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Molar Absorption Coefficients of Porphyrin Esters in Chloroform Determined by Copper Titration

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Chromatographically pure porphyrin esters free from metalloporphyrins were titrated with Cu^{2+} to determine the molar amount of porphyrin present. The end point of the titration was defined by a t.l.c. method detecting traces of metal-free porphyrin ester in admixture to its copper complex. From spectrometric measurement at the Soret maximum and the molar amounts found by the titration, ε_M was calculated for proto-, copro-, penta-, hexa- and hepta-carboxylic and uro-porphyrin permethyl esters. The values for uroporphyrin showed perfect agreement with those of previous workers, whereas those for coproporphyrin were about 5% lower and those for protoporphyrin more than 20% lower. The implications of the findings are discussed. Determinations of ε_M of some higher esters (ethyl to pentyl) and some partial methyl esters with one carboxyl group free are also presented.

Since Rimington's (1960) studies on the absorption coefficients of porphyrins in the Soret-band region, which have since then functioned as a basis for quantitative spectrophotometric determinations, few similar studies have been published. Mass spectrometry and t.l.c. have disclosed in thoroughly recrystallized porphyrin esters traces of metalloporphyrins as well as partially esterified matter. As metalloporphyrins (e.g. complexes with Cu and Zn) exhibit considerably higher spectral absorption coefficients than do the corresponding porphyrins, even minor contamination of porphyrins with their metal chelates could result in erroneously high absorption coefficients. Further, errors from solvents trapped in the crystals and minor contamination with partially esterified matter have to be taken into account. We therefore followed a suggestion of C. Rimington (personal communication), that the copper titration method of Oliver & Rawlinson (1951) could be used to obtain exact measurements of molar absorption coefficients if used on thoroughly demetallized porphyrin ester preparations. We also removed the partially esterified matter appearing in the sub-uro region on t.l.c. Oliver & Rawlinson (1951) used copper titration for quantitative determination of porphyrin *a*, but mentioned that it could be used for calculation of molar extinction coefficients. Their studies were confined to the visual spectral region and based on points of intersection between the spectral curves of the porphyrin and its copper complex as indications of the end point of titration. We developed a t.l.c. method for demonstration of traces of metal-free porphyrin ester present in the ester of its copper complex and used this to determine the end point of the titration.

Materials and Methods

Materials

Crystalline copro- and uro-porphyrin I esters were prepared from bovine porphyria excreta as described previously (With, 1958, 1969). The coproporphyrin preparations from this source regularly contained about 10% pentacarboxylic porphyrin. The esters were hydrolysed with conc. HCl, and the free porphyrin acids were precipitated at pH3–4. The precipitate was collected on a sintered-glass filter (type G3), washed, dried and dissolved on the filter by sucking conc. H_2SO_4 through it. In this way metalloporphyrins were split. Then the alcohol in question, usually methanol, was added to make the H_2SO_4 concentration 5% (v/v), and the mixture left overnight at room temperature for esterification. Esters with higher alcohols (e.g. pentanols) can also be prepared in this fashion (With, 1969, 1973). To avoid re-entry of metals on neutralization, extraction was made by adding an equal volume of chloroform with subsequent repeated washings by 5–10vol. of demineralized water until the washings became neutral. Preparations containing less than 0.5% metalloporphyrin were thus obtained (With, 1976*a*,*b*). To remove partially esterified matter, which often constitutes 5–10% of the esters prepared in this way, all our preparations were subjected to t.l.c. purification with benzene/ethyl acetate/methanol (17:2:1, by vol.), a modification of the method of Doss (1969) (cf. Rimington & With, 1974). In this system the partial esters remain in the sub-uro region, clearly separated from the per-esters. Coproporphyrin and pentacarboxylic porphyrin were separated by three-

times-repeated preparative t.l.c. with benzene/ethyl acetate/ethanol (90:20:1.5, by vol.), a modification of the system of Demole (1958, Fig. 7, p. 28), giving a wide separation of copro- and pentacarboxylic porphyrin. Hexa- and hepta-carboxylic porphyrins were prepared by heating dry uroporphyrin as the free acid to 150°C in air (With, 1975) followed by esterification and repeated t.l.c. The porphyrin methyl and higher alkyl esters were crystallized in the usual way from chloroform/methanol without methylation of the higher esters (cf. With, 1973, pp. 21–22 and Fig. 10).

Finally, the preparations were subjected to quantitative mass spectrometry and found to contain less than 0.5% (molar) of metalloporphyrin (cf. With, 1976*a*,*b*; Larsen *et al.*, 1977).

The mesoporphyrin ester used was batch no. 58697 from Koch–Light Laboratories (Colnbrook, Bucks., U.K.), and was found to contain less than 0.1% (molar) of metalloporphyrin on mass spectrometry. The protoporphyrin ester used was batch no. 17409 from Koch–Light. The latter preparation was not de-metallized, as protoporphyrin is oxidized to haematoporphyrin by conc. H_2SO_4, but was instead purified by preparative t.l.c. on silica gel with the modified Demole (1958) system, which showed two green zones, which were removed, below the proto zone. The preparation remained unstable, however, with the same green bands appearing on further t.l.c.

Titration procedure

About 1 mg of the ester was dissolved in 10 ml of analytical-grade chloroform. From this stock solution a 1:50 dilution was prepared and its absorbance at the Soret maximum was read, with $CHCl_3$ as blank, in a Beckman model 25 double-beam u.v. spectrophotometer. For titration, 1 ml of the stock solution was transferred to the bottom of 20 cm × 4 cm Pyrex test tube and mixed with 1 ml of analytical-grade acetic acid. For esters of dicarboxylic porphyrins, hydroxy porphyrins and partially esterified porphyrins it was necessary to add 2 drops of the detergent Triton X-100 (Technicon Chemical Co., Orco, Belgium; product no. T21-0188-17) to increase the reaction velocity (C. Rimington, personal communication; cf. also Lowe & Phillips, 1961). The titration was performed on a boiling-water bath under a gentle current of N_2 by adding first a 10 mM and subsequently a 1 mM aqueous solution of analytical-grade copper acetate; these additions did not cause phase separation of the chloroform/acetic acid mixture. Oliver & Rawlinson (1951) used a methanolic solution, but we preferred aqueous solution because the reaction with Cu^{2+} ions is not significantly retarded by the presence of small amounts of water (Phillips, 1963, p. 55), and the pipettes used were calibrated for aqueous solutions. First, the amount of copper solution to be added was calculated from the absorbance of the solution, by using the recommendations of Rimington (1960), i.e. ε_M approx. $2.15 \times 10^5 cm^{-1}$ for uroporphyrin ester and approx. $1.80 \times 10^5 cm^{-1}$ for coproporphyrin ester in chloroform.

The procedure is best illustrated by an example. A coproporphyrin stock solution showed, after 1:50 dilution, a Soret extinction of 0.432 (1 cm path length). A 1000 µl constriction pipette and a 50 ml measuring flask were used. Calibration of the pipette by weighing the chloroform (in weighing glasses with tight-fitting lids to prevent evaporation) showed 1.44 g instead of the 1.47 g expected from the specific gravity, for which only 0.98 ml of stock solution was diluted to 50 ml instead of 1.00 ml. Accordingly, the absorbances read had to be corrected by a factor of 1.02. As ε_M is about $1.8 \times 10^{-3} cm^{-1}$ the absorbance in question (0.432×1.02) corresponds to a content of about 120 nmol/ml of stock solution. Therefore 12 µl of the 10 mM solution was added to a test tube containing 1 ml of stock solution in chloroform and 1 ml of acetic acid, and the solution was heated on a boiling-water bath for 5 min to complete the formation of the copper complex. Then the solution was inspected in u.v. light in darkness, and if it still exhibited red fluorescence more Cu^{2+} solution was added. If no red fluorescence was visible the solution was subjected to t.l.c. as described below to separate the porphyrin ester from its copper complex. The former was identified by the presence of red fluorescence on its position on the chromatogram. If no fluorescence was visible on the porphyrin ester position another 1 ml portion of the stock solution was treated with 11 µl of 10 mM-Cu^{2+} solution plus 9 µl of 1 mM-Cu^{2+} solution. In this way the titration was continued until two consecutive amounts of Cu^{2+} differed by 1–2 nmol, one giving a trace of fluorescence on the chromatogram and the other no such trace. If 12 µl of 10 mM-Cu^{2+} solution plus 8 µl of 1 mM-Cu^{2+} solution was insufficient and 13 µl of 10 mM-Cu^{2+} solution was sufficient, the amount of porphyrin present must be between 128 and 130 nmol. The Soret extinction of the porphyrin stock solution was $0.432 \times 50 \times 1.02 = 22.03$, corresponding to between 128 and 130 nmol/ml of solution. Further, 130 nmol/ml is equal to 130 µmol/litre, and 1.30×10^{-4} mol/litre, which corresponds to an absorbance of 22.03 from which it is calculated that 1 mol/litre corresponds to an absorbance of $22.03/(1.30 \times 10^{-4}) = 1.69 \times 10^5$. Thus ε_M lies between $2.203/(1.30 \times 10^{-4}) = 1.69 \times 10^5$ and $2.203/(1.28 \times 10^{-4}) = 1.72 \times 10^5 cm^{-1}$.

We used a fresh 1 ml stock solution for every amount of Cu^{2+}, and did not add more Cu^{2+} to a mixture already treated because the titration includes removal of a sample for t.l.c.

T.l.c. indicator method

For separation of the porphyrin esters from their copper complexes, t.l.c. on silica gel was performed with the system benzene/ethyl acetate/methanol/acetic acid (17:2:1:4, by vol.), which separates the copper complexes of the esters from the metal-free porphyrin esters, widely for coproporphyrin, narrowly for uroporphyrin and intermediately for 5-, 6- and 7-carboxyl porphyrins. The R_F of Cu–coproporphyrin ester was 0.95 and that of the metal-free ester 0.53; for uroporphyrin ester the corresponding R_F values were 0.88 and 0.83. By running a marker of the metal-free porphyrin ester on the same plate its position was defined, and a close study in u.v. light in darkness of the chromatogram of the solution after the Cu^{2+} titration will reveal any fluorescence left on this position. The silica-gel plates were prepared from suspensions of silica gel H (Merck, Darmstadt, Germany) in methanol and were ready for use in 1 h without further activation (With, 1973; Rimington & With, 1974). The method worked only with such plates and not with prefabricated SiO_2/foil plates.

Preparation of partial esters

Purified uro- or copro-porphyrin I was dissolved in a known volume of conc. H_2SO_4; then 8 vol. of analytical-grade methanol and 1 vol. of demineralized water were added and the mixture was left overnight for esterification. Extraction with $CHCl_3$ and washing with water without previous neutralization was performed as described above. The $CHCl_3$ solution was dried and evaporated, and the partial esters with one and two free carboxyl groups were isolated by preparative t.l.c. with the system benzene/ethyl acetate/methanol (17:2:1, by vol.), which gives a broad suburo region. Partial esters of uroporphyrins to dicarboxylic porphyrins with one free carboxyl group are located between uro ester and the starting point (With, 1973, Fig. 15A, p. 31; Rimington & With, 1974). The esters were eluted with $CHCl_3$/methanol (4:1, v/v) and purified by three consecutive preparative runs, and the dry residue was used for titration without previous crystallization.

Results and Discussion

Seven titrations were performed with uroporphyrin methyl ester, three with heptacarboxylic ester, three with hexacarboxylic ester, three with pentacarboxylic ester and eight with coproporphyrin methyl ester, two with mesoporphyrin ester and two with protoporphyrin ester. The highest lower limits and the lowest higher limits found for ε_M are presented in Table 1.

We tried to supplement the titration by demonstrating surplus copper not bound to porphyrin, but even the most sensitive tests of Feigl (1954, vol. 1) were not adequate to detect the nanogram amounts on the t.l.c. plates.

The values for uroporphyrin agree perfectly with those from the literature, whereas those for coproporphyrin are about 5% lower and those for protoporphyrin as much as about 20% lower.

With regard to penta-, hexa- and hepta-carboxylic porphyrins, the only existing values are those of Doss (1969) and Dowdle *et al.* (1970). They gave no crystal melting points. Dowdle *et al.* (1970) controlled their preparation by mass spectrometry. Their

Table 1. *Molar absorption coefficients of porphyrin methyl esters in the Soret-band region*

Porphyrin	Crystal m.p. (°C)	Soret maximum in $CHCl_3$ (nm)	$10^{-5} \times \varepsilon_M$ in $CHCl_3$ (cm^{-1}) Lower limit	Upper limit	$10^{-5} \times \varepsilon_M$ from literature (cm^{-1})
Proto IX	230	407–408	1.315	1.332	1.71 at 407.5 nm (Rimington, 1960) 1.65 at 407.5 nm (Doss, 1969)
Meso IX	221	401	1.496	1.592	—
Copro I	246	400–401	1.705	1.710	1.80 at 400 nm (Rimington, 1960) 1.80 at 399.5 nm (Doss, 1969) 1.81 at 400 nm (Dowdle *et al.*, 1970)
Penta I	215	403	1.831	1.833	1.89 at 401 nm (Doss, 1969) 1.98 at 403 nm (Dowdle *et al.*, 1970)
Hexa I	235	404	1.683	1.805	1.98 at 402 nm (Doss, 1969) 2.03 at 404 nm (Dowdle *et al.*, 1970)
Hepta I	242	405	1.833	1.843	2.07 at 404 nm (Doss, 1969) 2.06 at 405 nm (Dowdle *et al.*, 1970)
Uro I	294	406	2.165	2.180	2.15 at 405–406 nm (Rimington, 1960) 2.26 at 405 nm (Doss, 1969) 2.16 at 406 nm (Dowdle *et al.*, 1970)

preparations cannot have been very pure, however, because they mention peaks of both metalloporphyrins and porphyrins with lower numbers of carboxyl groups. As the vapour pressures of metalloporphyrins are lower than that of corresponding porphyrins, it is most likely that their preparations contained at least a small molar percentage of metalloporphyrins, especially because their spectra may well have been rather weak, i.e. with peaks of low abundancy. Some of the differences between the results obtained by Dowdle *et al.* (1970) and ourselves may have been due to the fact that their esters were of porphyrins from human symptomatic porphyria urine. They apparently only performed one t.l.c. purification, whereas we performed three consecutive runs. We found about 5% lower values for penta-, about 15% lower for hexa- and about 11% lower for hepta-carboxylic porphyrin. These differences are compatible with metalloporphyrin contamination of the preparations used by previous researchers. Thus Doss (1970) found ε_M of the zinc chelates to be about twice that of the corresponding porphyrins, and we found that, after conversion of a porphyrin ester into the copper complex, with the technique described above, the absorbance at the Soret maximum increased by about 80%. A contamination with 5% (molar) copper or zinc complex in the preparations used by previous investigators would therefore easily explain the 5% higher values for ε_M.

As shown in Table 2, ε_M of coproporphyrin trimethyl ester was practically identical with that of coproporphyrin tetramethyl ester, whereas that of uroporphyrin heptamethyl ester was 4% higher than that of uroporphyrin octamethyl ester. This difference is so small that contamination with partial esters cannot explain the difference between our values and those of former investigators.

As ε_M values of higher porphyrin esters have not been reported, we performed a few measurements on such esters (Table 2). We found for coproporphyrin tetraethyl ester an ε_M value about 5% lower than that of the methyl ester, its prop-2-yl ester about 2% lower, and its pentyl ester about the same ε_M as the methyl ester. For uroporphyrin the ethyl ester showed values about 2% higher, the prop-2-yl ester about 8% lower and the pentyl ester about 9% lower than that of the methyl ester. These few observations are irregular, showing that ε_M of higher esters has to be determined experimentally in every case.

The greatest difference between our results and those of previous workers are those for protoporphyrin, our values being as much as about 20% below published values. Our low values can be due to the necessity of modifying the technique by the use of a detergent to get the copper to react with the dicarboxylic porphyrin. Although for uro- to coproporphyrin the red fluorescence of the solution rapidly disappeared on heating the porphyrin/metal acetate mixture on a boiling-water bath, the fluorescence persisted in the case of protoporphyrin, mesoporphyrin, hydroxyporphyrins, higher esters and partial esters, unless detergent was added. It is therefore reasonable to suppose that in these cases an excess of Cu^{2+} ions is necessary to complete the formation of the porphyrin complex. This would result in lower ε_M values. The same could also be the case to a smaller degree with the tetracarboxylic coproporphyrin, but this is not in agreement with our findings, as we found 5% lower values for copro- and penta-carboxylic porphyrin, but 15% lower for hexa- and 11% for hepta-carboxylic porphyrin and the same for uroporphyrin as reported in the literature. Also, one would expect higher values for coproporphyrin by titration with addition of detergent if the four carboxyl groups of coproporphyrin were the cause of the 5% lower ε_M found by copper titration. We therefore performed two titrations of the same stock solution of coproporphyrin ester, one with addition of Triton X-100 and one without this addition. This gave exactly identical results, in contrast with protoporphyrin ester, where the fluorescence did not disappear during heating with Cu^{2+} before detergent was added.

Table 2. *Molar absorption coefficients of higher and partial esters of porphyrins in the Soret-band region*

Porphyrin preparation	M.p. (°C)	Soret maximum in $CHCl_3$ (nm)	$10^{-5} \times \varepsilon_M$ in $CHCl_3$ (cm^{-1})	
			Lower limit	Upper limit
Copro I ethyl ester	214	400–401	1.630	1.635
Copro I prop-2-yl ester	215	401	1.673	1.683
Copro I pent-2-yl ester (with 2-methylbutan-1-ol)	185	401	1.729	1.735
Copro I partial methyl ester (one CO_2H free)	215	400–401	1.685	1.717
Uro I ethyl ester		405–406	2.222	2.424
Uro I prop-2-yl ester	230	406	1.974	1.998
Uro I pentyl ester (with 2-methylbutan-1-ol)	110	406	1.940	1.978
Uro I partial methyl ester (one CO_2H free)		405–406	2.200	2.230

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References

Demole, E. (1958) *J. Chromatogr.* **1**, 24–34
Doss, M. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 499–502
Doss, M. (1970) *Z. Klin. Chem. Klin. Biochem.* **8**, 208–211
Dowdle, E., Goldswain, P., Spong, N. & Eales, L. (1970) *Clin. Sci.* **59**, 147–158
Feigl, F. (1954) *Spot Tests, Vol.* 1*: Inorganic Applications*, pp. 77–90, Elsevier, Amsterdam
Larsen, E., Egsgaard, H., Møller, J. & With, T. K. (1977) *Biomed. Mass. Spectrom.* in the press
Lowe, M. B. & Phillips, J. N. (1961) *Nature (London)* **190**, 262–263
Oliver, I. T. & Rawlinson, W. A. (1951) *Biochem. J.* **49**, 157–162
Phillips, J. N. (1963) *Compr. Biochem.* **9**, 34–72
Rimington, C. (1960) *Biochem. J.* **75**, 620–623
Rimington, C. & With, T. K. (1974) *Enzyme* **17**, 17–28
With, T. K. (1958) *Biochem. J.* **68**, 717–720
With, T. K. (1969) *Dan. Med. Bull.* **16**, 268–279
With, T. K. (1973) *Scand. J. Clin. Lab. Invest. Suppl.* **134**, 1–80
With, T. K. (1975) *Biochem. J.* **147**, 249–251
With, T. K. (1976*a*) *Ann. Clin. Res.* **8**, *Suppl.* **17**, 144–150
With, T. K. (1976*b*) *Int. Porphyrin Meet. 1st: Porphyrins in Human Diseases*, pp. 481–485, Karger, Basel