Spectral Absorption and Fluorescence of Coproporphyrin Isomers I and III and the Melting-points of their Methyl Esters

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(*Received* 26 *March* 1945)

Both the determination and the identification of the naturally occurring coproporphyrin isomers I and III depend upon the exactness of the data relating to their physical properties and to those of their methyl esters. Quantitative estimation of these isomers involves spectrophotometric or fluorimetric comparisons with standard solutions usually prepared from the crystalline tetramethyl ester of isomer I. The accuracy of such determinations depends upon the purity of this ester and also upon the knowledge of the spectral absorption of the isomers or of the relative fluorescence efficiencies of the two molecules. Differential identification of the coproporphyrin isomers is achieved through differences in the properties of their tetramethyl esters, particularly their solubility in organic solvents and their melting-points. A clean-cut separation of the esters is not easily effected. This difficulty must be borne in mind in considering the melting-points of the methyl esters isolated from natural sources, unless careful recrystallizations have been done. In studies of porphyrin metabolism, an obvious essential is the definition of those properties which form the basis of quantitative methods of estimation and of identification. This paper presents data obtained in an attempt to correlate the spectral absorption and fluorescence of the isomers and the m.p.'s of their esters, so that their additive and constitutive properties may be more closely defined.

The necessity for such data became apparent when we found that solutions prepared from synthetic coproporphyrin I tetramethyl ester (as received from Prof. H. Fischer) gave extinction coefficients, $E_{1\,\text{cm.}}^{1\%\,(w/v)}$, as much as 12% lower than those made up from the same specimen after recrystallization. Recrystallization, however, caused no appreciable change in m.p.'s, which were throughout within the generally accepted range of good values for this substance (Table 1 and Fig. 4). Similar observations were made on synthetic coproporphyrin III tetramethyl ester and on several natural specimens. This experience led us to investigate the criteria for the purity of the tetramethyl esters of coproporphyrins I and III, and to attempt to define more closely the additive properties of standard solutions of coproporphyrins I and III.

EXPERIMENTAL

Materials

Sources. The synthetic tetramethyl esters of coproporphyrins I and III were those of Prof. Fischer (Münich): for the latter sample we are indebted to Dr C. Rimington, as also for two specimens of the tetramethyl ester of coproporphyrin I isolated by him, and for several specimens of the corresponding coproporphyrin III ester obtained by decarboxylation of uroporphyrin III made from its copper complex, turacin (Rimington, 1939). Another specimen of coproporphyrin I tetramethyl ester was isolated by us from faeces of a patient with haemolytic anaemia.

Purification. Coproporphyrin I tetramethyl ester was recrystallized from chloroform-anhydrous methanol mixtures, the crystals being removed while the volume of mother liquor was still fairly large.

Owing to its high solubility, good yields of the corresponding coproporphyrin III ester could not be obtained from chloroform-methanol mixtures. This ester was first crystallized twice from anhydrous diethyl ether, followed by slow crystallization in the ice-chest from chloroform-hexane mixtures, and a final crystallization from anhydrous ether. Grinstein (1941) has used chloroform-benzene mixtures for the same purpose.

Crystals of the esters were dried *in vacuo* over conc. H_2SO_4 and paraffin wax for 2 days, then at 80° for 1 hr., and afterwards cooled *in vacuo*. No change in weight of 5 mg. of synthetic coproporphyrin I tetramethyl ester (as received) was observed on drying at 60°/0·3 mm. Hg for 1 hr. It was therefore concluded that the low extinction coefficient recorded for this specimen (Table 1) was not due to the presence of solvent.

Methods

Absorption curves. A Hilger E316 medium quartz spectrograph and Spekker photometer were used, with a 108 W. ribbon filament lamp providing a continuous source from 300 mμ. into the far red. For precise observation of the sharp peaks of the Soret bands of porphyrin compounds, this lamp is preferable to the tungsten-steel spark with its scarcity of emission lines in this region. The plates obtained were matched on a photoelectric microphotometer. The spectrograph slit-widths were—for ultra-violet range (u.v.) 0·03 mm. (≡0·12 mμ. at λ = 400 mμ.)—for visible 0·015 mm. (≡0·12 mμ. at λ = 550 mμ.). No differences were observed in the form of the sharp peaks of the Soret bands until the slit width was increased above 0·1 mm. (≡0·4 mμ. at λ = 400 mμ.).

Table 1. *Extinction coefficients of coproporphyrins I and III in* 0·1–0·15 N-HCl, *and the m.p.'s of their tetramethyl esters*

Isomer		Specimen of tetramethyl ester	m.p. of ester	Extinction coefficients of coproporphyrins $E^{1\%}_{1\,cm.}$, 401 mμ.	$E^{1\%}_{1\,cm.}$, 548 mμ.
I	(*a*)	*Synthetic:*			
		As received	252–254°	6880–7100	232
		Recryst. × 2*	253–254·5°	7940	—
		Recryst. × 3	256–258°	8080	259
		Recryst. × 5	256–257·5°	8140	—
	(*b*)	*Natural:*			
		From calf meconium, recryst. × 2	254°	8190	261
		From cow faeces, recryst. × 1	251°	8100	—
		From haemolytic anaemia faeces, recryst. × 2	252°	7980	—
		Standard solution, 1 mg./ml. (Rimington, 1942)	—	8140	(262)†
III	(*a*)	*Synthetic:*			
		As received	129–130/150–161°	7300	236
		Recryst. × 3, chloroform-hexane	129–130°	—	—
		Recryst. × 3, chloroform-hexane, × 2, anhydrous ether	128/158–162°	8120	261
	(*b*)	*Natural:*			
		T. Turaco and *T. Gall.* Recryst. × 2	153/178–179°	8100	259·5
		T. Corythaix. Recryst. × 2	148/173–176°	8080	258·5
I		$E_{mol.}$ therefore defined as		531×10^3	$17{\cdot}0 \times 10^3$
III		$E_{mol.}$ therefore defined as		530×10^3	$16{\cdot}97 \times 10^3$

* I.e. twice recrystallized.

† Value by Rimington (1942) and, independently, Kench *et al.* (1942), on Nutting Hilger spectrophotometers.

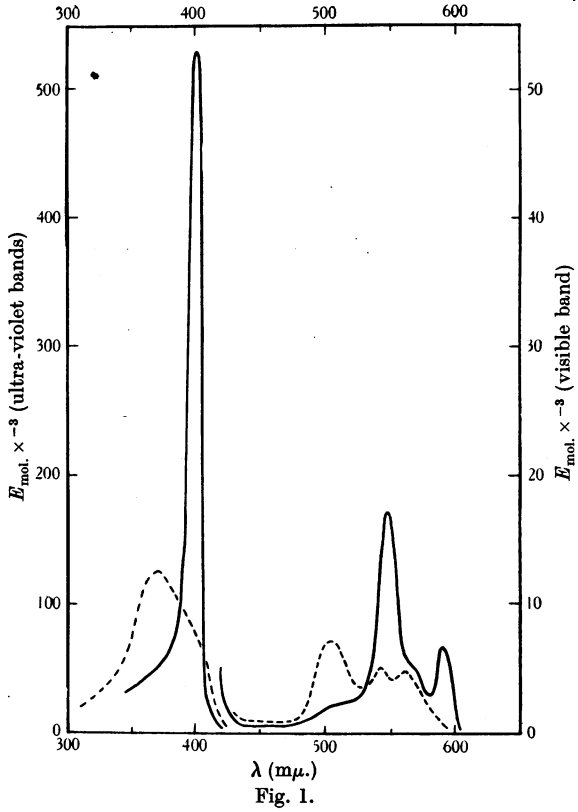

Fig. 1.

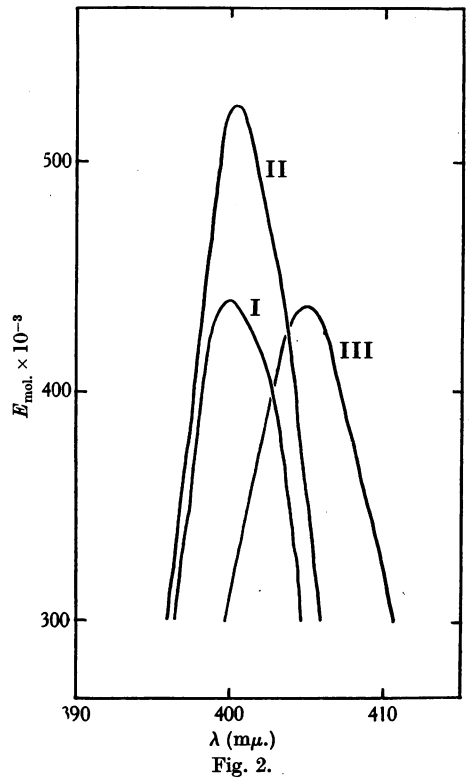

Fig. 2.

Fig. 1. Spectral absorption curves of coproporphyrins I and III, in 0·15 N-HCl (——) and 0·1 N-NaOH (------). (N.B. The curves in the visible region are magnified × 10 in comparison with the Soret bands.)

Fig. 2. Variations in the form of the Soret band peak of coproporphyrins I and III with different HCl concentrations. Curve I, 0·02 N-HCl; curve II, 0·1 N–0·15 N-HCl; curve III, 7 N-HCl. The curves for intermediate HCl concentrations lie between these extremes.

Fluorescence. A Zeiss Pulfrich photometer was used with an L3 filter and 2 cm. 'Hysil' cells filled with 6 ml. of liquid exposed to air. A Hanovia S500 mercury vapour lamp with Wood's glass filter, vertically above the cells, provided an exciting source. Such an arrangement is only suitable for porphyrin solutions of low concentration, below 2 mg./l. A Hilger Spekker photoelectric fluorimeter has also been used. Fluorescence intensities of solutions of coproporphyrin I and III isomers were compared one against the other.

Preparation of solutions for spectrophotometry and fluorimetry. Weighed amounts of the coproporphyrin tetramethyl esters were hydrolyzed by standing in conc. HCl (sp.gr. 1·16; about 1 ml./1 mg. ester) for 24 hr., and diluting with distilled water to the required HCl concentration. Hydrolysis was usually performed in silica tubes; Monax and soft glass tubes were equally satisfactory. No decomposition was observed on increasing the time of standing in conc. HCl up to 60 hr. Hydrolysis was, however, complete after 10 hr. at room temperature.

Stability of coproporphyrin solutions. Whereas in 0·1–0·2 N-HCl these porphyrins were stable at room temperature for at least 12 months when kept in diffuse daylight, in 1·5 N-HCl they deteriorated after 2 months under the same conditions. This was shown by periodic examination of the absorption curves of the solutions.

If the concentration of coproporphyrin I exceeded 20 mg./l., fine needles of the hydrochloride were deposited after 1 month. This crystallization was not observed with solutions of coproporphyrin III.

Melting-points. A Köfler apparatus was used which permitted microscopic observation. Examination by polarized light was especially useful in observing the double m.p.'s of coproporphyrin III tetramethyl ester (Rimington & Symons, 1938). The temperature of the stage was changed at a rate of 1°/min.

For the m.p.-composition curve (Fig. 4) the following specimens of tetramethyl esters were used: coproporphyrin I —synthetic, recrystallized three times; coproporphyrin III —from decarboxylation of uroporphyrin III from turacin (Rimington, 1939, p. 115, specimen analyzed). These were made up in chloroform solutions of equal concentration, and 3 or 4 drops of known mixtures of these were allowed to crystallize on a microscope slide. After the initial melt, resolidification and remelting temperatures were observed four times on each specimen, though only very slight changes from the initial values were noted.

RESULTS

Spectral absorption curves. Data for the two chief bands of coproporphyrins I and III are given in Table 1, stated as extinctions of 1 % (w/v) solutions in 1 cm. layers, $E^{1\%}_{1\,cm.}$. The isomers gave identical absorption curves (Fig. 1). The bottom lines of the table contain the values of the molar extinction, $E_{mol.}$, of the free coproporphyrins, which were derived from the $E^{1\%}_{1\,cm.}$ values.

Absorption curves of these porphyrins were found to depend greatly upon the nature of the solvent and the pH (Fig. 2). Between the intense spectral types characterizing acid solvent and neutral or alkaline solvent, there is a region of considerably reduced absorption near the isoelectric point of the porphyrins, which is probably due to an aggregation of the molecules forming a colloidal solution. The considerable effect of different pH values on the Soret band is shown for HCl solutions in Fig. 2. In contrast, the effect upon the absorption curves in the visible region of varying the HCl concentration from 0·06 to 3 N was very small. The 548 mμ. band of 0·06 N-HCl solutions shifted only 2 mμ. towards the red when the HCl concentration was increased to 3 N and the extinction coefficient remained unchanged.

The 548 mμ. band can be conveniently isolated by an Ilford 'spectrum yellow green' filter, no. 605 (peak transmission at 550 mμ.). Using a Pulfrich photometer fitted with this filter, we obtained a value of 0·645 ± 0·015 for the extinction coefficient of a solution containing 10 mg. coproporphyrin in 1 l. in a 5 cm. cell.

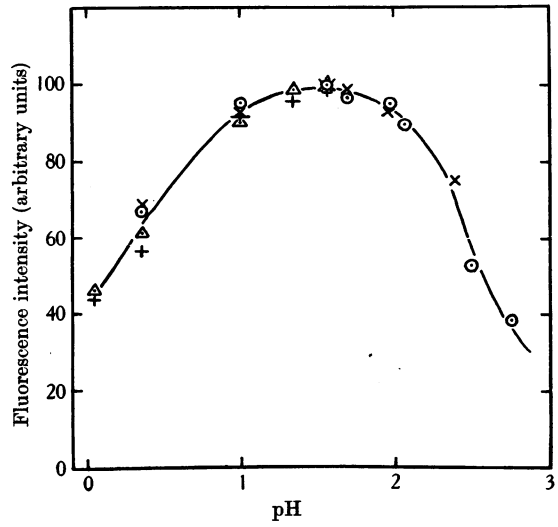

Fig. 3. Fluorescence of natural and synthetic coproporphyrins I and III in HCl solutions of different pH values. The fluorescence intensity is reduced to equivalent concentrations of all four porphyrin solutions used.

⊙ Coproporphyrin I, 0·6 mg./l. } Purified natural
× Coproporphyrin III, 0·545 mg./l. } specimens
△ Coproporphyrin I, 0·48 mg./l. } Purified synthetic
\+ Coproporphyrin III, 0·48 mg./l. } specimens

Fluorescence. By direct comparison of solutions of known concentrations, the fluorescence of coproporphyrins I and III was found to be of equivalent intensity within ± 2 %. A similar result was obtained by Rimington (1943) for uroporphyrins I and III. Below pH 2 the same changes of fluorescence intensity with pH were observed with both isomers (Fig. 3). Rimington (1943) has found a similar relation between pH and fluorescence for coproporphyrin I in acid solutions. In the range pH 2–6,

the differences between pH-fluorescence curves of coproporphyrins I and III observed by Hoerburger (1933) were confirmed.

Melting-points of coproporphyrin tetramethyl esters. Determinations of the m.p. of the coproporphyrin I ester were straightforward and the results agreed with those of other workers (Table 1). With the coproporphyrin III ester there are complications due to its 'double m.p.' Its lower and upper m.p.'s have been previously recorded as lying between the wide limits of 135–153 and 158–178° respectively. The upper m.p. is observed by reheating a specimen which has melted at the lower m.p. and has been kept molten before being allowed to cool. We have detected no differences in crystal form accompanying this change. Rimington (1939) has illustrated the two forms in which coproporphyrin III tetramethyl ester occurs. Both clusters and needles may melt indiscriminately at either m.p.

In our experience the behaviour of synthetic coproporphyrin III tetramethyl ester during melting was substantially the same on four separate determinations. Our observations through the microscope were as follows: At least one bunch of crystals coalesced at 129–130°, another softened and coalesced at 150–157°, and the remaining mass melted sharply at 161°. After cooling and solidification, which occurred at 120°, on heating, some of the crystals softened with loss of double refraction at 129°, and the remainder coalesced fairly sharply at 158°. Intensive drying of the crystals *in vacuo* had no effect upon these changes. After three crystallizations from a chloroform-hexane mixture and drying *in vacuo*, this synthetic specimen, although spectrophotometrically pure porphyrin, coalesced at 129–130° and could not be persuaded to solidify or to exhibit double refraction on cooling; this prevented an observation of its behaviour on remelting. After two more crystallizations from anhydrous ether, the specimen softened with loss of double refraction at 128° and gradually coalesced as the temperature rose to 150°. On cooling, good crystals appeared; on reheating, these melted at 158–168°.

Some natural specimens of coproporphyrin III tetramethyl ester examined showed a slight loss of double refraction at 130°, but the real lower m.p. was usually 138–153°, and the upper m.p. as high as 173–179°.

Melting-point composition curve for mixed tetramethyl esters of coproporphyrins I and III. This is given in Fig. 4. The vertical thick lines represent for each mixture the temperature range over which softening of crystal outline and loss of double refraction were observed; its upper limit represents the temperature at which the whole mass coalesced. 'Solidification point' is here taken as the temperature at which double refraction reappeared on cooling. All these mixtures recrystallized well on cooling, those containing 25, 50 and 75% of the coproporphyrin I ester showing crystal forms hybrid between those typical of the esters of coproporphyrins I and III, and the other mixtures showing forms more like those of the major constituent.

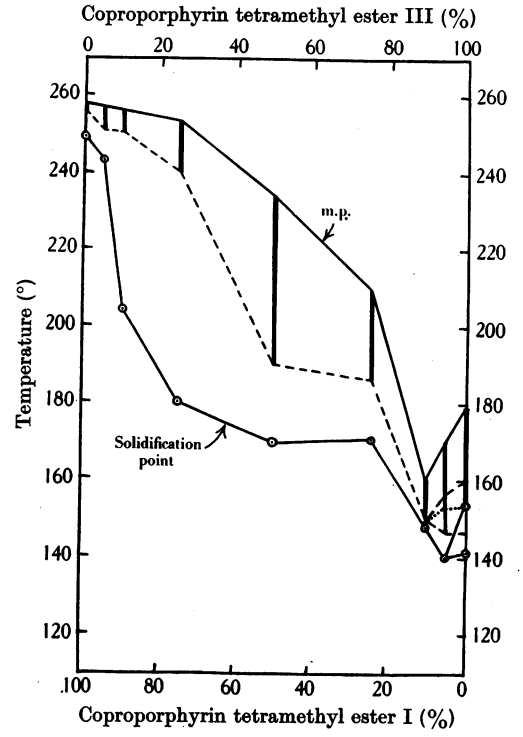

Fig. 4. Melting-point composition curve for tetramethyl esters of coproporphyrins I and III.

- ---- Temperature at which softening and loss of double refraction begins, on heating
- .... Lower m.p. characteristic of coproporphyrin III tetramethyl ester
- —— Melting-point
- ⊙——⊙ Solidification point

DISCUSSION

Spectral absorption curves. A comparison of the scanty data in the literature for the extinction coefficients of 1% solutions of coproporphyrin with our own is given in Table 2. Paic (1936) gives absorption curves for coproporphyrin I in the u.v. obtained by examination of a standard solution supplied by Hans Fischer, and his low value for the extinction coefficient agrees with our findings on solutions made up from Fischer's synthetic coproporphyrin I tetramethyl ester before recrystallization.

Our data for the 548–550 mμ. band agree with those of Kench, Gillam & Lane (1942) and Rimington (quoted by Kench *et al.* 1942), who examined the

Table 2. *Comparison of data of spectral absorption of coproporphyrins*

Reference	Normality of HCl solution	Max. λ in mμ.	$E^{1\%}_{1\,cm.}$
	Ultra-violet		
Present authors	1·5	403·5	7450
Paic (1936)	1·5	403·5	6830
	Visible region		
Present authors	1·5–3·0	549–550	260
Kench *et al.* (1942)*	1·5	549	262
Rimington (1942)†	1·5	549	262
Stern & Wenderlein (1934):			
Coproporphyrin I	3·0	548	254·5
Coproporphyrin III	3·0	548	246

* These authors examined the same solution.
† Quoted by Kench *et al.* (1942).

same solution made up from recrystallized natural coproporphyrin I tetramethyl ester on two Nutting Hilger spectrophotometers. We have examined this same solution in the u.v. (Table 1), but insufficient was available for recording in the visible. Stern & Wenderlein (1934) record values for $E^{1\%}_{1\,cm.}$, $\lambda = 548 m\mu.$, below ours by 2·1 and 5·4% for coproporphyrins I and III respectively. Our observations indicate, however, that these two coproporphyrins have quantitatively equivalent absorption curves over the range $\lambda = 300$ to $\lambda = 650 m\mu$.

Fluorescence. For equal concentrations of coproporphyrin and HCl the fluorescence intensity of coproporphyrin I has been shown equivalent to that of coproporphyrin III. As their energy absorption curves are also equivalent (Table 1 and Fig. 1), the molecules of these two porphyrins have therefore the same fluorescence efficiency, though it is not possible to derive an absolute value from the data at present available.

The fluorescence-pH curves of the coproporphyrin isomers figured by Fink & Hoerburger (1933, 1935) and reproduced by Fischer & Orth (1937, p. 596), suggest that there is a difference between the fluorescence efficiencies of the coproporphyrin I and III molecules. These curves are misleading, as the ordinates are not comparable for each isomer. When Hoerburger's original data (tables in Hoerburger (1933), and Fink & Hoerburger (1933)) are plotted with comparable ordinates, although there are no values below pH 1·8, the curves do suggest that coproporphyrins I and III would be found to have equivalent fluorescence in the dilute acid range where estimations are carried out.

Melting-points of the tetramethyl esters. The m.p.'s we have observed for coproporphyrin I tetramethyl ester agree with those recorded by other workers. With coproporphyrin III tetramethyl ester the phenomenon of 'double m.p.' leads to poor agreement among the values obtained by different workers. Although Fischer and his collaborators (1927, 1929, 1931) have synthesized coproporphyrin III tetramethyl ester by three different processes and have followed the changes in m.p. through eight crystallizations, they have not succeeded in reaching agreement between the m.p. data of synthetic specimens and those from natural sources. Our results tend to confirm this discrepancy between synthetic and natural specimens, and at present there are no means of deciding which of the two represents the behaviour of coproporphyrin III tetramethyl ester.

Criteria of purity of the tetramethyl esters. Although most workers rely upon m.p. data, no m.p.-composition relationship has been previously reported for the system of tetramethyl esters of coproporphyrins I and III, or an attempt made to assess the influence of possible impurities. The m.p.-composition relation (Fig. 3) shows that with coproporphyrin I tetramethyl ester m.p. determination is misleading, allowing the presence of up to 10% of coproporphyrin III tetramethyl ester, unless it is taken in conjunction with resolidification point (not usually stated) and with observation of m.p. after further recrystallization. Such data, considered together with the absorption curves, can provide a reasonable definition of identity and homogeneity of coproporphyrin I tetramethyl ester. With the coproporphyrin III ester the usual m.p. criteria taken alone allow the presence of up to 15% of coproporphyrin I tetramethyl ester, and the position is more complex owing to the 'double m.p.' and the greater difficulty of recrystallization. This 'double m.p.' can be eliminated by converting to the Cu complex, but this introduces a further process, and the Cu complexes have not yet received adequate study (Mertens, 1937; Völker, 1938).

The m.p.-composition relationship is of importance in the estimation of the ratios of I and III isomers in coproporphyrin specimens from natural sources, since the m.p.'s of the tetramethyl esters are usually taken alone as criteria of identity and purity of each isomer. The present work suggests that the m.p.'s must be studied in conjunction with solidification points, and perhaps absorption curves, before such ratios can be investigated accurately. Further work on this subject is in progress.

While the absorption curve may be a good indication of the total porphyrin present, it shows no distinction between the numerous porphyrins, either isomeric or those with different substituents in the porphyrin nucleus, which have very similar absorption curves. Effective separation of these porphyrins is therefore an essential preliminary to the study of their absorption curves and fluorescence properties. Different porphyrins can usually be separated by means of their varying partitions between ether-aqueous HCl, but with isomers it is necessary to rely upon the solubilities of their methyl

esters in different organic solvents. Coproporphyrin I tetramethyl ester, for instance, is insoluble in anhydrous ether or methanol, whilst coproporphyrin III tetramethyl ester is appreciably soluble (Rimington, 1939). So far we have not achieved separation of these isomers by chromatography, and have been unable to confirm the results of Watson & Schwarz (1940), who adsorb the mixed tetramethyl esters of coproporphyrins I and III on Brockmann alumina (Merck Inc.) and elute with varying acetone-water mixtures.

The most important implication of the above results is that the usually accepted criteria of purity and identification of coproporphyrins I and III are inadequate. Thus, quantitative estimations of coproporphyrin may be misleading because the physical constants of standard solutions have not previously been adequately defined. Specimens of coproporphyrin I tetramethyl ester require to be freshly crystallized before they are used for standard solutions. Only by achieving uniformity in this respect can comparable data be obtained by different workers on normal and pathological excretion of coproporphyrin. In the estimation of total coproporphyrin, standard solutions have usually been made from coproporphyrin I tetramethyl ester on the assumption, hitherto unsupported by experimental data, that under the conditions of estimation the absorption curves and fluorescence efficiencies of these two isomers were equivalent. The observations reported here show that this assumption is in fact correct.

SUMMARY

1. The spectral absorption curves of coproporphyrins I and III, in the range 300–650mμ., were found to be identical, and on the acid side of pH 2 show identical changes with varying pH.

2. At acidities below pH 2, the fluorescence efficiencies of these two molecules were found quantitatively equivalent.

3. Although their m.p.'s were satisfactory, the synthetic coproporphyrin I and III tetramethyl esters gave spectral absorption values 12% below those of purified natural specimens, but were brought into agreement by recrystallization. The m.p. of the coproporphyrin esters is alone a poor criterion of purity.

4. The m.p.-composition curve of the tetramethyl esters of coproporphyrins I and III shows that the presence of 10–15% of one isomer may remain undetected in the other, if the m.p. is the sole criterion: the resolidification point is the more sensitive index of purity.

We wish to express our thanks to Prof. R. A. Peters for his interest in this work, to Dr E. R. Holiday for the use of his spectrographic equipment, and to Dr C. Rimington for specimens of porphyrins, for the use of a Köfler melting-point apparatus and for discussion.

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