chotic acid secretion, but that the amount of carbonic anhydrase required is only a very small proportion of the total potential activity.

We wish to thank Prof. H. A. Krebs, F.R.S., for advice during the course of this work. One of us (J.E.) is indebted to the Agricultural Research Council for a grant.

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Isolation of Unequivocal Uroporphyrin III

A FURTHER STUDY OF TURACIN

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No authentic specimen of uroporphyrin III has until now been prepared either synthetically or from natural material.

The first uroporphyrin to be isolated was that from urine of the congenital porphyria patient Petry. Fischer (1915) originally considered it to be a heptacarboxylic porphyrin, but later (Fischer & Hilger, 1925) established that it was an octacarboxylic acid which could be partially decarboxylated, losing four acid groups and yielding coproporphyrin I, by heating with hydroxide acid in acetic acid or with 1% hydrochloric acid at 180–190° (Fischer, 1916; Fischer & Zezweck, 1924). This reaction established that it belonged to the isomeric series derivable from aetio- or porphyrin I, and Fischer concluded that it had the structure, porphyrin-1:3:5:7-tetra-acetic acid-2:4:6:8-tetrapropionic acid since comparison with a synthetic iso- or uroporphyrin I having four methyl and four methylene groups would show the presence of a predominant side chain in the porphyrin III and, since the side chain was unique, could be taken as evidence for the presence of 4-methyl groups. Fischer concluded that the side chain was attached to C-5 and C-8 and Heidegger (1936) and, after correction by Feldberg (1944), that the side chain was attached to C-6 and C-9. This point has been confirmed in recent work.

β side chains and with a similar porphyrin having succinic acid instead of methylmalonic acid groups (Fischer & Zischler, 1937; Fischer & Hofmann, 1937) eliminated these structures as constitutional possibilities.

Uroporphyrin I, isolated from bones and urine of congenital porphyria patients and from pearl oyster shells, Pteria vulgaria (Fischer & Haarer, 1932), and the pigment from turacin, stated to be uroporphyrin I (Fischer & Hilger, 1924), was the only uroporphyrin known (although the melting points of the methyl esters of different preparations were widely variable from about 273 to 293° when almost simultaneously, Waldenström, Fink & Hoerburger (1935; see also Waldenström, 1934, 1935, 1936) and Mertens (1936, 1937) claimed that the urine of sufferers from acute porphyria contained another uroporphyrin, the octamethyl ester of which melted at about 258°, and in the products of the decarboxylation of which...
coproporphyrin III could be identified. It was thus regarded as uroporphyrin III belonging to the aetioporphyrin III series of position isomers.

About the same time, Rimington (1936) reported the study in South Africa of a living bovine case of congenital porphyria and the chromatographic separation upon alumina with dioxan as solvent of its urine and bone uroporphyrins into a fraction with m.p. 293° and another melting at 260–261°. It was suggested that the latter might be uroporphyrin III.

The homogeneity of the Waldenström ester from acute porphyria urines was disputed by Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945) who claimed that in spite of an apparently constant melting point of the crystalline material, it could in some cases (Type A Waldenström ester) be resolved by chromatography upon calcium carbonate into uroporphyrin I (ester m.p. 284°), yielding coproporphyrin I on decarboxylation, and a heptacarboxylic porphyrin (ester m.p. 208°) which apparently belonged to the III series, since on decarboxylation it yielded coproporphyrin III. Even in the case of esters which were not resolvable by chromatography (Type B Waldenström esters), it was claimed that decarboxylation indicated the same mixture or molecular association of I and III series porphyrins. Support for these claims has been proffered by Prunty (1948) and McSwiney, Nicholas & Prunty (1950). It followed that the porphyrin described by Waldenström and Mertens was not to be regarded as uroporphyrin III. Fischer & Hofmann (1937) applied adsorption chromatography, after Waldenström's publication, to a large quantity of 'uroporphyrin I' from Petry urine and claimed to have separated it into a uroporphyrin I ester melting at 302° (corr. 311°) and an ester of m.p. 261° which they regarded as identical with Waldenström's uroporphyrin III (mixed melting point determination). It was not further characterized.

The uroporphyrin copper complex 'turaecin' (Church, 1889), which forms the red pigment in the flight feathers of several Turacos and related species of birds, was studied by Fischer & Hilger (1923, 1924), who concluded that it was a derivative of uroporphyrin I (comparison of melting point with that of uroporphyrin I; decarboxylation not performed). A re-investigation of turaecin by one of the present writers (Rimington, 1939) produced unequivocal evidence that it yielded coproporphyrin III on decarboxylation and had therefore to be regarded as a series III isomer.

The ester of the uroporphyrin obtained by removal of copper from turaecin was very difficult to crystallize, as Fischer & Hilger had found previously, but it is clear that if it could be properly purified it would provide for the first time a reference sample of authentic uroporphyrin III.

The present investigation was undertaken with two objects in view: (1) to obtain pure uroporphyrin III from turacin and to characterize it chemically and physically, and (2) to search for other porphyrins accompanying uroporphyrin III in turacin preparations.

Below, we present evidence that the main porphyrin derivable from turacin (mixed from several species of bird, but see Rimington, 1939) is indeed uroporphyrin III, melting point of octamethyl ester 264° and yielding coproporphyrin III on decarboxylation; it is accompanied by another porphyrin analysing as an 8-carboxyl porphyrin, but with octamethyl ester of melting point 210°, which we suggest may be a porphin octa-acetic acid. When decarboxylation of this pigment was attempted, employing the usual conditions, it proved to be unstable, the reaction mixture being colourless (except for some suspended material) and exhibiting only a trace of fluorescence.

The preparation contained, in addition, small quantities of a dichroic porphyrin, resembling spectroscopically a phylloporphyrin, and a chlorin-like material. These are regarded as chemical artifacts arising during the sodium-amalgam reduction and re-oxidation employed to remove the copper from turacin. Similar products have been obtained by subjecting a synthetically prepared copper uroporphyrin I complex to the same procedures.

MATERIALS AND METHODS

Feathers. These were obtained from the same material as used by Rimington (1939). Since all eleven species of birds had been shown to contain turacin, yielding coproporphyrin III on decarboxylation, the feathers from the different specimens were mixed.

Paper chromatography was performed according to Nicholas & Rimington (1949, 1951).

Adsorbents and solvents were prepared according to Nicholas (1951). Unless otherwise stated, the MgO used was grade III, and the Al₂O₃, grade IV.

Melting points were recorded upon an electrically heated micro-melting point stage using a calibrated thermometer.

Spectral absorption was measured with a Beckman photometric spectrophotometer, model DU, or on a Beck- Hartridge reversion spectroscope.

Decarboxylations were carried out in sealed Pyrex tubes in a thermostatically controlled, covered oil bath.

EXPERIMENTAL

Preparation of turacin. The red portions of the feathers were defatted by extraction with ether in a Soxhlet apparatus. Weight 6–68 g. They were steeped in successive changes of 1% (w/v) aqueous NH₃ until the extract was practically colourless. The deep-red turacin solution showed two bands only; there were no bands in the region of 635 m."
(see later). By addition of dilute HCl to pH 3-1, the pigment was precipitated. It was collected on the centrifuge and reprecipitated twice more under the same conditions. The last supernatant fluid was of pale straw colour.

Removal of copper. The turacin was dissolved in 0.1-n-NaOH and 200 g. of 2-5% sodium amalgam added in small portions at a time, shaking on a machine between each addition. The colourless mixture was filtered through a folded paper and the porphyrinogen re-oxidized by drawing a stream of air through the liquid overnight. By adjustment to pH 3-1, the uroporphyrin was precipitated; it was purified by reprecipitating twice. The first and second supernatants were greenish brown in colour and showed a strong, broad absorption band at about 620 m.μ. The third supernatant contained a trace only of this component.

Isolation of a chlorin-like pigment

The supernatant fluids, referred to above, from precipitation of the uroporphyrin were combined and shaken with kieselguhr which adsorbed the substance having the strong absorption band in the red. The kieselguhr was washed, then the pigment eluted with dilute ammonia, affording a greenish solution. Shaking with ethyl acetate removed the pigment from the acidified solution (pH 3-1) and from this solvent it passed back into dilute HCl. It was readorsorbed upon a small quantity of kieselguhr (pH 3-1) and, after drying, this was treated with methanol saturated with HCl to esterify the pigment. The ester was extracted with CHCl₃ in the usual way and crystallized from CHCl₃-methanol. Recrystallization from benzene-light petroleum afforded greenish-brown needles. The spectral absorption of the free pigment was measured in 0.1-n-NaOH and in dilute HCl (see Fig. 1). The high band in the red in both acid (619 m.μ.) and alkaline (635 m.μ.) media suggests a chlorin structure for this material. Paper chromatography revealed the presence of material with 8 carboxyl groups. In addition to the two fluorescent spots characteristic of uroporphyrin (Nicholas & Rimington, 1949) this pigment and many of the others encountered in the present study of turacin exhibited a third faint spot lying below but very close to the uroporphyrin pair, i.e. there was a triple rather than a double spot system. The third spot was not sufficiently far down to correspond to a 7 carboxyl porphyrin.

Formation of a chlorin-like pigment by reduction of synthetic copper uroporphyrin

As it seemed likely that the chlorin could have been formed during the reduction of natural turacin, a similar experiment was carried out upon chemically prepared copper uroporphyrin. 1.50 g. of 2.5% sodium amalgam was added to the pigment dissolved in 25 ml. of 0.1-n-NaOH. After filtration, air was drawn through the solution until reoxidation had occurred and the porphyrin was precipitated at pH 3-1. The supernatant fluid was greenish and contained the same chlorin-like material as previously encountered (red band at 635 m.μ. in 0.1-n-NaOH) together with some unprecipitated uroporphyrin.

Esterification and preliminary purification of the crude porphyrin

The precipitated porphyrin was dried in vacuo and left for 12 hr. in contact with methanol saturated with HCl. An equal volume of CHCl₃ was then added, together with sufficient saturated aqueous sodium acetate (rapidly) to neutralize the mixture to Congo red. Much water was then added, and after thorough shaking the CHCl₃ layer removed, washed with dilute ammonia, then water, filtered and evaporated to dryness. The residue of crude ester (475-3 mg., m.p. 263°) was chromatographed upon a column of MgO packed in CHCl₃, using CHCl₃-methanol (100:0.5) for development. A small, dark residue was left on the top of the column and discarded, the porphyrin passing through with an indication of separation into two zones. It was recovered from the solvent, and recrchromatographed upon a large MgO column (1 ml. adsorbent/mg. porphyrin) again using 100:0.5 CHCl₃-methanol. Three zones appeared: I, a lower band containing the majority of the pigment; II, an intermediate, rather diffuse band; and III, an upper zone surrounded by a small layer of non-porphyrin material on the surface of the adsorbent. Each of these fractions was further purified, as described below (see also Fig. 2).
Table 1. Absorption maxima and relative intensities of turacin porphyrin esters A and B compared with those of uroporphyrin I ester and a Waldenström ester

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th></th>
<th>Band II</th>
<th></th>
<th>Band III</th>
<th></th>
<th>Band IV</th>
<th></th>
<th>Soret band</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>m(\mu)</td>
<td>Relative intensity</td>
<td>m(\mu)</td>
<td>Relative intensity</td>
<td>m(\mu)</td>
<td>Relative intensity</td>
<td>m(\mu)</td>
<td>Relative intensity</td>
<td>m(\mu)</td>
</tr>
<tr>
<td>Turacin porphyrin A ester, m.p. 264(\degree) (uroporphyrin ester III)</td>
<td>625</td>
<td>0.275</td>
<td>570</td>
<td>0.431</td>
<td>535</td>
<td>0.587</td>
<td>502</td>
<td>1.000</td>
<td>406</td>
</tr>
<tr>
<td>Uroporphyrin ester I, m.p. 293(\degree), from bone</td>
<td>625</td>
<td>0.258</td>
<td>570</td>
<td>0.445</td>
<td>535</td>
<td>0.606</td>
<td>500</td>
<td>1.000</td>
<td>406</td>
</tr>
<tr>
<td>Waldenström ester, m.p. 260(\degree), from acute porphyria urine</td>
<td>625</td>
<td>0.252</td>
<td>570</td>
<td>0.445</td>
<td>535</td>
<td>0.594</td>
<td>501</td>
<td>1.000</td>
<td>406</td>
</tr>
<tr>
<td>Turacin porphyrin B ester, m.p. 210(\degree)</td>
<td>625</td>
<td>0.268</td>
<td>570</td>
<td>0.491</td>
<td>535</td>
<td>0.625</td>
<td>502</td>
<td>1.000</td>
<td>406</td>
</tr>
</tbody>
</table>
light petroleum, m.p. 264°. This, the main constituent of the crude turacin porphyrin ester, weighed 258·2 mg. (54·3 % of the total crude porphyrin ester), and after hydrolysis behaved on paper chromatography as a single 8 carboxyl porphyrin indistinguishable from uroporphyrin I (see Fig. 4).

Turacin porphyrin B. The material from the main band crystallized poorly from hot CHCl₃-methanol. In CHCl₃ it had a-band at 625·4 m.μ. It was rechromatographed once more upon MgO, on which it behaved as a single band,
octa-acetic acid ester than with that of a uroporphyrin ester (porphin-tetra-acetic acid tetracarboxylic acid ester). The behaviour of this material on decarboxylation (see below) affords some support for this suggested structure. The spectral absorption of the methyl ester (see Fig. 7) was not significantly different from that of uroporphyrin ester.

![Fig. 5. Spectral absorption of ester of turacin porphyrin A (uroporphyrin III octamethyl ester). Solvent, chloroform. Concentration 28.13 mg./l. for range 630–430 m.μ., 2.81 mg./l. for Soret band.](image)

![Fig. 6. Spectral absorption of uroporphyrin I octamethyl ester, m.p. 293° (see Rimington & Sveinsson, 1950). Solvent, chloroform. Concentration 65.8 mg./l. for range 630–440 m.μ., 3.29 mg./l. for Soret band.](image)

![Fig. 7. Spectral absorption of octamethyl ester of turacin porphyrin B. Solvent, chloroform. Concentration approx. 30 mg./l. for range 630–430 m.μ., 3 mg./l. for Soret band.](image)

![Fig. 8. Spectral absorption of dichroic pigment (phylloporphyrin type) separated during purification of turacin porphyrins. Solvent, chloroform.](image)

### Table 2. Micro-analysis of turacin porphyrin esters A and B

<table>
<thead>
<tr>
<th></th>
<th>C (%)</th>
<th>H (%)</th>
<th>N (%)</th>
<th>MeO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turacin porphyrin A ester (uroporphyrin III ester)</td>
<td>61.9</td>
<td>6.4</td>
<td>5.8</td>
<td>26.5</td>
</tr>
<tr>
<td>Required for uroporphyrin octamethyl ester C₄₅H₅₀O₁₈N₈</td>
<td>61.1</td>
<td>5.8</td>
<td>5.9</td>
<td>26.3</td>
</tr>
<tr>
<td>Turacin porphyrin B ester</td>
<td>59.5</td>
<td>6.1</td>
<td>6.1</td>
<td>28.1</td>
</tr>
<tr>
<td>Required for a porphin octa-acetic acid methyl ester C₄₆H₅₂O₁₈N₈</td>
<td>59.6</td>
<td>5.2</td>
<td>6.3</td>
<td>28.0</td>
</tr>
</tbody>
</table>

a green band with red upper and lower edges moved down, leaving a small red-brown residue on the surface of the adsorbent. This residue was discarded and the green band eluted with methanol. Transferred again to CHCl₃, it had an α-band at 624-6 m.μ. and was distinctly dichroic (red-green). It crystallized from CHCl₃-methanol in feathery greenish-brown crystals, m.p. 212°. On recrystallization (three times) from benzene-light petroleum, the melting point became constant at 240°. The paper chromatogram of the ester after hydrolysis was similar to that of a uroporphyrin, but the second spot had a greater relative intensity. Spectral absorption in CHCl₃, measured on the Beckman spectrophotometer, revealed a curve of phyllo-type (Fischer & Orth, 1937), band II being broad and intense; order of intensity IV, II, III, I (see Fig. 8). The α-band maximum lay at 625 m.μ. We consider that this pigment is probably a phyllo-photosensitive with 8 carboxyl groups which may have arisen from the turacin porphyrin during the chemical manipulations employed to remove copper from the feather pigment.

### Dichroic pigment

The greenish-brown band obtained in the purification of zone II was rechromatographed upon Al₂O₃, packed in benzene. Development only began with pure CHCl₃ when
Purification of zone III

This zone on the second MgO chromatogram of the total crude porphyrin was eluted by methanol containing 1% (v/v) of H₂SO₄. It was transferred to CHCl₃ and purified by two successive chromatograms upon MgO. The main porphyrin band was rather diffuse and left a small greenish zone upon the adsorbent. An unsuccessful attempt was made to secure crystalline material from benzene-light petroleum. From these solvents, the melting point rose continuously from 155°. A paper chromatogram showed similarity with turacin porphyrin B. In CHCl₃ it had α-band at 625-7 mμ. The amount was small.

Decarboxylation of turacin porphyrins

The acetic acid residue as a substituent on the pyrrole ring is unstable. The same is true to a less marked extent in the macrocyclic porphyrin system. Exploiting this property, Fischer & Zerweck (1924) showed that the uroporphyrin from Petry urine, when heated with 1% HCl in a sealed tube at 180-190° for 3 hr., lost 4 mol. of CO₂ and yielded coproporphyrin I. Since all four coproporphyrins have been synthesized, this reaction affords an excellent means of establishing to which astiroporphyrin series the parent substance belongs; uroporphyrin III would yield coproporphyrin III.

Turacin porphyrin A. The uroporphyrin ester from turacin porphyrin A (m.p. 264°) (3-8 mg) described above, was decarboxylated by heating with 10 ml. of 1% HCl under the prescribed conditions. It was found most convenient to weigh out the ester, leave it-to hydrolyse in 0-4 ml. of 25% HCl for 24 hr. and then to transfer the solution to the combustion tube with 9-6 ml. of water.

The reaction mixture contained some dark material which was filtered off and treated with glacial acetic acid to remove any adsorbed porphyrin, this extract being added to the main bulk from which the porphyrin was extracted with ether in the usual way, then from the latter into HCl. After precipitation and esterification, the total porphyrin in CHCl₃ was passed through a column of MgO and the purified coproporphyrin crystallized from a very small volume of CHCl₃ by addition of hot methanol. The crystals which separated had the habit of coproporphyrin III and m.p. 153°, remelt 179°. Recrystallization did not alter these melting points. By concentration of the first mother liquor, a further crop of similar crystals was obtained also melting at 163°, remelt 179°. Paper chromatography after hydrolysis showed only a 4 carboxyl porphyrin. The solution thus contained only coproporphyrin III, indicating that the parent uroporphyrin was the series III isomer alone. The yield of coproporphyrin III isolated was 68% of theory.

Turacin porphyrin B. On account of the small quantity of pigment available, only 2-5 mg. was taken for decarboxylation under the conditions described above. Examination at the end of the experiment showed that the substance had been degraded beyond the porphyrin stage, there being only a trace of fluorescence in the colourless solution.

DISCUSSION

The work reported here shows that by the removal of copper from turacin a mixture of porphyrins and porphyrin-like materials is obtained. These, by the aid of chromatography, have been separated. The ester of the main constituent, turacin porphyrin A, has m.p. 264°, remaining constant upon recrystallization. In elementary analysis, methoxyl content, spectral absorption and (after hydrolysis) behaviour on paper chromatography, it corresponds to the octamethyl ester of a uroporphyrin.

Decarboxylation afforded a single coproporphyrin, identified as coproporphyrin III by paper chromatography, spectral absorption and the melting point behaviour of its tetramethyl ester. That only coproporphyrin III was present was shown by the fact that there was no difference in melting point between the first crop of crystals separating and those obtained by further concentration of the mother liquors; moreover, their melting points were not changed by recrystallization of the respective fractions.

The main constituent of turacin is thus uroporphyrin III copper complex (confirming the earlier result of Rimington, 1939) and the ester, m.p. 264°, we have prepared provides for the first time an authentic specimen of uroporphyrin III octamethyl ester.

Accompanying this porphyrin and separable from it by chromatography is a second porphyrin, turacin porphyrin B, the ester of which we have obtained with constant m.p. 210°. Although this material resembles uroporphyrin octamethyl ester in such characteristics as paper chromatographic behaviour and spectral absorption, its elementary and methoxyl analysis agrees more nearly with the requirements of a substance containing four CH₂ groups less than uroporphyrin ester, such as, for example, the octamethyl ester of porphin octacetic acid. Difference from uroporphyrin is shown by the infrared spectrum and by the greater instability of this porphyrin under the conditions usually employed for decarboxylation. Only a trace of material exhibiting red fluorescence in ultraviolet light was recoverable from the reaction mixture. An acetic acid grouping attached to a β-position in the pyrrole ring is known to be unstable. Fischer & Müller (1937) synthesized 1:4:5:8-tetramethylporphin-2:3:6:7-tetra-acetic acid which proved to be less stable than uroporphyrin. On decarboxylation under the usual conditions, octamethyl porphin was obtained, the yield of crude material being 20% of theory.

In the present instance, more complete degradation of the molecule appears to take place. Elucidation of the exact structure of turacin porphyrin B will be attempted by a synthetic approach. It is of interest to recall that from the urine of an atypical case of porphyria, Gray, Rimington & Thomson (1948) obtained an ether-insoluble porphyrin which suffered extensive degradation to colourless products when decarboxylation was attempted in acid solution. The presence of turacin porphyrin B,
together with uroporphyrin III in turacin from feathers, no doubt accounts for the extreme difficulty of crystallizing the methyl ester of the crude porphyrin obtained by the removal of copper from the natural pigment. In their first paper on turacin, Fischer & Hilger (1923) report that their porphyrin ester would not crystallize; in a later paper (Fischer & Hilger, 1924) they state that from 0.1 g. turacin they obtained a uroporphyrin ester in very small needles, but do not record the yield. The melting point was given as 295°, not depressed by admixture with uroporphyrin I octamethyl ester. This finding is difficult to understand.

During the purification of turacin porphyrins A and B, we also isolated a chlorin-like pigment and a dichroic pigment (ester m.p. 240°) with a phyllo-type spectrum; both possessed 8 carboxyl groups by paper chromatography. We regard these as chemical artifacts arising during the reduction necessary to remove the copper from turacin. A similar chlorin-like pigment was obtained as a by-product by subjecting some uroporphyrin I copper complex to the same treatment with sodium amalgam as was accorded to turacin.

Now that an authentic specimen of uroporphyrin III octamethyl ester is available as a reference standard, it is hoped that some of the uncertainty surrounding the nature of the Waldenström porphyrin may be experimentally resolved. This pigment is excreted by sufferers from acute porphyria and was regarded as uroporphyrin III (see Waldenström, 1937) until Watson et al. (1945) disputed its homogeneity. Experiments with such an object in view have already been commenced.

**SUMMARY**

1. The porphyrin mixture resulting from the removal of copper from turacin has been examined chromatographically.

2. The main constituent has been isolated (ester m.p. 264°) and identified as uroporphyrin III. Decarboxylation afforded only coproporphyrin III. This provides for the first time an authentic specimen of uroporphyrin III.

3. Another porphyrin, present in about one-tenth of the amount, has been isolated as the methyl ester (m.p. 210°). From the results of analysis and paper chromatography it is suggested that this material may be porphin octa-acetic acid. When decarboxylation was attempted, it proved to be less stable than uroporphyrin, degradation into non-porphyrin products taking place.

4. In addition to these porphyrins, two materials having chlorin-type and phyllo-type spectra respectively have been isolated in very small quantity. They are, however, regarded as chemical artifacts.

The Beckmann photoelectric spectrophotometer used in this investigation was purchased out of a grant from the London University Research Grant Fund. One of us (R.E.H.N.) was in receipt of a grant from the Medical Research Council. Our thanks are due to Miss P. A. Miles for valuable technical assistance. The turacin was obtained from material given to one of us (C.R.) in 1938 by the British Museum (Natural History).

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