Hepta-, Hexa-, and Pentacarboxylic Porphyrins of Porphyria Cutanea Tarda*

I. ISOLATION AND PROPERTIES OF THE PORPHYRINS

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Ever since the introduction of paper chromatography to the study of porphyrins, many new members other than coproporphyrins and uroporphyrins have been detected in various porphyrin materials as well as biosynthetic preparations (1-12). However, their low concentration, and unsatisfactory methods of isolation and purification have delayed an extensive study of these porphyrins. A preliminary study has shown that patients with porphyria cutanea tarda excrete relatively more of them. This report describes the separation and properties of the hepta-, hexa-, and pentacarboxylic porphyrins of both I and III series, which have been isolated from urine samples of a cutanea tarda patient. The identification of these products may permit a better understanding of the metabolism of porphyrins.

EXPERIMENTAL

Materials and Methods

Urine samples used for this study were collected from a female patient with porphyria cutanea tarda. Now in her early fifties, she has been afflicted for years with the illness. Her symptoms are characterized by red patches on skin and bullae or blisters all over the upper part of her body including arms and hands with some old scars, but no abdominal pains or nervous symptoms. The brownish urine showed a reddish violet fluorescence under ultraviolet light and had absorption bands (538, 578 mp) with some old scars, but no abdominal pains or nervous symptoms. The dried sample was then introduced onto a packed Hyflo column, followed by another layer of plain Hyflo. A column 18 x 3.5 cm in diameter may be used for the separation of a sample of 3 to 5 mg, depending on its composition. When the major constituents of a sample are closely adjacent on the chromatogram, as in this case, it is advisable to start with a smaller amount. A typical primary Hyflo chromatogram shown in Fig. 1A was achieved in 5 minutes by using chloroform-petroleum ether (1:2 by volume) as the developer. As a result of suction, the lower zones especially of larger columns may be somewhat curved like cones.

Other adsorbents such as MgO, Al₂O₃, MgCO₃ and CaCO₃ were also used in trial tests according to known procedures, and found to be unsatisfactory for the separation. The controversial problem of the so-called Waldenström porphyrin (17, 18) was highly complicated by the use of CaCO₃ or MgO as the adsorbent (12).

Fluorescence-pH Curves—The variation of fluorescence intensity of a porphyrin with the pH of a solution was studied by the use of a Coleman 14 universal spectrophotometer with the fluorescence attachment. Stock solutions of the porphyrins were prepared by hydrolysis of esters in 5% HCl solution containing 5% acetic acid. They were allowed to stand at room temperature for 24 hours or more for complete hydrolysis. The amount of each ester was so adjusted that 0.2 ml of the stock solution, when diluted to 10 ml with McIlvaine's standard buffer A (0.1 M citric acid), should possess approximately the same fluorescence intensity as the calibration standard. In this case 2 µg of pure Copro P in 10 ml of 1% HCl solution was set at 100 as the standard. A blank solution was set at 0. For each determination, 0.2 ml of the stock solution was thoroughly mixed with 9.8 ml of a buffer or hydrochloric acid of a proper concentration, as the case might be. On the alkaline side, an equivalent amount of sodium hydroxide solution was added to neutralize the stock solution before the addition of 9.6 ml of sodium hydroxide of a proper concentration. A Beckman G pH meter was used to check the final pH reading.

Absorption Spectra—The absorption curves of methyl esters of these porphyrins were determined with a Beckman DU spectrophotometer equipped with a photomultiplier. For measurements in the visible region, a solution of 0.003 to 0.004% sample

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1 The abbreviations used are: 7 I III, 6 I III, and 5 I III, hepta-, hexa-, and pentacarboxylic porphyrins of both I and III series; 6 I and 6 I, those of the I series; Uro I and Uro III, uroporphyrin I and III; Copro I and Copro III, coproporphyrin I and III.
Hepta-, Hexa-, and Pentacarboxylic Porphyrins. I

TABLE I
Urinary porphyrins from cutanea tarda patient (daily averages)

<table>
<thead>
<tr>
<th>Period</th>
<th>Volume</th>
<th>Total porphyrin</th>
<th>Copros*</th>
<th>5 I III</th>
<th>6 I III</th>
<th>7 I III</th>
<th>Uros</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml/day</td>
<td>μg/day</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>1, Jan. and Feb. '55; 33</td>
<td>960</td>
<td>4552</td>
<td>1.5</td>
<td>3.3</td>
<td>2.4</td>
<td>22.0</td>
<td>67.2</td>
<td>3.6</td>
</tr>
<tr>
<td>2, Apr. '55; 10</td>
<td>955</td>
<td>3485</td>
<td>1.8</td>
<td>2.4</td>
<td>2.7</td>
<td>22.2</td>
<td>66.7</td>
<td>4.2</td>
</tr>
<tr>
<td>3, Aug. '55; 3</td>
<td>1100</td>
<td>4745</td>
<td>1.1</td>
<td>1.5</td>
<td>2.1</td>
<td>25.2</td>
<td>68.0</td>
<td>1.6</td>
</tr>
<tr>
<td>4, Oct. '55; 4</td>
<td>958</td>
<td>4053</td>
<td>1.9</td>
<td>1.8</td>
<td>1.7</td>
<td>25.0</td>
<td>68.0</td>
<td>1.6</td>
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<tr>
<td>5, Aug. '56; 4</td>
<td>1160</td>
<td>1874</td>
<td>0.8</td>
<td>2.0</td>
<td>2.2</td>
<td>24.0</td>
<td>66.6</td>
<td>4.4</td>
</tr>
<tr>
<td>6, Feb. '57; 5</td>
<td>890</td>
<td>4288</td>
<td>2.1</td>
<td>3.4</td>
<td>4.5</td>
<td>24.7</td>
<td>61.1</td>
<td>4.2</td>
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<td>7, Dec. '58; 2</td>
<td>1460</td>
<td>4745</td>
<td>1.5</td>
<td>2.0</td>
<td>1.9</td>
<td>23.1</td>
<td>64.3</td>
<td>7.1</td>
</tr>
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</table>

Averages……………… 980 4110

<table>
<thead>
<tr>
<th></th>
<th>%</th>
<th>%</th>
<th>%</th>
<th>%</th>
<th>%</th>
<th>%</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>66 μg</td>
<td>124 μg</td>
<td>165 μg</td>
<td>925 μg</td>
<td>2752 μg</td>
<td>140 μg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(15-125)</td>
<td>(37-190)</td>
<td>(41-105)</td>
<td>(443-1196)</td>
<td>(1330-3170)</td>
<td>(63-275)</td>
<td></td>
</tr>
</tbody>
</table>

* Copros and uros, for copro- and uroporphyrins; 5 I III, 6 I III, and 7 I III for penta-, hexa-, and heptacarboxylic porphyrins of both I and III series.

RESULTS AND DISCUSSION

Zone C (Fig. 1A) of the developed column contained methyl esters of Copro I and III. Zones 5, 6, and 7 contained esters of 5 I III, 6 I III, and 7 I III porphyrins, respectively. Zone U contained those of Uro I and III, whereas Zone O consisted of esters of at least two unidentified porphyrins. One of them closely resembles uroporphyrins in absorption, but differs markedly from the latter in its behavior on decarboxylation. The methyl ester crystallized in fine needles, m.p. 238°. A comparison of its infrared spectrum with those of Dr. MacDonald's synthetic uroporphyrins gave no hint of its identity to any of them.

It is noted from Table I that the composition of the porphyrins excreted daily by this patient was rather constant throughout a course of about 4 years, although a significant reduction in volume was noted.

 Hình 1. Chromatograms of urinary porphyrins. A, Hyflo column chromatogram; B, C and D, paper chromatograms of different solvent systems, with solvent fronts as marked for Rf calculations at 21-22°.

The solvent system KC-KD consisted of 3:3 mL of kerosene-chloroform, followed by 4:4:1.8 mL of kerosene-dioxane (12); LW, 3:3:2.7 mL of 2,6-lutidine-water (20); and KC-KP, 3:3 mL of kerosene-chloroform followed by 5:0.9 mL of kerosene-n-propyl alcohol (12). Methyl esters were used as samples in A, B, and D; and free porphyrins in C.

The solvent system T stands for total porphyrins isolated; E, for the ether-extracted portion; C, coproporphyrins; 5, 6, and 7, porphyrins with penta-, hexa-, and heptacarboxyl groups; Cg, Cs, and Ct, coproporphyrins obtained from decarboxylation of 5, 6, and 7 porphyrins, respectively; U, uroporphyrins; Cg, Cs, and Ct, coproporphyrins and residual pigments, respectively.

Fig. 1. Chromatograms of urinary porphyrins. A, Hyflo column chromatogram; B, C and D, paper chromatograms of different solvent systems, with solvent fronts as marked for Rf calculations at 21-22°.

For measurements of the Soret band, a solution of 10 times the dilution was used. Each determination was made at 22° against the pure solvent. Several solvents have been tested.

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Dr. S. F. MacDonald, private communications.
quantity was noticed during the summer of 1956. During that period, the absorption bands of porphyrins in the urine were scarcely visible, and the skin lesions of the patient were also reduced to a minimum. However, the symptoms recurred in the following winter.

Methyl esters of the porphyrins obtained from primary columns were repeatedly chromatographed on secondary columns for purification.

The esters of coproporphyrins crystallized from chloroform and methanol as needles, m.p. 232-236°, which were found by paper chromatography (12) (Fig. 1D) to consist of about equal parts of I and III isomers.

The esters of uroporphyrins isolated from all urine samples, including those collected during the summer of 1956 (Table I, Period 5), had the same melting point range of 275-280°. After repeated purification with long Hyflo columns, the product from Period 1, 1955, was found to consist of 78% of Uro I, m.p. 295°, and 22% of Uro III, m.p. 260° ((12) Patient 2), whereas that from Period 5, 1956, consisted of 77% of Uro I and 23% of Uro III. The pure esters of Uro I and its decarboxylation product, Copro I, were used as references for all experiments below. The possible existence of uroporphyrins II and IV in nature, as was pointed out by MacDonald and Michl (19), would raise some doubt as to the purity of all the uroporphyrins thus far isolated from natural materials.

The crude porphyrins from Zone 5 (Fig. 1 A) closely resemble a pentacarboxylic porphyrin on the lutidine-water paper chromatogram (20). This is supported by other findings described below. The methyl esters crystallized from chloroform-methanol as needles with a melting point range of 210-216°. The melting point was raised to 216-220° and further to 220-224° by repeated chromatography on secondary columns with chloroform-petroleum ether (1:3.5) as the developer. It was later found that they are isomers of the I and III series. Therefore, the tentative name 5 I III has been proposed for the mixture. Likewise, 6 I III and 7 I III are used for isomeric mixtures of hexa- and heptaacarboxylic porphyrins, respectively.

The melting point of porphyrin esters from Zone 6 was also raised from 185-190° to 194-196° by repeated chromatography with chloroform-petroleum ether (1:2.5) as the developer. The variation of m.p. was again due to the change of isomeric compositions by repeated chromatography. Decarboxylation experiments (21) further indicated that 6 I III might contain more isomers than either 5 I III or 7 I III porphyrins. Photomicrographs of crystals of these products are presented together with those of pure isomers in Paper II of this series (21).

The esters of 7 I III porphyrins, the most abundant fraction next to uroporphyrins, crystallized less readily from most organic solvents. However, they were obtained from chloroform-methanol in fine wool-like crystals after chromatography on secondary columns with chloroform-petroleum ether (1:2) as the developer. The m.p. was raised from 210-214° to 217-218° after repeated chromatography.

The paper chromatograms of the porphyrin esters are shown

### Table II

<table>
<thead>
<tr>
<th>Porphyrin*</th>
<th>M.p.</th>
<th>( \lambda_{\text{max}} )</th>
<th>HCl number†</th>
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<tr>
<td></td>
<td>(°C)</td>
<td>1, Dioxan; 2, chloroform; 3, ethyl acetate; 4, 2N HCl</td>
<td>Ether: HCl</td>
</tr>
<tr>
<td>Copro I</td>
<td>254</td>
<td>1, 403 (11.5) 498 (1) 531 (.651) 568 (.455) 622 (.333)</td>
<td>(a) 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2, 499 533 568 622</td>
<td>(b) 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3, 498 530 568 622</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4, 550 575 593</td>
<td></td>
</tr>
<tr>
<td>5 I III</td>
<td>220-224</td>
<td>1, 400 (12.2) 499 (1) 532 (.608) 569 (.466) 623 (.300)</td>
<td>(a) 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2, 500 535 569 623</td>
<td>(b) 2.6</td>
</tr>
<tr>
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<td></td>
<td>3, 498 531 569 623</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4, 551 576 594</td>
<td></td>
</tr>
<tr>
<td>6 I III</td>
<td>194-196</td>
<td>1, 402 (12.4) 499 (1) 533 (.695) 569 (.460) 624 (.307)</td>
<td>(a) 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2, 500 535 570 624</td>
<td>(b) 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3, 499 531 569 624</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4, 552 576 595</td>
<td></td>
</tr>
<tr>
<td>7 I III</td>
<td>217-218</td>
<td>1, 403 (12.8) 500 (1) 534 (.624) 570 (.450) 625 (.264)</td>
<td>(a) 0.03</td>
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<td></td>
<td></td>
<td>2, 501 537 571 625</td>
<td>(b) 4.0</td>
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<td></td>
<td></td>
<td>3, 499 532 570 625</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4, 553 570 596</td>
<td></td>
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<tr>
<td>Uro I</td>
<td>295</td>
<td>1, 404 (13.2) 500 (1) 534 (.578) 571 (.428) 626 (.257)</td>
<td>(a) 0.01</td>
</tr>
<tr>
<td></td>
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<td>2, 406 536 572 626</td>
<td>(b) 5.0</td>
</tr>
<tr>
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<td>3, 500 532 571 625</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>4, 554 577 607</td>
<td></td>
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</table>

* See Table I for abbreviations of porphyrins.
† The concentration in per cent of HCl, which will extract ‡ of the porphyrin from an equal volume of porphyrin solution in the organic solvent; (a) free porphyrins; (b) methyl esters.
in Fig. 1, B and D. Brief heating of the ester at the melting point did not change its behavior on a paper chromatogram. The solubilities of these crystalline esters in several organic solvents are generally of the same order, most soluble in chloroform, less in ethyl acetate, benzene, methanol and least in petroleum ether.

**HCl Number**—The partition of the porphyrins between an organic solvent and a dilute hydrochloric acid solution, generally expressed by the HCl number (22), was measured. The HCl numbers of porphyrin esters were also determined. Both ethyl ether and ethyl acetate were chosen as the organic solvents. The results are given in Table II.

In the case of free porphyrins, the HCl numbers in ether are very close to that of coproporphyrin. The conventional method for the determination of the latter by ether-HCl extraction
Fig. 5. Absorption spectra of methyl esters of urinary porphyrins in dioxane. See Fig. 3 for abbreviations of porphyrins.

would, therefore, involve much contamination. As shown in Fig. 1, the ether extract, E, of the urine sample prepared according to the known method for coproporphyrins, has almost as many spots as the extract of total porphyrins, T. The relative sizes of the spots corresponding to Copro at top, followed successively by 5 I III, 6 I III, and 7 I III porphyrins, indicate a considerable extent of contamination in this particular case. Since these porphyrins are present in all types of porphyrin materials and even in normal urine samples (16), they may cause a higher result in such a determination of coproporphyrine.

With ethyl acetate as the organic solvent, the HCl number of 5 I III is again identical with that of coproporphyrin, and the value of 7 I III is very close to that of uroporphyrin. Therefore, extractions of porphyrins from porphyric samples with ether or ethyl acetate would not result in pure copro- or uroporphyrins.

On the other hand, the HCl numbers of the esters in ethyl acetate revealed a linear relationship with the number of ester groups of the porphyrins (Fig. 2A).

Fluorescence-pH Curves—The results are presented in Fig. 3.

In Fig. 4, a group of five curves of Uro I is given to show the relation of the fluorescence yield with respect to the concentration of the solution. Their concentration ratios are as follows:


where Curve U is the same curve of Fig. 3.

Similarly, other porphyrins in solutions of different concentrations also give different fluorescence curves.

From these figures, it is obvious that copro- and uroporphyrins have different pH-fluorescence patterns. This is primarily due to the fact that the latter contains four more ionizable groups of acetic acid instead of methyl groups. The pH-fluorescence patterns of hepta-, hexa- and pentacarboxylic porphyrins, with decreasing number of acetic acid groups, fit right between those of uro- and coproporphyrins. Besides the regular shifts around the isoelectric points, there are other empirical relationships between the fluorescence and the number of carboxyl groups of the molecule. When the ratio of fluorescence intensity of each porphyrin in 5% sodium hydroxide to that in 20% solution was calculated, there was found a linear relationship between these ratios and the number of carboxyl groups of the respective porphyrins. Similar relationships were also found on the acid side as shown in Fig. 2, B and C. Since the fluorescence decreases gradually on standing, readings should be taken right after the mixing of solutions.

The direct proportionality between the number of carboxyls

...
and fluorescence ratios on the alkaline side was also observed with some dicarboxylic porphyrins such as meso- and deuteroporphyrins, but not with proto- and hematoporphyrins. These observations support the view that the 5 I III, 6 I III, and 7 I III porphyrins may not contain any reactive functional groups (hydroxy, vinyl) such as protoporphyrin or hematoporphyrin do.

**Absorption Spectra**—The results are presented in Table II. The regular shifts of absorption maxima according to the number of carboxyl groups are best shown in Fig. 5, where the absorption curves of the porphyrins in dioxane are given. The gradual shift of peaks from Copro to Uro indicates that these intermediate porphyrins have structures very similar to those two porphyrins. Assuming that the molecular extinction coefficients of these intermediate porphyrins lie somewhere between those of Copro, \( \epsilon \) (500 m\( \mu \)) = 1.82 \( \times 10^4 \), and Uro, \( \epsilon \) = 1.38 \( \times 10^4 \), we can calculate their approximate molecular weights by dividing the value of \( \epsilon \) by \( \gamma^t \) of the corresponding values of \( \epsilon_{570} \), thus,

\[
\begin{align*}
5 & \text{ I III} = 1.45 \times 10^4 + 18.8 = 771, \\
6 & \text{ I III} = 1.45 \times 10^4 + 17.6 = 825, \\
7 & \text{ I III} = 1.45 \times 10^4 + 16.1 = 900.
\end{align*}
\]

These calculated values receive strong support from the experimental results discussed below.

**Decarboxylation and Possible Molecular Structures**—Experiments on decarboxylation were carried out according to the procedure of Edmondson and Schwartz (23). The results are summarized in Table III. The esters prepared from decarboxylation products of the porphyrins behave in all respects as those of coproporphyrins. Approximately 200 \( \mu \)g of each ester were used for the experiment. The porphyrins of 5 I III gave an over-all yield of 92\% of coproporphyrins (methyl ester, m.p. 248-250°). The paper chromatographic analysis (Fig. 1D) revealed its composition as 80\% of Copro I and 20\% of Copro III, when densities of the spots were considered. The coproporphyrins (m.p. 152/167°) from 6 I III consist of 90\% of Copro III and 10\% of Copro I, and those from 7 I III also consist of 90\% of Copro III and 10\% of Copro I. Supporting evidence for the estimation of these ratios of isomers (±5\%) can be obtained from the melting points of the products. Other data concerning the decarboxylation products of 7 I III, including a photomicrograph of the product, were given in the previous paper (12) under the name “7III.” These experiments have strongly indicated that penta-, hexa- and heptacarboxylic porphyrins are isomeric mixtures of the I and III series. Furthermore, the infrared spectroscopy of the esters (24) has confirmed these findings.

The elementary analyses of the methyl esters of the 7 I III porphyrins, listed in Table III, agree very well with the molecular formula, \( \text{C}_{46}\text{H}_{52}\text{O}_{14}\text{N}_4 \), of the heptamethyl ester of methylporphin-triacetic-tetrapropionic acid. Similar analyses were not done for the esters of 5 I III and 6 I III for lack of material. However, the analyses for methoxyl of pure samples of 5 I and 6 I porphyrins (21) were in agreement with the calculated values for the methyl esters, respectively, of trimethyl-porphin-monosuccinic-tetrapropionic acid (\( \text{C}_{46}\text{H}_{52}\text{O}_{14}\text{N}_4 \)) and dimethyl-porphin-diaesic-tetrapropionic acid (\( \text{C}_{46}\text{H}_{52}\text{O}_{14}\text{N}_4 \)).

On the assumption that the decarboxylation of uroporphyrin is a mole-to-mole reaction, the molecular weights calculated for 7 I III, 6 I III, and 5 I III porphyrin esters would be 885, 879, and 769, respectively. These values agree well with those derived from the absorption data.

**SUMMARY**

1. The urinary porphyrins of a cutanea tarda patient have been studied in detail.
2. The structures of the porphyrins with five, six and seven carboxyl groups are discussed. The possibility of the existence of several isomers of the hexacarboxylic porphyrin is indicated.
3. A linear relationship has been found to exist between the number of carboxyl groups of porphyrins and the ratios of their fluorescence intensities in sodium hydroxide solution of varying concentration.
4. It is suggested that these porphyrins may be present as contaminants in copro- or uroporphyrins prepared from biological materials by conventional methods involving extraction with ether or ethyl acetate.

**REFERENCES**
