Attempted Isolation of Haem \( a \) and Porphyrin \( a \) from Heart Muscle

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Haem \( a \) is the name which has been given (Rawlinson & Hale, 1949) to the haem which is apparently the prosthetic group of at least some of the cytochromes \( a \). Studies of the visual spectrum of this haem, and of its iron-free derivative, porphyrin \( a \), in comparison with the spectra of other porphyrins and haems of known structure (Lemberg & Falk, 1951) have led to the postulation of two possible structures, consistent with all the data available.

In addition to visual spectra an extensive study has been made of the infrared spectra of most natural and many synthetic porphyrins and haems (Falk & Willis, 1951) in the hope that these might provide an analytical tool through which further details of the structure of haem or porphyrin \( a \) may be made clear. The infrared spectra of porphyrins and haems provide a useful means of identification, not only of individual porphyrins, but also of certain side chains on porphyrins. When the infrared spectrum of pure haem or porphyrin \( a \) can be obtained, some further light may be thrown on its structure by this means. Unfortunately, the isolation of pure material has not yet been achieved. The study reported below concerns further work on the isolation of the compound, for the purpose of obtaining pure material for measurement of its infrared spectrum and, if it could be obtained in sufficient quantity, for direct chemical study. Haem \( a \) of doubtful purity has been obtained in very small quantities from horse heart (Negelein, 1933; Roche & Bénévent, 1936) and from both ox heart and the cells of Corynebacterium diphtheriae (Rawlinson & Hale, 1949). The haemochromogen band of this haem, at about 587 m\( \mu \), is easily observed with the hand spectroscope in pyridine extracts of many tissues (heart muscle, pigeon-breast muscle, insect thoracic muscle) after dilution with water and reduction with \( \text{Na}_2\text{S}_2\text{O}_4 \). Attempts to isolate the haem are complicated, however, by four factors: (1) The lability of the haem itself, particularly in the presence of tissue components such as cysteine (Rawlinson & Hale, 1949). (2) The concurrent extraction of much protohaem (from haemoglobin, myoglobin, catalase, etc.). (3) The concurrent extraction of a lipid material which it is extremely difficult to remove completely. (4) The relatively minute amount of the haem present in the tissues.

Negelein's (1933) method depended on the extraction of the total haems from water-washed, minced muscle with acetone acidified with hydrochloric acid. The yield of material with 'a very weak protohaemochromogen band at 557 m\( \mu \) was 12 mg. from 5 kg. of fresh mine. The Soret band was at about 430 m\( \mu \).

Negelein considered that the haemochromogen of haem \( a \) had a single visual band (at 587 m\( \mu \)). Roche & Bénévent (1936), on repeating Negelein's procedure, obtained a compound with haemochromogen bands at 587 and 530 m\( \mu \). By a modification of Negelein's process they obtained a compound, completely free from protohaem, with only a single band in the visual region, at 587 m\( \mu \); the Soret band was at 425 m\( \mu \). Roche & Bénévent were unable to crystallize this compound satisfactorily; they presented evidence which led them to believe that the compound with the two-banded haemochromogen (587 and 530 m\( \mu \)) was the true haem \( a \), and the compound with a single visual haemochromogen band an artifact.

In 1949 Rawlinson & Hale developed a new method for the separation of haem \( a \) from protohaem. After extraction of the haems from the tissues by acetone-hydrochloric acid, they were transferred to ether, adsorbed on a column of aluminium oxide, some lipid removed by washing the column with ether, the haems eluted with hot glacial acetic acid, and transferred again to ether. On extracting this ether solution several times with an aqueous pyridine-hydrochloric acid buffer the protohaem was completely removed, leaving haem \( a \) in the ether phase. The haem so obtained was contaminated with lipids, but its haemochromogen had only a single absorption band in the visual region (at 587 m\( \mu \)). Rawlinson & Hale found that the haem could react with compounds such as cysteine to yield a substance which gave a haemochromogen with visual bands at 553 and 525 m\( \mu \), and that such reactions could occur during isolation by unsatisfactory procedures. They considered, and it now appears acceptable, that the natural haem \( a \) is the compound which has a haemochromogen with the single visual band (at 587 m\( \mu \)). Though Rawlinson & Hale's process was a great improvement on Negelein's, involving far simpler and fewer manipu-
lations, the yield was very small, and the product, which was contaminated by lipids, was unstable. Rawlinson & Hale (1949) prepared a porphyrin from this haem; the visual spectrum of the porphyrin prepared in this manner was used as a basis for some of the work of Lemberg & Falk (1951).

Negelein had earlier (1932a) reported the isolation of a porphyrin from pigeon-breast muscle which he called 'cryptoporphyrin'. The haem prepared by the introduction of iron into this porphyrin gave a haemochromogen with bands (about 582 and 531 m\(\mu\)) recalling those of some other early haem \(a\) preparations, and at first he thought that this was possibly the porphyrin of the prosthetic group of the cytochromes \(a\). Shortly afterwards, however, Negelein (1932b) reported evidence which led him to believe that this porphyrin was an artifact arising from protoporphyrin through the action of hydrochloric acid during the isolation; indeed, in the original paper he reported that the porphyrin could be obtained from crystallized, but not recrystallized, haemin from blood. No cytochrome \(a\) has ever been identified in blood, and there is thus good evidence that the porphyrin was an artifact. This was further discussed by Lemberg & Falk (1951).

It was now sought, after extraction of the haems from ox heart and conversion of these to porphyrins, to prepare porphyrin \(a\) in greater quantity and in a pure state. A process was indeed found by which relatively large amounts of porphyrin, free from protoporphyrin, can be prepared conveniently in ordinary laboratory apparatus. It has been shown, however, that porphyrin \(a\) prepared by this method, and presumably by any method so far available, is a mixture of closely similar substances. Evidence is presented which shows that these substances arise, during the isolation, from one, or at most relatively few precursors.

The cause of the degradation of the original substance(s) has been found to be the action of acid and no process has been found in which this can be avoided entirely. Until such a process is devised, the problem appears to be insoluble.

**MATERIALS AND METHODS**

Absorption spectra were measured with a Beckman photoelectric spectrophotometer.

Ether was treated to remove peroxides.

Hydrochloric acid concentrations. Because the familiar HCl number (Willstätter no.) widely used in the purification of porphyrins is stated in terms of \(\% (w/v)\) HCl, this form is used instead of normality.

Reaction with hydroxyamine. To a solution of the porphyrin in pyridine, excess of a mixture of equivalent amounts of solid hydroxyamine hydrochloride and \(\text{Na}_2\text{CO}_3\) was added, the mixture refluxed gently for 5 min., cooled and filtered.

**Preparation of porphyrin \(a\). Method \(A\)**

1. **Extraction of haems.** Fresh ox heart (4-6 kg.), dissected free of macroscopic fat, yielded 3-2 kg. of minced muscle; this was washed twice with acetone at 0\(^\circ\), pressing out each time, and air dried (800 g.). Of this dried mince, 500 g. were extracted at 3\(^\circ\) for 2 hr. with 2 l. acetone containing 40 ml. conc. HCl. The extract was filtered from the tissue residue; so little haem remained in the tissue that a second extraction was not profitable. The filtrate was mixed with an equal volume of ether, and the acetone and HCl washed out with 2\% NaCl to minimize emulsions.

2. **Preliminary defatting.** The ether solution was now run through a column (10 \times 3 cm.) of MgO grade III (Nicholas, 1951) packed in ether; the haems were adsorbed as a very deeply coloured layer at the top of the column. The column was then washed with ether (about 2 l.) until the ether running through no longer left a fatty residue on evaporation. The dark zone containing the haems was separated from the column, and the haems eluted with glacial acetic acid. Since MgO dissolves in glacial acetic acid, the elution was quantitative and could be done at the melting point of acetic acid.

The acetic acid solution was mixed with about 2 l. ether, and the acetic acid and the magnesium acetate washed out with 2\% NaCl; the ether was then removed in vacuo.

3. **Removal of iron from haems.** The residue was dissolved in 100 ml. hot glacial acetic acid, and this solution treated in 20 ml. portions as follows. The haem solution was brought quickly to the boil, and while refluxing gently, about 5 ml. of a boiling saturated solution of ferrous acetate in acetic acid (prepared under CO\(_2\)) and 2 ml. conc. HCl were added. The resulting porphyrin solution was cooled as quickly as possible under the tap. This is the process of Warburg & Negelein (1932), modified so as to use the least possible amount of heat.

The several lots of porphyrin solution so prepared were combined, mixed with 2 l. ether, the acetic acid neutralized with sodium acetate, and the ether solution of the porphyrins washed several times with 2\% NaCl.

4. **Removal of protoporphyrin from the porphyrin mixture.** The ether solution was shaken with 500 ml. portions of 4\% (w/v) HCl until no more protoporphyrin was removed. This was usually achieved in six or seven extractions; very little porphyrin \(a\) was extracted at the same time, but most of it remained in the ether phase, where its absorption bands could be seen with the hand spectroscope at 648, 582, 560, 518 m\(\mu\) approx. Emulsions were broken when necessary by centrifuging.

5. **Removal of lipids by treatment with 25\% hydrochloric acid.** The ether solution, besides the porphyrin \(a\), still contained much lipid material. It was found that this could be quantitatively removed as follows. The ether was removed in vacuo, and the residue shaken with 25\% HCl at -10\(^\circ\). After standing for about an hour at this temperature the porphyrin was in solution and the lipids which had solidified were easily separated by gravity filtration at -10\(^\circ\) (Whatman no. 54 paper). The filtrate was clear and olive-green in colour. Ether was added, the mixture diluted with water, and on neutralization with sodium acetate the porphyrin was transferred to ether. The ether solution could now be washed with water; indeed, after this treatment, no more emulsions occurred at all. No more fatty material could be removed by repeating the 25\% HCl treatment. The porphyrin now appeared to be stable if kept in ether or pyridine.
solution. No change could be detected by spectrophotometric measurements in material stored for several months at 3°C.

From 500 g, dried mince (equivalent to 2000 g, fresh muscle) yields of 18–20 mg of this porphyrin were regularly obtained in the course of a working day. Its spectroscopic properties were very close to those of the porphyrin prepared by Rawlinson & Hale (1949) (cf. Table 2).

**Preparation of porphyrin a. Method B**

The haems were extracted from the aceton-dried tissue as in method A, step 1, and gross fat removed as in step 2, except that a column of Al₂O₃ (Savory & Moore) was used instead of MgO, and the haems eluted by several lots of hot glacial acetic acid.

After the elution the haems were again taken into ether, and the ether solution shaken repeatedly with an equal volume of pyridine-HCl buffer (30 vol. pyridine, 0.15 x HCl to 100 vol.; cf. Rawlinson & Hale, 1949) until no more protohaem remained in the ether phase. The ether solution of crude haem a was then evaporated to dryness in vacuo. A portion now dissolved in pyridine, diluted with 2 vol. of water, and reduced with Na₂S₄O₆ gave a haemochromogen curve identical with the curve published by Rawlinson & Hale (with a single visual band at 587 μμμμ).

The haem was now dissolved in glacial acetic acid, and the iron removed as in step 3 above; the porphyrin obtained was treated with 25% HCl as in step 5.

**RESULTS**

**Porphyrin prepared by Method A.** Spectrophotometric curves of the material before and after the treatment with 25% HCl, and of the fatty residue, are shown in Fig. 1. The ratios of the intensities of the absorption bands I–III to that of band IV provide a useful means of comparing such curves (Table 1). As may be seen from Fig. 1, with the removal of the strong absorption in the blue region due to the fat, the intensities of bands I–III increase relative to IV, though the positions of the maxima are hardly changed.

**Porphyrin prepared by Method B.** This process is essentially the same as that used by Rawlinson & Hale (1949) for the preparation of their porphyrin a; the main difference is that instead of the treatment with 25% HCl they removed some fatty material from the porphyrin by repeated transfers between HCl and ether.

Absorption curves of the porphyrin before and after the 25% HCl treatment were similar to those shown in Fig. 1. Indeed, the material obtained by this method behaved in all respects like that from Method A. The manipulations were much more troublesome, however, and the yields much smaller and for most of the experiments reported below material prepared by Method A was used.

![Fig. 1. Visual absorption spectrum of the porphyrin: a, before, and b, after the treatment with 25% HCl (stage 5, Method A); ---, the fatty residue. Solvent, pyridine.](image-url)

**Preliminary ether-HCl fractionation of porphyrin a**

Rawlinson & Hale observed (personal communication) that the HCl number of their porphyrin apparently became lower as transfers between HCl and ether were repeated. We made similar observations. Thus before the 25% HCl treatment (step 5) it was possible to remove the protoporphyrin with 4% HCl (step 4) without appreciable loss of porphyrin a. After the treatment, however, even 1% HCl extracted significant amounts of porphyrin a-like material from ether.

We found, on preliminary fractionation of our material, that 6% HCl removed a considerable fraction, and when no more porphyrin was removed by acid of this strength, a further fraction at least as large could be extracted by 15% HCl. Absorption data (in pyridine) for typical 6 and 15% fractions are shown in Table 2, where the measurements of Rawlinson & Hale (1949), calculated to the same form, are included for comparison. The positions of the bands in all the materials were very similar, but band I (about 650 μμμμμμ) in the 15% fraction was more intense than in the fractions extracted by weaker HCl solutions.

It was evident that our porphyrin a, which spectrophotometrically was virtually identical with that described by Rawlinson & Hale, was a mixture. It

| Table 1. Positions of absorption maxima, and ratios of intensities of the porphyrin (in pyridine solution) a, before, and b, after the treatment with 25% HCl (cf. Fig. 1) |
|---|---|---|---|---|
| Positions of maxima (μμμμμμ) | Band | IV | III | II | I |
| a | 517 | 560 | 582 | 647 |
| b | 517 | 560 | 583 | 648 |

| Intensities, relative to band IV | Band | IV | III | II | I |
|---|---|---|---|---|
| a | 1.0 | 1.197 | 0.852 | 0.295 |
| b | 1.0 | 1.525 | 1.148 | 0.328 |
 HAEM a FROM HEART MUSCLE

Table 2. Positions of maxima and ratios of intensities of absorption bands of porphyrin preparations (see text); solvent, pyridine

<table>
<thead>
<tr>
<th>Band</th>
<th>Positions of maxima (mμ)</th>
<th>Intensities, relative to band IV</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IV</td>
<td>III</td>
<td>II</td>
</tr>
<tr>
<td>Porphyrin</td>
<td>516</td>
<td>559</td>
<td>582</td>
</tr>
<tr>
<td>prepared</td>
<td>517</td>
<td>560</td>
<td>583</td>
</tr>
<tr>
<td>by Method</td>
<td>518</td>
<td>562</td>
<td>583</td>
</tr>
<tr>
<td>Fraction</td>
<td>517</td>
<td>562</td>
<td>584</td>
</tr>
<tr>
<td>extracted</td>
<td>6 % HCl</td>
<td></td>
<td>Hale’s pyridine buffer treatment). Further, the absorption curves (cf. Fig. 1) before and after the 25 % HCl treatment suggested only that this treatment caused a fall in the absorption at the region of 500–520 mμ, relative to that at about 650 mμ, and not a specific increase in intensity of the band at 650 mμ.</td>
</tr>
<tr>
<td>by Method</td>
<td>15 % HCl</td>
<td></td>
<td>The hypothesis that the substance of higher HCl number was an artifact was thus not directly proved or disproved.</td>
</tr>
<tr>
<td>Fraction</td>
<td></td>
<td></td>
<td>A possible alternative hypothesis was that it was the substance with lower HCl number and band I of lower intensity which was the artifact. If this were the case, the experiments described above should have provided evidence about it just as well as the original hypothesis they were designed to test. The one process not yet tested was the action of aqueous HCl. The reason which made it appear unlikely that some effect of HCl could have been the cause of the appearance of the substance of higher HCl number (observation of the band before any aqueous HCl had been used) argues not against, but for the possibility that the converse process was taking place, namely, some change was caused by aqueous HCl as a result of which the substance of lower HCl number was derived from the substance of higher HCl number.</td>
</tr>
<tr>
<td>extracted</td>
<td></td>
<td></td>
<td>Evidence about this was sought by careful fractionation, with HCl, of ether solution of porphyrin prepared by Method A, and refractionation in the same way of the fractions so obtained.</td>
</tr>
<tr>
<td>by Method</td>
<td></td>
<td></td>
<td>Full fractionation and refractionation of the porphyrin preparation</td>
</tr>
<tr>
<td>Fraction</td>
<td></td>
<td></td>
<td>An ether solution of the porphyrin (free from protoporphyrin) prepared by Method A was extracted with successive portions of HCl as shown in Fig. 2a. The volume of the ether phase was kept constant by the addition of fresh ether as required, and the volume of HCl used at each extraction was equal to that of the ether. The position and intensity of maximum absorption in the Soret region (about 410 mμ) was determined in each HCl extract. Beer’s law was obeyed at the concentrations used, and the density readings were proportional to the porphyrin content of each fraction. For this purpose</td>
</tr>
<tr>
<td>extracted</td>
<td></td>
<td></td>
<td>was at first thought that the material with higher HCl number and increased intensity of band I might be an artifact which had arisen during the manipulations. Artifacts with such characteristics are not uncommon in porphyrin chemistry. Controlled experiments showed that the proportion of this material obtained was not influenced by:</td>
</tr>
<tr>
<td>by Method</td>
<td></td>
<td></td>
<td>(1) The length of time for which the acetone-dried mince was stored (at 3°) before extraction. There did not appear to be any significant spectrophotometric difference between the product obtained from one half of a batch of acetone-dried ox-heart mince which was extracted at once, and the products from the other half, which was extracted after it had been stored for 24 days at 3°.</td>
</tr>
<tr>
<td>Fraction</td>
<td></td>
<td></td>
<td>(2) The length of time the mince stood with acetone-HCl for extraction of the haems. A batch of acetone-dried ox-heart mince was halved. One half was extracted with acetone-HCl for 1-5 hr. and the porphyrin a prepared by Method A immediately. The other half was extracted for 18 hr. and the porphyrin prepared in the same way. There was no significant difference in the spectrophotometric properties, nor in the relative amounts of the porphyrins extractable by 6 and by 15 % HCl in each experiment.</td>
</tr>
<tr>
<td>extracted</td>
<td></td>
<td></td>
<td>(3) The use of the magnesium oxide or aluminium oxide columns. A batch of the porphyrin was prepared essentially by Method A, the preliminary defatting on the column (step 2) simply being omitted. The procedure was rendered rather more difficult by emulsion formation, but the 25 % HCl treatment removed the fat completely. The product was extracted exhaustively with 6 %, and then with 15 % HCl; two crude fractions were again obtained, their spectrophotometric properties being similar to those reported above (Table 2).</td>
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<tr>
<td>by Method</td>
<td></td>
<td></td>
<td>There remained as a possible cause of degradation the treatment with aqueous HCl—at the stages of ether-HCl fractionation, and the 25 % HCl treatment for the removal of fat. Most known porphyrins are quite stable to such treatment, and in addition the strong band at about 650 mμ was observed with the hand spectroscope in preparations which had never been treated with aqueous HCl (protohaemin having been removed by Rawlinson &amp; Hale).</td>
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</table>
it was not necessary to adjust the HCl concentration to the same value in every fraction.

The distribution of porphyrin in the successive fractions is shown in Fig. 2a. It is obvious that perfect separation into true fractions was not achieved, but there is good evidence for the presence of several (at least four) components. For the refractionation, the combined 2, 3, 4 and 6 % extracts (b), the 8 and 10 % extracts (c), and 12, 15 and 20 % extracts (d) were transferred to ether, and the ether (c) on refractionation yielded significant fractions to 1, to 8 and to 10 % HCl, but virtually no porphyrin remained after this (fluorescence under ultraviolet light hardly visible). This meant that the fractions which passed from the original into 15 and 20 % HCl were not present in fraction (c), nor did they arise during the refractionation. The 1 % fraction of the original should equally have been excluded from fraction (c), but apparently more of this material arose during the refractionation. This direction of

Fig. 2. a, the relative yield of porphyrin (determined at the Soret maximum, see text) at successive extractions with HCl. Each step on the histogram represents one extraction with HCl of the concentration shown on the abscissa. b–d, the relative yields on refractionation, in the same way, of b, the combined 2, 3, 4 and 6 %; c, the combined 8 and 10 % (readings x 4); and d, the combined 12, 15 and 20 % (readings x 8) fractions from the fractionation shown in 2a.

solutions (b–d) extracted with successive lots of HCl in the same way as in the original fractionation. The results of these refractionations (Fig. 2 b–d) were very interesting. It was evident that shaking the ether solutions with aqueous HCl caused a degradation. The direction of this degradation was from products with higher, to products with lower, HCl numbers.

Thus fraction (d) could hardly have contained any of the original material which was extracted by HCl concentrations lower than 8 %, yet on refractionation it yielded a pattern of fractions very like that from the original porphyrin solution. Again fraction the degradation, towards products with lower HCl numbers, is strikingly confirmed by the results of refractionation of fraction (b).

Absorption spectra of fractions. The first HCl extract at each HCl concentration (cf. Fig. 2a), as the fraction least likely to be contaminated with material of higher HCl number, was transferred to ether, washed well, the ether removed in vacuo, and the porphyrin dried and dissolved in pyridine. Absorption curves in the visual region were taken on each of these pyridine solutions; the positions of the maxima and the ratios of the intensities of the bands are shown in Table 3. It was found useful to
introduce a graphical method for comparison of the spectroscopic properties of these rather similar materials. The changes in position of the maxima are readily appreciated from a consideration of the table, but the pattern in the ratios of intensities of the bands in the different fractions is made much clearer by plotting them as in Fig. 3.

The similar data from the refractionation of fractions (b) and (d) are shown in Fig. 4, in which the pattern of the ratios of intensities of the bands in the original fractions is strikingly reproduced. This similarity leaves no doubt that really different materials are contained in these different fractions.

It is noteworthy that all the compounds from refractionation of fraction (d) (Fig. 4 a) have the relative intensities of bands II and III depressed in comparison with the corresponding original fractions (Fig. 3). For example, the porphyrin extracted by 6% HCl (Fig. 3) has a true oxorhodo type spectrum, while the 6% fraction (Fig. 4 a) has only a weak rhodo type spectrum. The accuracy of the determination of the ratios relative to band IV was approximately ± 0.02 unit for bands II and III and ± 0.05 unit for the much weaker band I. Band I was significantly more intense in the 15 and 20 % fractions, and this increased intensity was paralleled by a slight shift to longer wavelengths. This was the only consistent marked change in the positions of the maxima.

Fig. 3. Diagram showing the change of intensities of bands I–III relative to band IV; data from Table 3. The relative intensities after reaction with hydroxylamine are shown as –O–.

Fig. 4. The changes in relative intensities in the materials from: a, stage d, Fig. 2; b, stage b, Fig. 2. Relative intensities after reaction with hydroxylamine are shown as –O–.

Fractionations in the above manner were carried out on several samples of porphyrin, prepared by Method A from different batches of ox hearts, with consistent results.

Effect of heating the porphyrin with aqueous hydrochloric acid

Since it appeared that the changes were caused in some way by aqueous HCl, its effect was studied in more detail. It was hoped that the material might be degraded by HCl under more drastic conditions to a useful, single degradation product.

In a preliminary experiment some material prepared by Method A was fractionated as in Fig. 2a above. The combined 8 and 10 % fractions, and the combined 12, 15 and...
20% fractions were transferred to ether, the solution washed, the ether removed, and the residues dissolved in 25% HCl. These HCl solutions were each divided into four portions. Portion I was at once transferred to ether, washed, expected to shift towards shorter wavelengths; there were not any significant changes in the positions of the other bands. The changes in the relative intensities of the bands were, however, quite marked and these are shown in Fig. 5.

It is seen that the 25% HCl, even at -10°, caused a depression of the intensities of bands II and III relative to that of band IV. The depression of these bands was increased and hastened as the temperature increased. These measurements were on the whole samples, without fractionation. The depression of the bands in the separate fractions (Figs. 3 and 4a) was paralleled in the present experiment by the depression of the bands in the unfractonated material.

In another experiment, porphyrin prepared by Method A was treated, in HCl solution, as indicated in Fig. 6. The material, which in this experiment had been treated at 57° for 6 hr., was transferred to ether and fractionated with HCl. The pattern of relative intensities of the absorption bands of the fractions was similar to that shown in Fig. 4a. As in the previous experiment, the depression of the ratios of bands II and III increased both with time and temperature, though the band positions hardly changed. Even treatment of a 25% HCl solution at 95° for 5 hr., however, led to a change only from oxorhodo- to rho-type spectrum, band I remaining at 644 mμ.

Reactions with hydroxylamine

Various fractions were treated with hydroxylamine, and the spectroscopic properties of the products are indicated in Figs. 3, 4a and 6. It may be seen that in all fractions so treated, irrespective of the character of the spectrum before the treatment, oxime formation had taken place, demonstrating that the —CHO group was still intact. This is discussed below.

Evidence for degradation during removal of iron (step 3, Method A)

There is no doubt that HCl, during the HCl-ether fractionations, and also during the treatment with 25% HCl causes changes in the material. It appeared likely that similar changes would occur during the removal of iron.

Porphyrin mixtures after removal of iron from the haem were transferred immediately to ether and esterified with diazomethane. Chromatography of the esters on columns of aluminium oxide grade IV and of magnesium oxide grades III and IV (cf. Nicholas, 1951) showed the presence, apart from protoporphyrin, of a variety of porphyrin a-like materials which could not be satisfactorily resolved. It is only necessary to report briefly that materials with spectra similar to most of the fractions shown

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**Fig. 5.** The change in intensities of bands I–III relative to band IV, when: A, the combined 8 and 10% fractions and B, the combined 12, 15 and 20% fractions (Fig. 2a) stood in the dark in 25% HCl solution a, at zero time; b, after 50 hr. at 10°; c, after 30 hr. at 30°; and d, after 3 hr. at 70°.

**Fig. 6.** The change in relative intensities of the bands of porphyrin prepared by Method A, after standing in the dark in 25% HCl solution a, at zero time; b, after 50 hr. at -10°; c–h, after 1, 2, 3, 4, 5, 6 hr. at 57°; i, j, after 1-25 and 5 hr. at 95°. The relative intensities after reaction with hydroxylamine are shown as —O—.

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**Diagram**

[Diagram showing relative intensities of bands I–IV with different time intervals and temperature changes.]
in Fig. 3 were obtained from the chromatograms. It thus appeared that, during the removal of iron, materials similar to those which arise during treatment with HCl had appeared.

**Attempts to purify haem a**

There is no evidence yet that the haem, as such, is labile in acid conditions. This was borne out to some extent when, instead of acetone-HCl, cold pyridine was used to extract the haems from the tissue. The haems were transferred to ether, and without any attempt at removal of the large amount of lipid material the protohaem was removed by the aqueous pyridine-HCl buffer method of Rawlinson & Hale (1949). The haem a so obtained was grossly contaminated with fat, but its haemochromogen had only a single visual absorption band, at 587 mµ, apparently the same as the material prepared after acetone-HCl extraction of the tissue followed by elution from alumina by boiling acetic acid. This haem was eventually converted to porphyrin, and this treated by Method A. On fractionation of an ether solution of this porphyrin with HCl, fractions identical with those shown in Fig. 2a were obtained.

**Chromatography of haems**

The haems were extracted from the tissue and defatted as in steps 1 and 2 of Method A. The resulting ether solution of the haems was evaporated to dryness in vacuo. Neither light petroleum nor benzene extracted any fatty material from the dry residue. The dry material was soluble in butanol, which also dissolved the haem when shaken with a suspension of it in water brought to pH 4. The dried haems, or these, after esterification with diazomethane, were used for the following experiments.

The haem esters were chromatographed on columns of the following absorbents: Alumina grades II and IV, magnesium oxide grades II and III (cf. Nicholas, 1951); talc and kieselguhr (Hyflo supercel). The following solvents were used, singly and in pairs, in varying proportions: chloroform, methanol, ether, benzene, pyridine. Separation of both the haems and the haem esters was also sought on partition columns. The following solids were tried as supports: filter paper powder (Whatman, standard grade) and kieselguhr (Hyflo supercel). For 'reverse phase' chromatography, Hyflo treated with dichloromethylsilane was used (cf. Martin, 1949; Howard & Martin, 1950). The solvent systems tried for all three supports were lutidine-water, equilibrated and used at both 21° and 3° and ether-pyridine buffer (pyridine 30 vol., 0-15N-HCl to 100 vol.), at room temperature (18°).

There was no indication in any of these experiments that the haem a might be separated from the other materials. The coloured material in every case moved slowly down the column with the solvent front. Material which eventually ran through the columns had the same proportion of haem a to protohaem as the starting material.

**DISCUSSION**

From the experiments reported, it became clear that once the iron is removed from haem a, the porphyrin is very unstable in the presence of acid. The spectrum of the product obtained was the sum of the spectra of the degradation products, and it is clear that the proportions of these, and the resulting mixed spectrum, vary with both time and temperature during manipulations with acid.

The tendency of the material with higher hydrochloric acid number and band I at longer wavelengths to be changed to material with lower hydrochloric acid number and band I at shorter wavelengths, as well as the constant downward trend in the intensities of bands II and III relative to band IV, are consistent with the hypothesis that the changes are due to the destruction of 'rhodofying' groups (Lemberg & Falk, 1951). Among such groups are the —CHO group, the —CH:CH:COOH group (Formula I), and the unsaturated isocyclic ring (Formula II). These were suggested by Lemberg & Falk (1951) as possible structures for porphyrin a, consistent with the visual spectroscopic properties of haem a and porphyrin a in comparison with the properties of compounds of known structure. As a basis for this study, the spectra of haem a and porphyrin a described by Rawlinson & Hale (1949) and Rimington, Hale, Rawlinson, Lemberg & Falk, (1949) were used. The presence of a —CHO group was confirmed (Lemberg & Falk, 1951), but the exact nature of the other 'rhodofying' group is not known. These studies are considered not to be invalidated by the present work, since it seems likely that the undegraded, natural porphyrin a has spectroscopic properties very close to those of Rawlinson & Hale's (1949) material.
Since it is not clear how a —CHO side chain could be changed by HCl in such a way as to yield products with the characters described above, it was considered more likely that it was the other ‘rhodofying’ group which was being changed. That this was so became apparent when it was found that the material which had been heated to 95°C for 5 hr. in 25% hydrochloric acid solution was still able to react with hydroxylamine, the spectrum changing from rhodo to aetio type and band I shifting from 647 to 636 m\(\mu\).

These findings were parallel with those of Rawlinson & Hale (1949) and Rimington et al. (1949), who found that treatment of their porphyrin \(\alpha\) with diazoacetic ester or HI (double bonds in side chains) or hydroxylamine (carbonyl groups in side chains) led to a change from oxorhodo- to rhodo-type spectrum, band I hardly shifting in position. The action of both these types of reagents in succession, however, led to a product with aetio-type spectrum and band I at 625 m\(\mu\). The parallelism between the spectra of numerous fractions before and after reaction with hydroxylamine (Figs. 3, 4a, 6) showed, moreover, that the effect of the —CHO group on the spectrum was approximately equivalent in all the materials. Thus the differences between the fractions must be due to a series of changes in the other rhodofying group. It is evident that this group is gradually changed by acid towards an end state in which its rhodofying effect is completely lost. It is not possible, however, to postulate intermediate steps in this degradation which could account for the many apparent stages in the change.

Acid apparently caused degradation of the porphyrin even at step 3 (removal of iron). It is possible that the numerous products revealed by chromatography directly after this step were original components, but their similarity to the materials which were shown to be produced by hydrochloric acid at later stages makes it more likely that they arose in the same way. All methods for removing metals from metalloporphyrins (including the relatively mild method of Paul, 1950), feature strongly acid conditions except the sodium amalgam method (Fischer & Hilger, 1924). The latter was unsuitable, however, because the —CHO side chain and the side chain with an ethylenic double bond would be reduced. Thus no suitable alternative process is available for this step, nor could any be found for the other steps involving the use of acid. Fractionation of ether solutions of the haems with aqueous NaOH, Na\(_2\)CO\(_3\) or Na\(_2\)HPO\(_4\) was ineffective. Until suitable techniques are developed for all these steps, attempts at purification through the porphyrins must be unsuccessful.

It appeared more profitable to turn again to the separation and purification of the haem as such. There is no direct evidence that the haem is unstable in acid conditions, though this possibility cannot be ignored. It should be pointed out that the haemochromogen band at 587 m\(\mu\), in haem \(\alpha\) prepared by Rawlinson & Hale’s (1949) method, and, indeed, in direct pyridine extracts of tissues, is broad, and may include the bands of several similar compounds. It is quite possible that there exists more than one natural haem \(\alpha\), perhaps corresponding to different cytochromes \(\alpha\). Examination at very low temperatures (cf. Keilin & Hartree, 1949) of this haemochromogen band in pyridine extracts of tissues might allow the detection of such components, though the lipids extracted concurrently by pyridine would make such a study difficult.

There is little doubt that the failure to separate the haems by chromatography was due to the presence of lipids, which might be expected to change their partitioning properties. The gross fat can be removed without much trouble, but the lipid which is encountered in smaller but appreciable quantities in attempts to purify haem or porphyrin \(\alpha\) is still, perhaps, the greatest single factor hindering its isolation.

It appears now that the most fruitful approach to the problem might be to abandon the efforts to purify the natural compounds and instead to attempt to prepare a stable derivative of the haem through, for example, catalytic hydrogenation, fusion in resorcinol or the action of diazoacetic ester. Material obtained by the procedures of steps 1 and 2, Method \(A\), may be sufficiently free of gross contamination for this purpose. Unfortunately, it is not certain that the haem even at this stage has not already suffered some change.

**SUMMARY**

1. During attempts to find a method for the preparation, from ox-heart muscle, of porphyrin \(\alpha\) in quantities sufficient for direct chemical study, it was found that the porphyrin is very unstable in acid media.

2. Evidence is presented that the —CHO side chain of the porphyrin is not changed during this degradation. It appears that the other ‘rhodofying’ group is modified in a gradual manner, leading to a series of porphyrins with rather similar spectra. The degradation is hastened at raised temperatures, and in the end-state the ‘rhodofying’ properties of the group are lost completely. It was suggested (Lemberg & Falk, 1951) that this group may be an acrylic acid side chain. It is not yet possible, however, to interpret in terms of chemical structure the changes which occur during the degradation.

3. No procedure has been found by which the use of acid can be avoided entirely during the isolation of the porphyrin.

4. Haem \(\alpha\), as such, may not be unstable to acids, though there is no direct evidence on this point.
Attempts were made by the use of both absorption and partition chromatography to find a method for the isolation of relatively large amounts of haem a, but without success.

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REFERENCES


Some Properties of the Glutaminase of Clostridium welchii

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In previous papers from this laboratory (Krebs, 1948; Hughes, 1949, 1950) it was reported that cetyltrimethylammonium bromide (cetavlon) accelerates the decarboxylation of glutamine and glutamic acid in intact cells and extracts of *Clostridium welchii*. The present paper is a study of the mechanism of this effect. The glutaminase has been purified and the effect of cetavlon upon purified enzyme preparations has been investigated. Whilst cetavlon accelerates the rate of deamination of glutamine in intact cells and crude extracts it inhibits it in the purified extracts. These findings and the result of kinetic studies support the view put forward previously (Hughes, 1949) that the accelerating effect of cetavlon is due to the removal of an intracellular inhibitor normally accompanying the enzyme.

METHODS

Organisms. Three strains of *Clostridium welchii* (strains SR 12 and 1490 of the National Collection of Type Cultures, and a locally isolated strain) were maintained in Robertson’s meat medium. Through the courtesy of Dr B. C. J. G. Knight, two batches of about 500 g. (wet wt.) of strain 1490 were made available from the Wellcome Physiological Laboratories, Beckenham, Kent. These cells had been collected after 5 hr. growth on the papain digest described below.

Growth medium. The usual medium consisted of casein hydrolysate, meat, yeast extract and glucose (see Krebs, 1948). The papain digest meat medium was prepared according to Ainsworth, Brown, Marsden, Smith & Spilsbury (1947). A semi-synthetic medium was prepared from hydrolysed casein (Mollwain & Hughes, 1944) as described by Boyd, Logan & Tytell (1947).

Measurement of enzyme activity. In general, the activity of the glutaminase was estimated by determination of the rate of ammonia formation. A fresh solution (0·5 ml.) of glutamine (0·02M in 0·25M-sodium acetate buffer containing 0·025M-KCl) was placed in one arm of a branched test tube made from 20 mm. diameter Pyrex tubing in the form of an inverted Y. The other arm contained 1·5 ml. of the enzyme solution in acetate buffer (final concn. 0·2M) and KCl (final concn. 0·025M). A series of parallel tubes was placed in a water bath maintained at 40·0°, and after 1 min. equilibration the contents of the two arms were mixed without removing the tubes from the bath. At 5 min. intervals the tubes were removed from the bath and the reaction stopped by placing in ice water and adding 0·5 ml. 5H2SO4. N2H4 was determined according to Parmae. Blank N2H4 determinations were made on all reagents. In this way a time curve of the glutaminase activity was obtained. The initial rate of N2H4 formation was linear in intact cells and in crude extracts of *C. welchii* until approximately 50–60% of the added 224 µl. of glutamine was decomposed, except where the glutaminase activity was low, i.e. where less than 5% of the substrate was decomposed in 15 min. The enzyme dilution was therefore adjusted so that not more than 60% and not less than 10% of the glutamine was decomposed in 15 min. Under these conditions the initial rate of reaction was proportional to the dilution of the enzyme. Duplicate