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Preparation of Crystalline Porphyrin Esters from Bovine Porphyria Urine

By T. K. WITH

Central Laboratory, Svendborg County Hospital, Denmark

(Received 11 June 1957)

For studies of porphyrin metabolism in which chromatographic procedures are employed, pure porphyrins are often required as markers. With uro- and copro-porphyrins these are difficult to obtain. A simple method for preparing such markers is therefore desirable.

Preparation of porphyrins from urines very rich in them is simple, as the porphyrins are precipitated on acidification. Recently Ottolenghi-Lodigiani & Serchi (1951) prepared 200–300 mg. of crystalline porphyrin esters from 1 l. of human congenital-porphyria urine in this way; but if the porphyrin concentration is lower, the preparation is much more difficult (cf. Lemberg & Legge, 1949) and varied techniques have been employed. Rimington & Miles (1951) acidified 3 l. of human porphyria urine, collected the precipitate and adsorbed the porphyrins in the filtrate on kieselguhr; but Macgregor, Nicholas & Rimington (1952) preferred adsorption on talc at pH 3–4 and stated that the extraction of the total porphyrin from urine presents considerable difficulty.

The method described below was elaborated during work with bovine porphyria urine (cf. Jørgensen & With, 1955), but has been found to function equally well with human porphyria urines and even normal human urine. In the latter the red fluorescence is quenched, however, and appears only after the chromatographic purification. Volumes of 20 l. have been treated, and special difficulties were encountered because of the alkaline reaction of bovine urine. The only studies on bovine porphyria urine reported previously are those of Rimington (1936), in which the urine was removed from a single animal *post mortem*, and it seems that the porphyrin concentration of this urine was exceptionally high.

Because of the large amounts of porphyrins,

current methods of chromatography and crystallization had to be modified. For chromatography on aluminium oxide, mixtures of chloroform and benzene were employed instead of pure benzene, and crystallization was performed at room temperature.

The various steps of the procedure were followed with quantitative and qualitative analyses and the purity of the crystalline esters was checked by paper chromatography, melting-point determination and estimation of the extinction ($E_{1\text{cm}}^{1\%}$) at the maximum of the Soret absorption band.

MATERIALS AND METHODS

The urine was from the porphyric steer described by Jørgensen & With (1955). The CHCl_3 employed in the procedures was of pharmacopoeia quality (*Pharm. Danica*) unless otherwise noted. The MgO (British Drug Houses Ltd.) employed was the preparation for chromatography, and was found to correspond to grade III according to Brockmann & Schodder (1941). The commercial Al_2O_3 preparations (British Drug Houses Ltd. or Merck Inc.) were too strongly adsorbent and were therefore treated by suspension in water, then sucked dry on a Büchner funnel and dried at 60° for 1–2 days. Before use it was graded by the procedure of Brockmann & Schodder (1941), see also Williams (1946), an adsorbency corresponding to grade III–IV being required. It is important to remove the considerable quantities of non-porphyrin pigment present in all urines, both in porphyria and other conditions. These pigments are presumably dipyrrole pigments like the porphobilin of Waldenström & Vahlquist (1939), the urochrome B described by Stich & Stärk (1953) and the fuscins found in normal as well as pathological faeces (cf. With, 1954). The removal of such pigments was achieved by adsorption on talc, followed by elution with 5–10% (v/v) aqueous pyridine solution or N-HCl -acetone (1:9, v/v). The porphyrin was precipitated from the pyridine on acidification or from the acetone with ammonia; the precipitate was dried and esterified with 5% (v/v) H_2SO_4 in methanol (Fischer & Zerweck, 1924).

Collection and treatment of bovine urine

Collection of urine from cows is relatively simple, but collection from bulls is difficult. For large-scale collection a hole was dug in the stall under the hind part of the animal, and this hole was covered with a plastic-coated iron grate. From the hole a pipe of glazed clay allowed the urine to flow down into a 10 l. plastic bucket placed about 50 cm. below the floor of the stall. In this way the 24 hr. urine was collected conveniently without significant losses, and contact with metals was avoided. Contamination with a certain amount of dirt from the floor was inevitable, but this did not cause serious inconvenience during the following operations.

Acidification was carried out in 50 l. glass carboys because of liberation of CO_2 with violent foaming. The bovine urine required 50 ml. of conc. HCl /l. to reach about pH 2, which was found most convenient; human urine required only about 3 ml./l. to attain the same pH.

Adsorption on filters. The porphyrins of the urine were adsorbed on talc (*Pharm. Danica*) or crude kieselguhr by suction through filters of these materials on Büchner funnels. For large-scale work with 10–20 l. of urine, containing 20–40 mg. of porphyrins/l., portions (5 l. or less) were passed through layers of talc or kieselguhr, 2–3 mm. thick, prepared by pouring an aqueous slurry of the adsorbent on the funnels (25 cm. diam.). Foaming of the talc slurry was controlled by addition of ethanol, which was subsequently washed out with water. The urine was poured on after the filter had been sucked dry with vigorous suction. The passage of 5 l. often took several hours. A more complete adsorption of porphyrins may be achieved by passing the filtrate through a second filter of fresh talc or kieselguhr; this second passage is much more rapid and one filter is sufficient for 20 l. Talc is more convenient than crude kieselguhr because the fluorescence of the adsorbed porphyrin is clearly visible, whereas it is quenched on kieselguhr. However, kieselguhr does not give rise to foam and is less expensive than talc.

Elution and precipitation. The pooled adsorbents were extracted by suspension in N-HCl -acetone (1:9, v/v). Drying before this extraction is not necessary; residual moisture in the filters after suction does not influence the procedure. The HCl -acetone was removed from the adsorbent by suction through a Büchner funnel, the adsorbent was suspended in a fresh portion of HCl -acetone and this process repeated until complete. The combined HCl -acetone eluates were precipitated by addition of conc. aq. NH_3 soln. to about pH 8.5, when a precipitate was formed and simultaneously the red fluorescence of the eluate was quenched. The precipitate was collected on paper by filtration and dried on a radiator. It contained about 90% of the porphyrin present in the eluate.

Esterification and extraction of esters. The dried precipitate was pulverized in a mortar and treated overnight with H_2SO_4 -methanol (5:95, v/v), prepared immediately before use. For large-scale preparation commercial denatured methanol suffices, but for analytical purposes absolute methanol distilled over magnesium turnings was used. After standing overnight the suspension was filtered and the residue stirred and extracted on the Büchner funnel with CHCl_3 until completion. Then fresh H_2SO_4 -methanol was added and the CHCl_3 extraction repeated once more to completion. The pooled methanol- CHCl_3 extracts were neutralized (Congo red paper) with saturated sodium acetate in a separating funnel and water sufficient to bring

about separation was added. The aqueous phase was extracted with CHCl_3 to completion; the pooled extracts were then washed three times with water. A small sample was extracted with aq. 2N-NH_3 soln.; incomplete esterification shows itself by red fluorescence of the ammonia washings, but this was very rarely encountered. Finally, the CHCl_3 extract was dried by filtration through a double layer of filter paper wetted with freshly washed and dried CHCl_3 , and anhydrous CaCl_2 was added. After drying, the solution was separated from the CaCl_2 and the CHCl_3 evaporated at room temperature.

Chromatographic purification on magnesium oxide. This was performed on short columns, 2–3 cm. long. The technique of Nicholas (1951) was followed, the columns being prepared from suspensions of MgO in pharmacopoeia-quality CHCl_3 and the porphyrin esters were added to the column as a concentrated solution in the same solvent, which was also used for development (the ethanol present in the CHCl_3 used is of importance in this procedure). The non-porphyrin pigments adhere to the top of the column and the porphyrin esters are eluted. The size of the column depends on the amount of porphyrin to be handled. With 50 mg. or more, longer columns or passage through a second column after concentration of the first eluate by evaporation may be necessary.

Chromatographic fractionation on aluminium oxide. The procedure of Nicholas (1951) was employed, the columns being 7 cm. high and 1.5–2 cm. diam. Their tops were protected by a layer of acid-washed sea sand, 0.5–1 cm. high. The porphyrin esters were dissolved in as little freshly washed, dry CHCl_3 as possible, 9 vol. of pure analytical benzene were added and the porphyrins were adsorbed on a column prepared by suspending aluminium oxide in benzene- CHCl_3 (9:1, v/v). Crystals often separated from the concentrated CHCl_3 solution on addition of the benzene and were removed by filtration before applying the resulting filtrate to the column. The residue was mainly uroporphyrin ester, and was purified by recrystallization.

The columns were developed with benzene- CHCl_3 mixtures [analytical benzene, freshly washed and dried CHCl_3 (7:3, 5:5 and 3:7, v/v)], pure CHCl_3 and finally absolute methanol in CHCl_3 [beginning with 0.1% (v/v) and ending with 0.5 or 1% (v/v) methanol in CHCl_3]. As a rule the coproporphyrin began to be eluted with benzene- CHCl_3 (9:1 or 7:3, v/v), and passed quantitatively with pure CHCl_3 ; the uroporphyrin began passing with 0.1% methanol. The maximum amount of porphyrin which could be fractionated on one column was about 50 mg.

Crystallization. For large-scale work hot crystallization was not convenient, and the process was carried out at room temperature. The purified ester solutions were evaporated to dryness at room temperature, the dry ester was dissolved in as little CHCl_3 as possible and 3–4 vol. of absolute methanol were added and thoroughly mixed. In this way concentrated solutions of uroporphyrin I produce an immediate crystalline precipitate, uroporphyrin III (from turacin, cf. With, 1957b) shows a somewhat delayed crystallization and coproporphyrins only crystallize after some hours with much porphyrin remaining in the mother liquor. More rapid and complete crystallization of coproporphyrin was obtained by the use of light petroleum (b.p. below 50°) instead of methanol. The crystals were separated from the mother liquor by filtration and washed on the filter with absolute methanol (or light petroleum if this solvent was used for crystallization).

Analytical procedures

Ultraviolet light. The red fluorescence of porphyrin solutions was used when testing for the completion of the extractions and the elution from chromatograms. A lamp standing on the bench as described by With (1957a) was used.

Spectrophotometric determination of porphyrins. After appropriate dilution of the urine (at least tenfold) with 1.5N-HCl, readings were carried out at 380 and 430 m μ as well as at the maximum of the Soret band; the porphyrin content was calculated by the method of Rimington & Sveinsson (1950) with the constants given by With (1955). The porphyrin content of HCl-acetone eluates or H₂SO₄-methanol extracts was determined in a similar way after dilution 100- to 500-fold, after which the optical densities of the original solvents became insignificant. For CHCl₃ solutions of esters, 0.05 ml. was pipetted into a watch glass and evaporated, and the residue dissolved in an appropriate volume of 7N-HCl and transferred to a measuring flask, being simultaneously diluted to a concentration of about 1.5N-HCl. The identity of the porphyrin solutions was checked by determining the positions of the α bands in a Hartridge reversion spectroscope.

Analysis of crystals. The dry crystals (5-10 mg.) were weighed (± 0.1 mg.) and dissolved in 1-2 ml. of conc. H₂SO₄ in a small beaker. Solution was completed when necessary by cautious addition of about 1 ml. of water. The oily solution was quantitatively transferred to a 10 ml. measuring flask and diluted with 1.5N-HCl. After further dilution (300- to 500-fold) with 1.5N-HCl the Soret maximum was determined and $E_{1\text{cm}}^{1\%}$ at this wavelength calculated for the free porphyrin by assuming that uroporphyrin octamethyl ester contains 88% and coproporphyrin tetramethyl ester 92% of the porphyrin free acid.

Spectrophotometer. A Hilger Uvispek with quartz prism and quartz cells was used.

Paper chromatography in lutidine. The crystals were hydrolysed with 7N-HCl for 36 hr. and the HCl was removed *in vacuo*; the residue was dissolved in aq. 2N-NH₃ soln. and applied to Whatman no. 1 paper. Ascending paper chromatography with 2:6-lutidine, as described by Eriksen (1953), was used.

Melting-point determination. A Gallenkamp micro-melting-point apparatus was used.

RESULTS

During 3 months a total of about 1 g. of pure crystalline uroporphyrin ester and an equal quantity of coproporphyrin ester was prepared.

The crystals of uro- and copro-porphyrin isolated from the bovine urine were of varied purity. Sometimes preparations of high purity, giving values of $E_{1\text{cm}}^{1\%}$ at the Soret maximum about 6500 [corresponding to absolutely pure uro- and coproporphyrin (cf. Rimington & Sveinsson, 1950; Hilger & Watts Ltd., 1955; With, 1955; C. Rimington, personal communication)], were obtained at the first crystallization, but more often values of about 4000 were obtained. The uroporphyrin crystals usually showed only insignificant contamination with other porphyrins demonstrable on

chromatography in lutidine; at the most, traces only of 7- and 6-carboxyl porphyrin were present. The coproporphyrin was, on the other hand, regularly contaminated with 6-carboxyl porphyrin, and this porphyrin, which was constantly found in appreciable quantities in the urine of all the porphyric Danish cattle tested, was very difficult to remove completely from the coproporphyrin. By repeated crystallization it was found that the porphyrins with the higher number of carboxyl groups were found in increasing concentration in the crystals, whereas those with the lower number of carboxyl groups increased in concentration in the mother liquor. Therefore uroporphyrin crystals free from 6- and 7-carboxyl porphyrin were relatively easy to obtain whereas completely pure coproporphyrin was very difficult to obtain. Amounts of completely pure 6-carboxyl porphyrin sufficient to give reliable determinations of its absorption constants and melting point have not yet been prepared.

Complete chromatographic separation of the 6-carboxyl porphyrin from coproporphyrin and uroporphyrin was not achieved; fractions of uroporphyrin free from 6-carboxyl, and of coproporphyrin free from 6-carboxyl, could be obtained, but fractions of 6-carboxyl without uro- or coproporphyrin were not obtained in this way.

The characteristic crystal forms of uro- and copro-porphyrin obtained on crystallization from hot methanol-CHCl₃ did not usually appear on large-scale crystallization at room temperature, but when the crystals so obtained were recrystallized on a smaller scale the usual fine needles of uroporphyrin I and the coarser ones of coproporphyrin I were obtained.

The melting points of the uroporphyrin esters obtained on first crystallization varied between 275° and 295°, rising to 295-297° on recrystallization. For the coproporphyrin esters the corresponding figures were 230-255°, rising to 250-255°. The purest crystalline 6-carboxyl porphyrin ester obtained had m.p. 240-245°.

The fate of the porphyrins present in the bovine urine was followed by quantitative and qualitative analysis through the various stages of the purification procedure on several occasions. During the primary adsorption on to talc or kieselguhr about 80% of the total porphyrin was adsorbed on the first filter, and if two adsorptions in succession were employed less than 10% remained in the filtrate. The acetone eluate contained about 10 mg. of total porphyrin for every 30 mg. present in the urine, i.e. about two-thirds of the porphyrin present in the urine disappeared during the initial purification procedures. As only minor amounts were lost with the filtrate, the main loss must have been due to destruction on or incomplete elution from the

absorbent. The filtrate from the precipitated acetone eluate contained usually only traces of porphyrin. The esterification and ester extraction gave rise to heavy losses; 25–50% of the porphyrin present in the acetone eluates was found to be lost during this procedure, and only 15–20% of the porphyrin originally present in the urine was recovered as crude ester. The esterification is the most cumbersome part of the procedure, often giving rise to emulsions. The loss during the chromatographic purification on magnesium oxide amounted to about 20% and that during the chromatography on aluminium oxide to 5–10%. The method was also tried on a smaller scale on a few human porphyria urines, and the Waldenström esters were obtained in crystalline form without difficulty. Also, preparation of crystalline uro- and copro-porphyrin from 20 l. of normal human urine was achieved, but here no red fluorescence was visible before the chromatography.

DISCUSSION

The findings are consistent with current knowledge of the nature of the porphyrins present in congenital porphyria, since the uro- and copro-porphyrin isolated from the urine of the porphyric steer showed the crystal form and melting-point characteristic of the series I isomers. The constant occurrence of minor but significant amounts of a porphyrin which, according to the lutidine chromatography, contained six carboxyl groups is noteworthy. The same porphyrin was found in urines from all other Danish bovines with congenital porphyria. It may perhaps be characteristic of bovine congenital porphyria.

The urine of the porphyric steer contained between 2 and 4 mg. of total porphyrin/100 ml., when the animal was in the stable on a feed relatively poor in water. The total porphyrin was found to consist of about equal amounts of uro- and copro-porphyrin, mostly somewhat more copro- than uro-porphyrin. The sum of the concentrations of copro- and uro-porphyrin measured after extraction was always about 25% higher than the total concentration of porphyrin (calculated as uroporphyrin) found by direct spectrophotometric measurement of the diluted urine; this is presumably because the assumptions about the spectral-absorption curve in the region 380–430 m μ , on which the correction of Rimington & Sveinsson (1950) is based, were not valid for the diluted urine of the steer.

Another method for large-scale preparation of crystalline uroporphyrin has recently been published by Kennedy (1956). Although this method is relatively simple, our method is nevertheless more simple and convenient if porphyria urine rich in

porphyrins is available in quantity. But if this is not the case, Kennedy's method is valuable because his raw material, the shell of the pearl oyster *Pinctada vulgaris*, is commercially available.

SUMMARY

1. A method for large-scale preparation of uro- and copro-porphyrin I as crystalline esters from bovine porphyria urine is given.

2. About 10% of the porphyrin present in the urine is recovered as crystalline esters.

3. Besides uro- and copro-porphyrin I a 6-carboxyl porphyrin was found to be constantly present in minor quantities in bovine porphyria urine.

This study was supported by grants from Godsejer Viktor A. Goldschmidts Legat and from De Samvirkende Kvægavlsforeninger m.k.S. Veterinary assistance was given by practising veterinary surgeon J. Erdmann of Svendborg whose never-failing aid and encouragement have been of outstanding value. I also wish to thank chief veterinary surgeon S. K. Jørgensen of Thisted, as a result of whose interest the presence of porphyria in living Danish cattle was first discovered. Professor C. Rimington of London I want to thank for continuous friendly advice and help of the highest quality. Valuable technical assistance was given throughout the whole investigation by Mrs M. Rubin and in periods by Miss L. Hansen, Miss I. Westergaard, Miss B. Davidsen and Miss A. Pelck. I am most grateful to Mr Gordon Nielsen for taking care of the porphyric steer and collecting and acidifying the urine.

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