

tion of the structure of this material awaits the outcome of further work which is in progress.

The present work has demonstrated that powder patterns can be used to characterize porphyrins of established structure and to detect the presence of unusual porphyrins by departure from the standard patterns. Certain points of similarity between the powder patterns of various porphyrin esters are evident from Fig. 1. Full interpretation of the X-ray diffraction patterns of these substances will, however, have to wait upon the completion of studies on single crystals.

SUMMARY

1. The Debye-Scherrer powder patterns of pure porphyrin esters of known structure and configuration have been recorded.

2. A qualitative analysis of mixtures of porphyrin isomers has been made. It is shown that the powder pattern affords a very sensitive criterion of purity.

3. Powder patterns of methylesters of porphyrins from various natural sources have been compared with recorded reference patterns.

4. The nature of the 'Waldenström' porphyrin present in urines from cases of acute porphyria is discussed in the light of the present findings.

We wish to acknowledge the co-operation of Dr R. E. H. Nicholas in the early stages of this work.

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Studies on the 'Waldenström Porphyrin' of Acute Porphyria Urines

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Since the naturally occurring uroporphyrins have not yet been synthesized, conclusions as to their structure are based upon their chemical reactions, particularly their partial decarboxylation to the corresponding coproporphyrins. Identification of the coproporphyrin isomer indicates to which series the parent uroporphyrin belonged.

The first uroporphyrin (m.p. of octamethyl ester 290°), isolated by Fischer (1915) from the urine of a patient with congenital porphyria, yielded coproporphyrin I and was thus designated uroporphyrin I, and from supporting evidence (summarized by Nicholas & Rimington, 1951*a*) was assigned the structure porphin-1:3:5:7-tetraacetic acid-2:4:6:8-

tetrapropionic acid. No other uroporphyrin was known until Waldenström, Fink & Hoerbinger (1935, see also Waldenström, 1934, 1935, 1936) and almost simultaneously Mertens (1936, 1937) reported the presence in the urine of patients with acute porphyria of a new uroporphyrin, the octamethyl ester of which melted at 258° and yielded coproporphyrin III on partial decarboxylation. It was thus regarded as uroporphyrin III, porphin-1:3:5:8-tetraacetic acid-2:4:6:7-tetrapropionic acid. The occurrence of this new uroporphyrin was speedily confirmed and porphyrin octamethyl esters, prepared subsequently from material from similar cases and all melting within the range 255–262°, were referred to generally as 'uroporphyrin III'. Confusion arose when Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945) queried the existence of the series III isomer of uroporphyrin in Waldenström porphyrins and maintained that chromatography upon calcium carbonate showed these to be associations of uroporphyrin I, the ester of which was claimed to melt at 284°, and a heptacarboxylic porphyrin belonging to the III series and characterized by a melting point of the ester of 208°. Some support for this view was forthcoming from the work of McSwiney, Nicholas & Prunty (1950) and of Gray (1950) who all detected coproporphyrin I in the products of decarboxylation of the urinary uroporphyrins studied. The uroporphyrin ester of the former workers, it may be noted, had m.p. 271/274°.

Authentic uroporphyrin III, yielding only coproporphyrin III on partial decarboxylation, was isolated from turacin by Nicholas & Rimington (1951*a*, see also Rimington, 1939). Its octamethyl ester melted at 264°. Once this reference substance had been provided, a detailed investigation of the 'Waldenström' porphyrin became possible. The X-ray crystallographic diffraction patterns given by the octamethyl esters of pure uroporphyrins I and III and of mixtures of these in known proportions have been recorded for comparison with the patterns provided by Waldenström esters (Kennard & Rimington, 1953). Similarly, an investigation has been made of the melting-point curve of mixtures of these two reference substances. Falk & Benson (1953) have elaborated a technique for separating the uroporphyrin I and III esters by paper chromatography, while Falk & Willis (1951) have measured the infrared absorption spectra of a variety of porphyrin esters including those of uroporphyrins I and III. All these ancillary techniques have been used in the study now to be recorded. Chromatography of the pure porphyrins upon solid adsorbents (Nicholas, 1951) and upon paper (Nicholas & Rimington, 1949, 1951*b*) has ensured control of homogeneity and purity at every stage.

A brief summary of the present findings has appeared elsewhere (Rimington, 1952).

MATERIALS AND METHODS

Waldenström porphyrins. These were obtained from the urines of cases of acute porphyria in relapse, i.e. patients experiencing attacks of abdominal colic and neurological disturbances or both and passing porphobilinogen in the urine, but having no history of photosensitization or cutaneous fragility resembling epidermolysis bullosa or of jaundice accompanying the attacks. These criteria are necessary to exclude cases of porphyria cutanea tarda which may suffer attacks closely resembling those of acute porphyria (see Macgregor, Nicholas & Rimington, 1952). A further point of distinction is that in porphyria cutanea tarda, during remission, the stool porphyrin is raised much above the normal level, whilst cases of acute porphyria in remission exhibit a normal faecal porphyrin output. For comparison with these 'Waldenström' porphyrins, urinary uroporphyrins were also examined, one from a case of porphyria cutanea tarda and three from cases of cutaneous porphyria (see p. 112).

Preparation of porphyrins. Since porphobilinogen is the main excretory product in acute porphyria, the urines were either heated in a boiling-water bath for 20 min. at pH 4.2 or left at room temperature for several days until conversion of porphobilinogen to uroporphyrin was complete (see Westall, 1952). Total porphyrin was then adsorbed upon talc, esterified and chromatographed (Nicholas, 1951) the purity of the octacarboxylic fractions being checked by paper chromatography using lutidine (Nicholas & Rimington, 1949, 1951*b*). Melting points were observed on an electrically heated hot stage.

Decarboxylation. This was carried out in 1% (w/v) HCl in sealed tubes at 180–190° for 3 hr. The coproporphyrin fraction was isolated by chromatography of the esterified product.

Separation of isomers. The isomeric coproporphyrin esters were separated by the paper-chromatographic method of Chu, Green & Chu (1951) and uroporphyrin esters I and III by a similar technique due to Falk & Benson (1953). Approximate relative proportions were estimated visually from the intensity of fluorescence and area of the spots under ultraviolet light when compared with chromatograms of mixtures of the pure constituents in known proportions.

Standard reference substances. Uroporphyrin I, ester m.p. 293°, had been prepared from the urine of a patient with congenital porphyria (Rimington & Miles, 1951), further data concerning this material being given by Rimington & Sveinsson (1950). Uroporphyrin III, ester m.p. 264°, was obtained from turacin (Nicholas & Rimington, 1951*a*).

X-ray diffraction Debye-Scherrer patterns. The technique used to obtain these photographs and tables of interplanar spacings calculable from them are recorded in a separate communication (Kennard & Rimington, 1953).

RESULTS

Infrared absorption spectra

Falk & Willis (1951) record the infrared absorption spectra of the reference uroporphyrin esters I and III and also of a typical Waldenström porphyrin ester. The latter possessed characteristics suggesting a mixture of the two uroporphyrin isomers.

The melting-point curve of mixtures in known proportions of uroporphyrin esters I and III

Joje & O'Brien (1945) have shown that the melting-point curve of mixtures of coproporphyrin esters I and III has a pronounced minimum at a point corresponding to about 10% of the series I isomer plus 90% of the series III isomer. Solutions in chloroform of uroporphyrin esters I and III were mixed in known proportions, evaporated to dryness and the residue crystallized from benzene:light petroleum. The mother liquor in each case contained only a trace of porphyrin ester. These solvents were chosen, instead of chloroform:methanol mixtures, as it was important for the purpose of the investigation to obtain the whole of the material in each mixture in the solid crystalline form.

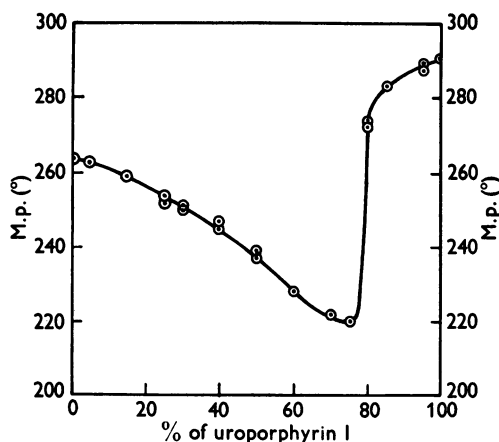


Fig. 1. Melting points of uroporphyrin esters I and III crystallized in known proportions.

The melting points of the mixtures are recorded in Fig. 1. There is a well-marked minimum at a eutectic mixture of approximately 75% I isomer and 25% III isomer. Addition of series I isomer to uroporphyrin III ester caused relatively little depression of melting point, so that a wide variation in composition is possible for mixtures melting between, say, 250 and 260°. But in the mixtures in which uroporphyrin I ester predominates, the reverse is the case. No obvious discontinuity in the curve could be detected around the region 75% series III plus 25% series I isomer.

X-ray Debye-Scherrer patterns

Kennard & Rimington (1953) report the X-ray Debye-Scherrer patterns given by the series of mixtures of known composition referred to above. They have also examined by the same technique some 'Waldenström' porphyrin esters including two used in the present study. The 'Waldenström'

porphyrin esters all afforded the same pattern which was identical with that of a specimen containing 75% of the III isomer and 25% of the I isomer of uroporphyrin. This pattern is characteristic and strongly suggests the occurrence of mixed crystal formation (see Kennard & Rimington, 1953, for further discussion).

Separation of isomers by paper chromatography

Uroporphyrin esters I and III are well separated by the method of Falk & Benson (1953). The 'Waldenström' porphyrin esters examined all exhibited the same two spots, that of the series III isomer being much the stronger. In some specimens, there was so little of the isomer of series I that its spot was only just detectable. The limit of detection, from experience with mixtures of known composition, is placed at about 10%. A specimen of the original 'new uroporphyrin' (ester m.p. 258°) isolated by Mertens (1936), when examined by paper chromatography, travelled at the same rate as pure uroporphyrin ester III. It will be recalled that both Waldenström and Mertens reported that their materials gave rise to coproporphyrin III on partial decarboxylation.

Results of partial decarboxylation

The entire coproporphyrin ester fractions obtained from the 'Waldenström' porphyrin esters were examined by the paper-chromatographic method of Chu *et al.* (1951) which revealed in each case the presence of both coproporphyrins I and III. Assessment of the proportions was made (a) from the relative intensities and areas of the spots and (b) from the melting point of the crystallized ester and reference to Joje & O'Brien's curve. The findings are recorded in Table 1 which also shows the assessment made from (c) the melting-point curve of the uroporphyrin esters and (d) the relative intensities of the spots by the Falk & Benson chromatographic method of the proportions of uroporphyrins I and III in the starting material.

The agreement of these results is reasonably good and shows clearly that the proportion of uroporphyrin III is usually around 75%, as indicated also by the X-ray diffraction results.

Examination of C. J. Watson's material

The results recorded above are at variance with the conclusions of Watson *et al.* (1945). Prof. Watson kindly supplied specimens of (a) a 'Waldenström' porphyrin ester 'type A' (i.e. separable into components by chromatography upon calcium carbonate), (b) his uroporphyrin I methyl ester, and (c) his heptacarboxylic porphyrin ester, m.p. 208°. Chromatography of the small quantity of 'Waldenström' ester upon calcium carbonate was not successful in producing a separation; nevertheless,

Table 1. *Estimation by different methods of the composition of urinary uroporphyrins*

Specimen	M.p. (°)	Proportion of uroporphyrin I from m.p. curve of uroporphyrin ester (%)	M.p. of coproporphyrin resulting from decarboxylation (°)	Proportion of uroporphyrin I from m.p. curve of coproporphyrin ester (%)	Proportion of uroporphyrin I from chromato- graphy of specimen on paper* (%)
Designation					
Beresf. ^A	253-254	15-20	204-208	20-25	ca. 20
Salisb. ^A	255-257	15-20	196-198	20	ca. 20
Glasg. ^A	260	15	180-190	15	Trace
McLaugh. ^A	257-258	15-20	200-205	20-23	ca. 10
L. Walk. ^T	256-260	15-25	202-210	20-25	ca. 25
Ance. ^C	272-278	80-82	—	—	80-95
Turp. ^C	272-279	80-82	—	—	80-90
Four. ^C	260	79 or 12	—	—	50-80

* Judged visually. Remainder uroporphyrin III in all cases.

^A Acute porphyria.

^T Porphyria cutanea tarda in relapse.

^C Cutaneous porphyria. Elderly patients with no history of abdominal or nervous symptoms.

two constituents were shown to be present by paper chromatography using lutidine, the major portion corresponding with an octacarboxylic porphyrin with a small quantity of a hexacarboxylic porphyrin. Falk & Benson's method showed the former to be mainly uroporphyrin I mixed with some uroporphyrin III. When the specimen of m.p. 208° was examined separately by the lutidine method it ran consistently as a hexacarboxylic and not a heptacarboxylic porphyrin. Watson's uroporphyrin I methyl ester contained only octacarboxylic porphyrin and was mainly the I isomer, but a trace travelling as uroporphyrin III ester was detectable by paper chromatography. When this specimen was observed on the electrically heated stage, it melted at 292-293° (Prof. C. H. Gray kindly examined this material independently and found m.p. 294°, substantially the same m.p. (293°) as found by us for our pure uroporphyrin I ester, and not at 284°, the figure repeatedly given by Watson *et al.* (1945) for the melting point of the series I ester. It may be noted, however, that 294° was the melting point recorded for the uroporphyrin I ester obtained from their case 5 (Grinstein *et al.* 1945).

Examination of urinary uroporphyrins from cases of cutaneous porphyria

Dr J. Canivet of Paris kindly placed at our disposal some specimens of uroporphyrin esters prepared by him from the urines of three cases of cutaneous porphyria (for a fuller definition of this condition, see Wells & Rimington, 1953). The patients were all of middle age and had exhibited some degree of photosensitivity and of formation of bullae during the past two or three years, but not previously. They had no symptoms of colic or of disturbances of the nervous system. They did not excrete porphobilinogen, but their urines were dark and contained uroporphyrin. Teeth, etc., were

normal. The esters were shown to contain octacarboxylic porphyrin only and had the following melting points: 'Four.' 260°; 'Turp.' 272-279°; 'Ance.' 272-278°. Examined by paper chromatography (Falk & Benson, 1953) and by X-ray diffraction (Kennard & Rimington, 1953), each was shown to consist of a mixture of uroporphyrins I and III. In specimens 'Turp.' and 'Ance.' the proportion of the I series isomer was 80-95%; in 'Four.' it was less than 80% but more than 50%. It is thus clear that in these cases of cutaneous porphyria the isomer of series I predominated.

DISCUSSION

The use of new techniques has made possible a much more exact analysis of porphyrin mixtures. By use of column and lutidine paper chromatography, fractions containing only octacarboxylic porphyrins may be prepared. Application to such fractions of the paper-chromatographic method of Falk & Benson (1953) and comparison of the X-ray crystallographic diffraction patterns obtained with such material with those of pure reference materials now makes possible the identification of the isomers present and allows a rough estimate of their relative proportions. Supporting evidence can be obtained from the melting point of the sample, and reference to the curve showing the melting points of mixtures in known proportions of uroporphyrins I and III, and also from the melting point and the paper-chromatographic behaviour of the coproporphyrin fraction separated from the products of partial decarboxylation of the parent uroporphyrins. All these methods, when applied to a group of typical 'Waldenström' porphyrins, demonstrate clearly that they contain predominantly uroporphyrin III accompanied by lesser amounts of uroporphyrin I. The uroporphyrin originally isolated by Mertens (1936) has been shown to behave as uroporphyrin

III, thus confirming her conclusion as to its structure. We have observed that the proportion of uroporphyrin III isomer excreted by patients with acute porphyria is somewhat variable, and on occasions the series I isomer may be detectable only as a trace, but as a rule the proportion is about 75 % uroporphyrin III to 25 % uroporphyrin I. It is of interest that it is just at this composition that X-ray crystallographic examination of artificial mixtures (Kennard & Rimington, 1953) shows a marked improvement of crystallization with the appearance of a new, characteristic pattern, strongly suggesting molecular compound formation. The ease of crystallization and recrystallization without change of melting point is a well-known property of the esters of 'Waldenström' porphyrins and is thus explained.

The urinary uroporphyrins of the cases of cutaneous porphyria are of especial interest. All had uroporphyrin I as the predominant isomer. For the specimens 'Turp.' and 'Ance.' with melting points 272–279° and 272–278° respectively, no other result could have been anticipated from comparison with the melting-point curve of known mixtures of uroporphyrins I and III. The specimen 'Four.' was deliberately chosen because of its low m.p., 260°, which could indicate a mixture of either 12 % series I plus 88 % series III or 79 % series I plus 21 % series III. Examination showed conclusively that series I isomer predominated. A melting point approximately the same as that of a typical 'Waldenström' porphyrin ester is thus possible in a uroporphyrin mixture in which there is much more uroporphyrin I than III.

Our contention that uroporphyrin III is the predominant porphyrin present in typical 'Waldenström' porphyrins is supported by the present work, and some comment is necessary concerning our disagreement with Watson *et al.* (1945) who deny the existence of uroporphyrin III in such materials. We can confirm from an examination of the specimen of their material which they sent to us, that it was predominantly uroporphyrin I with a much smaller quantity of uroporphyrin III, and another substance separable on paper as a hexacarboxylic porphyrin. This we regard as an incidental impurity, similar traces of hexa- or hepta-carboxylic porphyrins having been frequently observed by us as contaminants of the uroporphyrin fraction and having required careful chromatography for their complete removal (see, for example, Rimington & Miles, 1951). Watson's ester of m.p. 208° ran similarly as a hexa- and not a hepta-carboxylic porphyrin. Prior to lutidine chromatography, we hydrolyse our esters by contact with 7*N*-hydrochloric acid at room temperature for 36–48 hr. Partial decarboxylation may have occurred under these conditions, but this has never before been observed by us. Our disagreement over the melting

point of uroporphyrin I ester is difficult to understand but it is readily seen from the melting-point curve (Fig. 1) that a small inclusion of the series III isomer markedly lowers the melting point.

A careful reading of the papers of the American authors leaves some doubt as to the nature of the cases they studied. No mention is made of pre-treatment of the urines to convert porphobilinogen into uroporphyrin (cf. Westall, 1952), nor are full clinical descriptions of their cases given in the literature cited. We have found it difficult to separate cases of porphyria cutanea tarda in relapse from those of true acute porphyria. The former may experience colics and nervous symptoms and even excrete some porphobilinogen during their attacks (cf. Macgregor *et al.* 1952), but (a) their blood plasma exhibits porphyrin fluorescence during severe attacks, and (b) during remission their stool porphyrin is always markedly raised whilst the urinary porphyrin may be normal. Further, we have never classified as acute porphyria any case showing any degree of photosensitivity or of abnormal fragility of the skin (formation of bullae on slight trauma). The results recorded here of examination of uroporphyrins from Dr Canivet's three French cases show that in cutaneous porphyria, uroporphyrin I may predominate, even greatly, but that some such uroporphyrin mixtures may have melting points similar to those of true Waldenström esters, in the region of 260°.

SUMMARY

1. The behaviour of the methyl esters of some typical Waldenström porphyrins, obtained from carefully established cases of acute porphyria, has been compared with that of pure uroporphyrin esters I and III when examined by paper chromatography, infrared spectrophotometry, X-ray Debye-Scherrer photography and some other techniques.

2. The melting-point curve has been established for mixtures of uroporphyrin esters I and III crystallized in known proportions.

3. Deductions concerning composition from the melting point of the Waldenström porphyrin esters and of the esterified coporphyrin fractions obtained from them by decarboxylation, also from their paper-chromatographic behaviour and X-ray powder patterns, all indicate that the Waldenström porphyrins examined contain about 75 % of uroporphyrin III and 25 % of uroporphyrin I. The X-ray examination of artificially prepared mixtures has suggested that at this composition molecular compound formation takes place.

4. Some urinary uroporphyrin esters from cases of cutaneous porphyria have been examined by similar methods. They consisted mainly of uroporphyrin I, together with smaller amounts of uroporphyrin III. One such urinary uroporphyrin had ester m.p. 260°.

5. Specimens from Prof. C. J. Watson's laboratory have been examined. The uroporphyrin methyl ester I had m.p. 292–293° and behaved as uroporphyrin I containing a trace of uroporphyrin III on paper chromatography. The 208° ester behaved chromatographically as a hexacarboxylic porphyrin.

We wish to thank Dr Falk and Miss Benson for performing the chromatographic examinations by their method, and also Prof. C. J. Watson and Dr J. Canivet for kindly placing materials at our disposal. A generous grant to one of us (C.R.) from the Trustees of the Nuffield Foundation has made possible the establishment of a Unit for the Investigation of Pyrrole Pigment Metabolism and this grant is gratefully acknowledged.

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The Protein Components of the Isolated Myofibril

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The myofibrils are unique among the organized components of cells in that it requires a common substrate, adenosine triphosphate (ATP), for enzymic and mechanical activity. Both these properties survive when the myofibril is removed intact from the cell, and although it is true that the main protein component, actomyosin, has similar characteristics, extraction of this complex completely disorganizes the myofibrillar structure and results in modification of both enzymic and mechanical behaviour.

Schick & Hass (1951) investigated the solubility in salt solutions of varying ionic strength of myofibrils obtained by a procedure which involved tryptic digestion of the muscle tissue. These workers used an arbitrary method of microscopic examination to determine when solution was complete, but in view of the pronounced effect of trypsin on myosin (Gergely, 1950; Perry, 1951), and on proteins

generally, it is difficult to decide to what extent the constituent proteins were modified during the preparation of the myofibrils. Information about the protein components of the myofibril other than actomyosin is scanty; for example nothing is known about the nature of the various banded features, all of which must play a part in the function of the contractile unit. Earlier electron-microscope investigations (Rosza, Szent-Györgyi & Wyckoff, 1950; Perry & Horne, 1952) have provided evidence for the washing out of some band components with no apparent modification of the underlying basic filamentous structures.

The present communication is concerned with further investigations of the properties of myofibrils prepared without the use of enzymes. In particular, the nature of the protein components extracted from the myofibril under varying ionic conditions has been investigated.