann. Reports*, 1937, **34**, 386.
[15] Fischer, Mittenzwei, and Hever, *Annalen*, 1940, **545**, 154.
[16] Linstead and Whalley, unpublished work.

After one crystallisation from acetone, the pigment (1·02 g.) was methylated with diazomethane (from 0·8 g. of methylnitrosourea) in acetone (100 ml.), following Fischer and Spielberger.[17] After two crystallisations from acetone, methyl pyrophæophorbide-*a* was obtained as green needles with a blue reflex, m. p. 213—214° (445 mg.), phase test negative (Found: C, 74·4; H, 7·0; N, 10·3; OMe, 5·4. Calc. for $C_{34}H_{36}O_3N_4$: C, 74·4; H, 6·6; N, 10·2; OMe, 5·7%). Fischer *et al.*[18] gave m. p. 224°.

The phorbide-*a* pigment from the acid fractionation (3·3 g.) was boiled with pyridine (100 ml.) to complete the decarboxylation at $C_{(10)}$. The pyrophæophorbide-*a* (2·15 g.), isolated following Fischer, Riedmair, and Hasenkamp,[19] was crystallised from acetone (green needles, blue reflex) and converted into the oxime (Fischer and Siebel[20]), green needles (Found: C, 72·4; H, 6·5; N, 13·1. Calc. for $C_{33}H_{35}O_3N_5$: C, 72·1; H, 6·4; N, 12·7%), and into the methyl ester, m. p. 216—217°, not depressed by the methyl pyrophæophorbide-*a* prepared without the pyridine treatment. The methyl pyrophæophorbide-*a* had absorption maxima (log ε in parentheses) (in C_6H_6; Unicam SP 600 spectrophotometer) at 414·5 (5·05), 473 (3·61), 509 (4·05), 538·5 (3·98), 562 (3·46), 612·5 (3·46), and 669 mμ (4·70). Stern and Wenderlein[21] gave the following figures for the visible region for methyl pyrophæophorbide-*a*: 507 (4·07), 535·5 (3·97), 559 (3·45), 609 (3·89), and 667·5 mμ (4·71). All the phorbide used in oxidations was crystallised material with the correct light absorption.

Oxidation of Pyrophæophorbide-a.—The following experiment is typical. The finely powdered phorbide (2·0 g.) was stirred into 50% (vol.) sulphuric acid (90 ml.). Ice (90 g.) was added and the solution cooled to −10°. A solution of chromic oxide (4·5 g.) in water (40 ml.) was added in drops during 1 hr. The mixture was stirred for a further 4 hr. during which the bath-temperature rose nearly to that of the room; the bath was then removed and stirring continued for 2 hr. more. The deep green mixture was extracted with ethyl acetate (8 × 50 ml.), then with ether (2 × 50 ml.), and then continuously with ether for 24 hr. The extracts were dried (Na_2SO_4), combined, and evaporated to dryness.

Separation by Acid Fractionation.—The oily residue was taken up in warm water (50 ml.), cooled in ice, and made alkaline with 2N-sodium hydroxide. Insoluble material was filtered off and the alkaline filtrate reserved (A). A typical yield of insoluble product was about 60 mg. but more was obtained in oxidations carried out at lower temperatures. It was an amorphous solid sparingly soluble in most solvents. After three attempted crystallisations it melted indefinitely at about 115—120°. Sublimation lowered the m. p. greatly. Further oxidation of this material gave ether-soluble acidic products in which succinic acid and dihydrohæmatinic acid were identified by paper chromatography.

The alkaline solution (A) was extracted with ether (5 × 50 ml.), and the extracts were dried and evaporated to dryness. The oily residue weighed about 200 mg., but this yield varied considerably in different oxidations. A typical residue (250 mg.) on sublimation gave 93 mg. of ethylmethylmaleimide. This crystallised from *cyclo*hexane–benzene in colourless needles, m. p. and mixed m. p. 63—65° (Found: C, 60·1; H, 6·5; N, 10·2. Calc. for $C_7H_9O_2N$: C, 60·4; H, 6·5; N, 10·1%).

The alkaline solution after these ether extractions was acidified with concentrated hydrochloric acid to pH 4, and then extracted with ethyl acetate (5 × 50 ml.) and then ether (2 × 50 ml.), the pH being kept constant. The combined extracts were dried (Na_2SO_4) and freed from solvent. The yield of red-brown oil (crude dihydrohæmatinic imide) varied from 163 to 380 mg. in various oxidations, the yields being lower from experiments at low temperatures. The crude imide from one oxidation (380 mg.) was treated in ethyl acetate with a small excess of benzylamine. The solid benzylamine salt of (−)-*trans*-dihydrohæmatinic imide crystallised from ethyl acetate–methanol in colourless needles, m. p. 168° (290 mg.): for further characterisation see below.

The aqueous solution after this extraction at pH 4 was acidified to pH 2 with concentrated hydrochloric acid, extracted with ether (4 × 50 ml.) and then continuously with ether for 24 hr. The combined extract was dried and freed from solvent. The residue varied between 360 and 470 mg. and was often partly solid. Trituration of the residue of one experiment with dry ether at this stage yielded 33 mg. of oxalic acid dihydrate (m. p. and mixed m. p.), further identified by conversion into benzylamine oxalate (114 mg.), which after crystallisation from

[17] Fischer and Spielberger, *Annalen*, 1939, **519**, 146.
[18] Fischer, Filser, Hagert, and Moldenhauer, *ibid.*, 1931, **490**, 1.
[19] Fischer, Riedmair, and Hasenkamp, *ibid.*, 1934, **508**, 237.
[20] Fischer and Siebel, *ibid.*, 1932, **494**, 73.
[21] Stern and Wenderlein, *Z. phys. Chem.*, 1935, **174**, 81.

aqueous methanol had m. p. and mixed m. p. 196° (Found: C, 62·8; H, 6·7. Calc. for $C_{16}H_{20}O_4N_2$: C, 63·15; H, 6·6%).

In other experiments the mixed residue from the ether-extraction at pH 2 was taken up in ether and chromatographed on silica gel, wet ether being used as an eluant. Successive fractions of 3—4 ml. of ether were collected and examined separately by paper chromatography. The first 2 or 3 fractions yielded a crude solid (18 mg.), m. p. 180—202°, decomp. about 202°. This gave a strong Ehrlich reaction on gentle warming and had ultraviolet maxima at 279 and 222 mμ. Treatment with diazomethane followed by sublimation gave an oily intractable sublimate with maxima identical with those of dimethyl 3-ethyl-4-methylpyrrole-2 : 5-dicarboxylate (282 and 222 mμ). The intensities of these bands, however, were only about half those of authentic material, indicating the presence of non-absorbing impurity. The infrared spectra (CCl_4 solution) showed pyrrole maxima at 3425, 3279, and 1563 cm.$^{-1}$, together with bands at 1742, 1718, and 1705 cm.$^{-1}$. The fractions which followed the pyrrole acid gave a solid residue which, after trituration with dry ether, yielded crystalline succinic acid (10 mg.), identified by m. p., mixed m. p., and paper chromatography. The ether triturate contained traces of an acid which behaved on paper chromatography with two different solvent systems in the same manner as *rac-transoid*-dihydrohæmatinic acid and was presumably the lævorotary enantiomorph of this acid. The fractions which followed the succinic acid contained a little *trans*-dihydrohæmatinic imide which had not been completely extracted at pH 4. It was identified as the benzylamine salt. Other minor oxidation products are mentioned below.

Racemisation and Hydrolysis of "Natural" Dihydrohæmatinic Imide.—The crystallised benzylamine salt of the lævorotatory "natural" imide (280 mg.) was dissolved in dilute acid and the free imide recovered with ether. The imide was hydrolysed with boiling dilute hydrochloric acid for 5 hr. The product was evaporated to dryness, treated with a little water, and continuously extracted with ether for 10 hr. The ether extract was evaporated, and the residue dried in a vacuum-desiccator for 2 days and treated with dry distilled diazomethane in ether. After removal of the solvent, the ester was taken up in methanol and refluxed for 24 hr. with an excess of sodium methoxide. Water (5 ml.) was added and the mixture boiled for 5 hr. The product was acidified, evaporated to dryness, taken up in water (20 ml.), and continuously extracted with ether. The extract was dried and freed from solvent, an oily residue being left which soon crystallised (166 mg.). The solid was triturated with ether and the less pure, soluble material was purified by sublimation and hydrolysis of the anhydride. The combined solid acid was finally heated at 160° for 3 hr., the anhydride sublimed, and the sublimate hydrolysed with water. Removal of the water left solid transoid-*dihydrohæmatinic acid* which crystallised from ethyl acetate–benzene–methanol in needles, m. p. 140—142°; a mixture with the pure synthetic acid (m. p. 144°) melted at 141—143° (Found: C, 47·1; H, 6·05. $C_8H_{12}O_6$ requires C, 47·1; H, 5·9%). The infrared spectrum of the racemised acid from natural sources was identical with that of the synthetic *transoid*-dihydrohæmatinic acid.

Chromatographic Data and Separations.—(1) *Paper chromatography of imides.* 5% Solutions of the following imides in ethanol were used: ethylmethylmaleimide; hæmatinic imide; synthetic *rac-trans*-dihydrohæmatinic imide, m. p. 85°. These were spotted on paper and developed both with ethyl acetate and with butan-1-ol as the mobile phases. Imide spots were developed following Rydon and Smith [12] or Reindel and Hopper.[11] The following R_F values were obtained:

	Ethylmethylmaleimide	Hæmatinic imide	*trans*-Dihydrohæmatinic imide
Ethyl acetate	0·98	0·92—0·93	0·78
Butan-1-ol	0·89—0·82	0·56—0·57	0·39—0·40

Synthetic mixtures of these three compounds gave spots with the same R_F values. The procedure was applied to the total oxidation product of pyrophæophorbide-*a*. For this purpose the phorbide (310 mg.) was oxidised with chromic acid as before and the product was extracted thoroughly with ethyl acetate, without acid fractionation. A solution of the product in 10 ml. of ethanol run on paper gave only two clearly defined imide spots: in ethyl acetate R_F 0·98 and 0·78; in butan-1-ol R_F 0·89 and 0·42. These values correspond closely to those for ethylmethylmaleimide and *trans*-dihydrohæmatinic imide.

(2) *Partition chromatography of imides.* A mixture of ethylmethylmaleimide (63 mg.), hæmatinic imide (52 mg.), and *rac-trans*-dihydrohæmatinic imide (m. p. 85°; 68 mg.) was dissolved in ethyl acetate (5 ml.) and chromatographed on silica gel (57 × 2 cm.) saturated with water. The column was eluted with ethyl acetate and 4 ml. fractions were collected. Fractions 1—3 totalled 1·2 mg.; 4—10, 96·8 mg.; 11, 1·5 mg.; 12—26, 52·9 mg. Fractions 4—10

contained both ethylmethylmaleimide and hæmatinic imide. As indicated by the paper chromatograms these could not conveniently be separated by partition chromatography. These fractions combined were accordingly sublimed at 100°/20 mm. The sublimate was nearly pure ethymethylmaleimide, m. p. 53—60°, mixed m. p. 59—63°. The residue from the sublimation yielded the benzylamine salt, m. p. and mixed m. p. 156—158°, of hæmatinic imide (see following paper). Fractions 12—26, combined, yielded the benzylamine salt, m. p. 158—160°, of dihydrohæmatinic imide, not depressed on admixture with authentic material. The recoveries of crystalline material were : ethylmethylmaleimide, 41 mg. (65%); hæmatinic imide benzylamine salt, 59 mg. (72%); dihydrohæmatinic imide benzylamine salt, 73 mg. (69%). The separation of imides was not affected by the presence of oxalic acid which was retained on the column.

(3) *Partition chromatography of pyrophæophorbide oxidation product.* The total oxidation product from 773 mg. of phæophorbide-*a* was isolated by ethyl acetate extraction without acid fractionation. It was dissolved in ethyl acetate (5 ml.) and chromatographed on silica gel as described above, 4 ml. fractions being collected. The product separated as follows : Fractions 1—2, nil; 3—11, 234·2 mg.; 12—13, 16·3 mg.; 14—20, 75·0 mg.; further fractions totalling 236 ml. of solvent yielded only another 35·5 mg. Fractions 3—11, combined and cautiously sublimed under reduced pressure, yielded 72 mg. of ethylmethylmaleimide (m. p. and mixed m.p.). The residue from the sublimation gave no solid benzylamine salt. Fractions 14—20, combined, gave an oil with $[\alpha]^{21\cdot1}_{589\cdot3}$ $-50° \pm 2°$, $[\alpha]^{22\cdot1}_{640-690}$ $-37° \pm 2°$ in MeOH. It yielded *trans*-dihydrohæmatinic imide benzylamine salt, m. p. 164—165° $[\alpha]^{20}_{D}$ $-25° \pm 1°$ in MeOH (Found : C, 60·9; H, 6·9; N, 9·5. $C_{15}H_{20}O_4N_2$ requires C, 61·0; H, 6·9; N, 9·6%). The infrared spectrum (Nujol) showed maxima at : 1297 m; 1264 m; 1217 m; 1188 s; 1167 and 1159 w; 900 w; 888 w; 871 m; 823 m; 795 m; 772 m; 754 s; 741 m; 719 w; 696 m; 686 cm.$^{-1}$. These are identical within the limits of error with the maxima shown by the benzylamine salt of synthetic *rac-trans*-dihydrohæmatinic imide and show a number of differences from the corresponding *cis*-benzylamine salt (see following paper). Regeneration of the imide by treatment with dilute acid followed by ether extraction gave the lævorotatory imide as a colourless oil which failed to solidify, having $[\alpha]^{20\cdot5}_{589\cdot3}$ $-46° \pm 3°$ in MeOH.

(4) *Paper chromatography of acids.* Paper chromatography was used to identify two of the strong acids obtained by the final extraction of the oxidation product at pH 2. Two mixtures of solvents were found to be most suitable in these chromatograms : (*a*) Pyridine–ammonia–water 6 : 2 : 1; (*b*) butan-1-ol–formic acid–water 95 : 5, saturated with water.

R_F values obtained with reference compounds were :

			Dihydrohæmatinic acid	
Solvents	Succinic acid	Malonic acid	*cisoid*	*transoid*
(*a*)	0·36	0·23	0·28	0·23
(*b*)	0·70	0·57	0·84	0·78

The succinic acid obtained from the oxidation product had R_F values (*a*) 0·36 and (*b*) 0·70. The *transoid*-dihydrohæmatinic acid in the oxidation product had R_F values (*a*) 0·23 and (*b*) 0·78.

Minor Oxidation Products.—(i) From the extraction at pH 2 of the oxidation product of pyrophæophorbide-*a*, after chromatography on silica gel, an unidentified acid was separated in traces as a benzylamine salt, m. p. 165° (Found : C, 66·9; H, 6·4%) : infrared maxima at 3268, 1642, and 1620 cm.$^{-1}$. Ultraviolet : end absorption only.

(ii) The accumulated acid residues of earlier oxidations were chromatographed on silica gel from benzene solution. A single band was obtained which on treatment with benzylamine slowly gave a very small amount of a solid salt which, crystallised from ethyl acetate–methanol, had m. p. 127—128° (Found : C, 68·9, 69·9; H, 6·5, 6·5; N, 7·35%). Ultraviolet max. at 228—230 mμ; infrared maxima (Nujol) at 1701, 1678 (infl.), 1616, and 1555 cm.$^{-1}$; it took up hydrogen over Adams catalyst. These data, although inconclusive, indicate that the material is the benzylamine salt of an αβ-unsaturated acid, possibly the dibenzylamine salt of a rather simple *cis*-dibasic acid.

(iii) *Steam-volatile oxidation product.* The united extracts from a number of oxidations were distilled in steam, and the distillate treated with 2 : 4-dinitrophenylhydrazine. The yellow solid was chromatographed in chloroform containing 1% of ethanol on bentonite–kieselguhr, following the technique of Elvidge and Whalley.[22] The main band yielded an orange solid which after three crystallisations melted at 155°. The ultraviolet absorption showed maxima at 355 and 224 mμ with an inflexion at 248—250 mμ. There is a resemblance to the spectrum

[22] Elvidge and Whalley, *Chem. and Ind.*, 1955, 589.

of propionaldehyde 2 : 4-dinitrophenylhydrazone, which shows maxima at 357, 278, 256, and 228 mμ (Braude and Jones [23]) but the mixed m. p. shows a depression to 143—146°. Lack of material prevented further examination, but there is no doubt that a volatile saturated carbonyl compound is present in traces in the oxidation product.

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[23] Braude and Jones, *J.*, 1945, 498.