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Identification of the Acrylate Porphyrin S-411 from Meconium

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A new porphyrin isolated from calf and foal meconium has been characterized as a tris-(2-carboxyethyl)-mono-(2-carboxyvinyl)-tetramethyl porphin, that is as an analogue of coproporphyrin in which one propionate group has been replaced by an acrylate group. The porphyrin is mainly a type III isomer and its possible significance in haem biosynthesis is discussed.

Meconium is a valuable source of material for the biochemist since it represents the accumulated products of development and metabolism of the foetus, unchanged by the action of intestinal micro-organisms. It is rich in bile pigments and in porphyrins. The porphyrins of meconium have been studied by Garrod (1892), Günther (1922), Papendieck (1924), Waldenstrom (1936) and others. A comparative study of different animal species was made by Nicholas & Rimington (1951). Rimington (1939) found calf meconium to be a rich source of porphyrin, containing about 2mg/100g dry wt. Rimington also found that in human meconium coproporphyrin I comprises about 90% of the total coproporphyrin (C. Rimington, unpublished work). A pentacarboxylate porphyrin was detected in calf meconium in addition to coproporphyrin and was isolated by Nicholas (1951). Its methyl ester had m.p. 231°C, the α-band in chloroform at 623.1nm and it crystallized in curved needles.

In a study of human bile and meconium French & Thonger (1966), using their sensitive technique of microscale countercurrent distribution analysis, detected two unidentified porphyrins having Soret maxima in 5% (w/v) hydrochloric acid at 405–6nm and 410–11nm respectively. Of these, the latter (S-411) was present in the greater amount, approximating one-tenth of the accompanying coproporphyrin. The same porphyrins were present also in calf and foal meconium and in human bile. Porphyrin S-411 had a rhodo-type spectrum and ran as a tetracarboxylate porphyrin in the lutidine paper-chromatographic system described by Eriksen (1958).

In this paper we describe identification of porphyrin S-411 as a monoacrylate-tripropionate-tetramethyl porphin, that is, as a coproporphyrin in which one propionate β-side chain is replaced by an acrylate group. Its possible origin and relation to haem biosynthesis is discussed.

METHODS

Spectral absorption. This was measured on a Unicam SP.500 spectrophotometer or SP.800 recording spectrophotometer.

Paper chromatography. The method of Eriksen (1958) was used with the lutidine–water system.

Column chromatography. Alumina for chromatographic analysis (grade V; Savory and Moore Ltd., London W.1, U.K.) was activated by heating at 220°C for 3h and cooling in a desiccator. A weighed quantity was transferred to a well-stoppered jar and one-tenth of its weight of water was added. The jar was then closed and shaken vigorously until the contents were again a homogeneous fine powder. It was kept in a closed jar.

Adam's catalyst. This was prepared by passing H_2 into platinic oxide suspended in acetic acid.

Mass spectrometry. Mass spectrometry of the porphyrin esters was performed for us by Professor G. W. Kenner and Dr A. H. Jackson of the University of Liverpool. The instrument used was an A.E.I. MS-9 with direct inlet system operating at ionizing energy 70eV, ionizing current 50μA and source temperature up to 280°C.

Nuclear magnetic resonance. N.m.r. spectra were recorded by Professor J. A. Elvidge of the University of Surrey with an NMR 60 MH instrument.

RESULTS

The starting material for this investigation was the fraction S-411 obtained from calf and foal meconium by application of countercurrent analysis (French & Thonger, 1966). Evaporation of ether

from the fraction left a green mass (194mg) containing acetic acid. It was resuspended in 4ml of acetic acid, giving a solution in which red porphyrin fluorescence was masked by an intense green fluorescence due to non-porphyrin materials. Addition of 25ml of ether produced a precipitate containing almost all of the porphyrin.

For further purification, the precipitated material was washed with successive 2ml portions of ether and dried over sodium hydroxide *in vacuo* (yield 39mg). This residue was stirred with three successive 3ml portions of ice-cold acetic acid and the remaining 17.6mg was then extracted into acetic acid at 40°C. This gave a bright-red solution of porphyrin and a small white inorganic residue (9.3mg) which was discarded. The cold acetic acid extract exhibited spectral absorption similar to that of the porphyrin extracted by the warm acid but also contained much greenish-yellow impurity. This fraction was not studied further. Portions of the porphyrin solution in the warm acid were used for (*a*) measurement of electronic spectra in acetic acid, ether and 0.1M-hydrochloric acid, (*b*) catalytic hydrogenation and (*c*) preparation of the methyl ester. The ester was further purified for determination of melting point, absorption spectra and for mass spectrometry and n.m.r. studies.

Spectral absorption. The findings are recorded in Table 1. As reported by French & Thonger (1966) porphyrin S-411 has a rhodo-type spectrum in neutral solvents. The spectrum of the pure tetramethyl ester together with that of the product of its reduction is shown in Fig. 1.

Catalytic hydrogenation. Porphyrin S-411 (5.2mg) dissolved in acetic acid was shaken with reduced Adam's catalyst (10mg) in a current of H_2 at 20°C and uptake of gas was recorded (Fig. 2). Assuming a molecular weight of 652 for the porphyrin, H_2 uptake virtually ceased after absorption of 1 molecular equivalent (required at standard temperature and pressure: 0.18ml; found, 0.17ml).

At cessation of H_2 absorption the solution was filtered, diluted with 10vol. of water and the porphyrin was extracted into ether which was dried by filtration. Spectral absorption of the reduced porphyrin was measured in this solvent and in 0.1M-hydrochloric acid after transference by shaking the ethereal solution with the dilute acid. The values are recorded in Table 1. The spectrum in ether was of the aetio-type and similar to that of coproporphyrin. On lutidine paper chromatography about 85% migrated as coproporphyrin III, the remainder travelling to the coproporphyrin I position. In an earlier experiment in which a much smaller quantity of porphyrin S-411 was extracted from meconium, the product of catalytic reduction appeared to contain considerably more coproporphyrin I. The reason for this discrepancy remains obscure although it is possible that the separation of porphyrin S-411 and the coproporphyrin I in the meconium extract may have been incomplete.

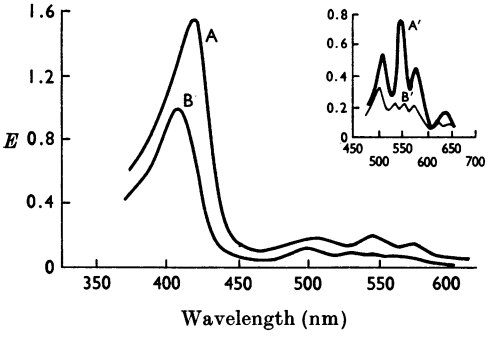


Fig. 1. Electronic absorption spectra, in chloroform solution, of S-411 porphyrin tetramethyl ester (A and inset A') and the ester of the porphyrin produced by catalytic reduction of S-411 porphyrin (B and inset B').

Table 1. *Spectroscopic characteristics of the unreduced S-411 porphyrin, its methyl ester, the product of its reduction with one molecular part of hydrogen and authentic coproporphyrin*

Compound	Solvent	Wavelength maxima (shoulders *in italics*) (nm)
Unreduced free porphyrin	Acetic acid	415, 559, 607, *390, 510–530, 575–590*
Unreduced free porphyrin	Diethyl ether	409, 507, 545, 575–585, 635
Unreduced free porphyrin	0.1M-HCl	408, 550
Tetramethyl ester of the unreduced porphyrin	Chloroform	413, 510, 553, 578, 640
Reduced free porphyrin	Acetic acid	410, 495, 505, 555, 580
Reduced free porphyrin	Ether	396, 495, 525–535
Reduced free porphyrin	0.1M-HCl	401, 490–510, 550
Tetramethyl ester of the reduced porphyrin	Chloroform	401, 500, 530, 575*, 630, 640
Authentic coproporphyrin III	Ether	396, 495, 525–535
Authentic coproporphyrin III tetramethyl ester	Chloroform	400, 498, 532, 567*, 622

* The difference between these two wavelengths is probably due to the presence of a trace of unreduced S-411 porphyrin.

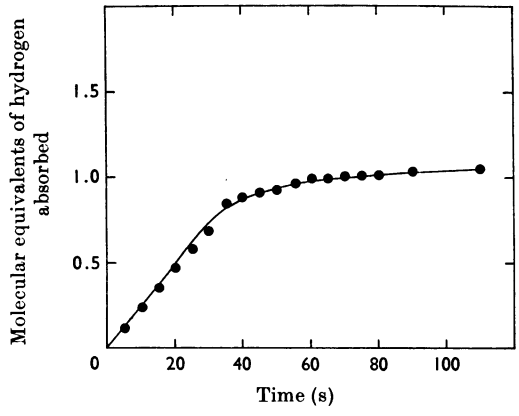


Fig. 2. Uptake of H_2 at 20°C and atmospheric pressure by 5.2mg of S-411 porphyrin in acetic acid and in the presence of 10mg of reduced Adam's catalyst.

Preparation of methyl esters. The reduced porphyrin and the remainder of the unreduced pigment were separately transferred to ether and esterified by an addition of an equal volume of methanol–sulphuric acid (19:1, v/v), the solution being kept overnight at room temperature in the dark. After addition of excess of water the porphyrin esters were extracted by shaking with chloroform. The extracts were washed by shaking with 0.5M-ammonia and then with water and were dried by filtration through paper. Spectral absorptions of the reduced and unreduced material are recorded in Table 1 and Fig. 1.

Crystallization of the unreduced porphyrin ester. The ester, obtained as described above, was purified by chromatography on a column of alumina (grade V) suspended in washed and dried chloroform. Development with chloroform eluted most of the pigment in a compact band (fraction 1) and left a small fluorescent residue (fraction 2) near the top of the column. This was eluted readily by chloroform containing 1% (v/v) of methanol. The two fractions had identical rhodo-type spectra. Fraction 1 was concentrated on a water bath (40°C) and the porphyrin esters were crystallized by addition of about 5vol. of hot dry methanol. It crystallized in rosettes of prismatic needles (Plate 1) and had m.p. 232°C. The small amount of material in fraction 2 was recovered by evaporation of the solvent.

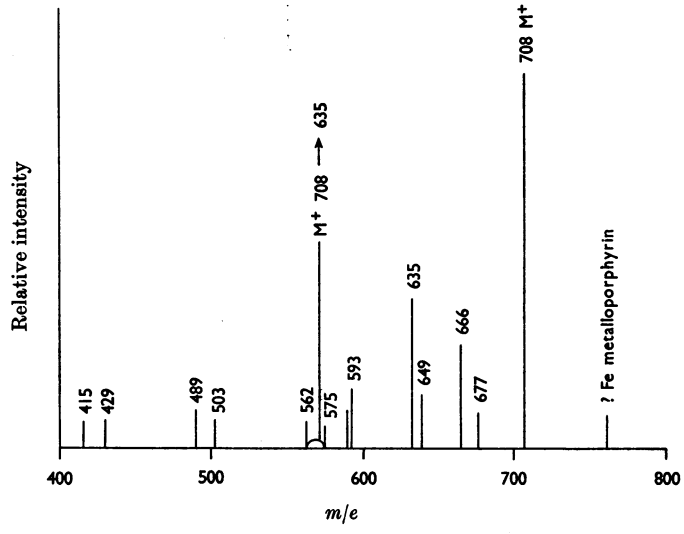
Mass spectrometry. The porphyrin methyl esters in fractions 1 and 2 and the corresponding products of their catalytic reduction were examined in the MS 902 spectrometer by Professor G. W. Kenner and Dr A. H. Jackson of the University of Liverpool. Both unreduced porphyrin esters showed major signals due to a parent ion of mass 708, which corresponds to the tetramethyl ester of a coproporphyrin analogue having one acrylate group replacing a propionate side chain. The general fragmentation pattern was compatible with such a structure, particularly in containing signals of mass 637 and 635 corresponding to scission of propionate and acrylate side-chains. The main material of fraction 1 but not fraction 2 showed in addition a signal of mass 914, also apparently due to a propionate porphyrin since it was accompanied by a P-71 signal due to loss of side chains. The mass 914 signal was not consistently observed, however, and may have been due to an artifact formed by a part-molecule transfer occurring during mass spectrometry. In an additional spectrum shown in a standardized form in Fig. 3 and obtained in a separate instrument by Mr D. W. Carter at the School of Pharmacy, London, this high-mass characteristic was completely absent.

Mass spectrometry of the ester of the reduced porphyrin showed the same general characteristics outlined above, but with displacement by +2 units of molecular weight. The main product of mass 710 was thus indistinguishable from coproporphyrin tetramethyl ester in its pattern but the anomalous heavier ion was still apparent in fraction 1, although now of mass 916 with its propionate-deficient derivative of mass 845.

Nuclear magnetic resonance. N.m.r. spectra of fractions 1 and 2 from the chromatography of the methyl ester of porphyrin S-411 were studied in deuterochloroform. The spectrum of fraction 1 contained a characteristic at 6.8p.p.m., probably due to methoxycarbonyl protons of the three propionate side chains, and a smaller one at 6.4 p.p.m. assumed to be due to protons of the methyl group of the esterified acrylate substituent. The spectrum of fraction 2 lacked all but vestiges of these characteristics probably owing to extensive hydrolysis of the ester group. The spectrum of the mobile fraction showed indications of two peaks at 1.4p.p.m. ($J = 14$Hz) and corresponding peaks were present in the spectrum of the immobile fraction at 1.7p.p.m. ($J = 16$Hz). These were assumed to be doublets arising from the olefinic protons of the acrylate group. If this assumption is correct the difference in spin-coupling constant would suggest that the mobile fraction contains the *cis* isomer and that the immobile fraction contains the *trans* isomer of porphyrin S-411.

DISCUSSION

Since meconium is a sterile material, the presence in it of the porphyrin S-411 described in this paper indicates that this pigment is a product of metabolism of the foetus. On lutidine paper chromatography it migrates as a tetracarboxylate porphyrin.

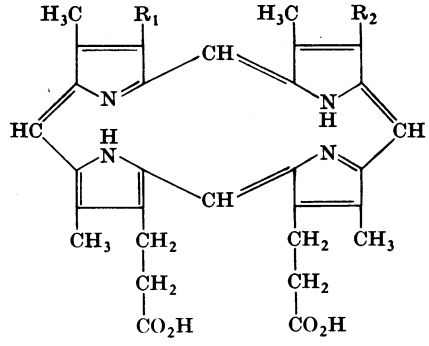


m/e	Fragments lost
677	$-O\cdot CH_3$
666	$-CH_2:C:O$
649	$-CO_2\cdot CH_3$
635	$-CH_2\cdot CO_2CH_3$
593	$-CH_2\cdot CO_2CH_3 + -CH_2\cdot CO$
575	$-CH_2\cdot CO_2CH_3 + -CO_2CH_3$
562	2 $-CH_2\cdot CO_2CH_3$
503	2 $-CH_2\cdot CO_2CH_3 + -CO_2CH_3$
489	3 $-CH_2\cdot CO_2CH_3$
429	3 $-CH_2\cdot CO_2CH_3 + 4\ -CH_3$
415	3 $-CH_2\cdot CO_2CH_3 + 4\ -CH_3 + CH_2$ (or 3 $-CH_2\cdot CO_2CH_3 + -CO_2CH_3 + -CH_3$)

Fig. 3. Normalized mass spectrum of the tetramethyl ester of S-411 porphyrin obtained in the MS 902 mass spectrometer (Mr D. W. Carter).

Mass spectrometry of the tetramethyl ester indicates a molecular weight of 708, two units less than coproporphyrin tetramethyl ester, and on catalytic reduction 1 mol of hydrogen is taken up with change of the spectral absorption from rhodo-type to aetio-type. Apart from a slight shift of the 567 nm absorption maximum of coproporphyrin to 575 nm for the reduction product the latter is indistinguishable from coproporphyrin III in molecular weight, spectral absorption and behaviour on lutidine paper chromatography. These properties are consistent with a mono-acrylate analogue of coproporphyrin in which a propionate side-chain at one of the positions 2, 4, 6 or 7 is replaced by an acrylate residue. Our investigation does not distinguish between these four isomeric possibilities; from general consideration of natural porphyrins, however, replacement at position 6 or 7 would seem less likely than at position 2 or 4 (see Formula I).

It is tempting to speculate on the origin of this porphyrin. One step in the biosynthesis of protoporphyrin and haem that is not fully understood is that between coproporphyrinogen III and protoporphyrinogen IX, the coproporphyrinogenase reaction. This reaction requires an oxidative decarboxylation and so far no hydrogen acceptor has been found capable of replacing molecular oxygen *in vitro*. The reaction is an unusual one. Porra & Falk (1961) found that the porphyrinogen went through a protein-bound stage involving a linkage that was presumably of the thioether type as in cytochrome *c* since it was split by silver salts (Paul, 1950) but not by simple treatment with acid.



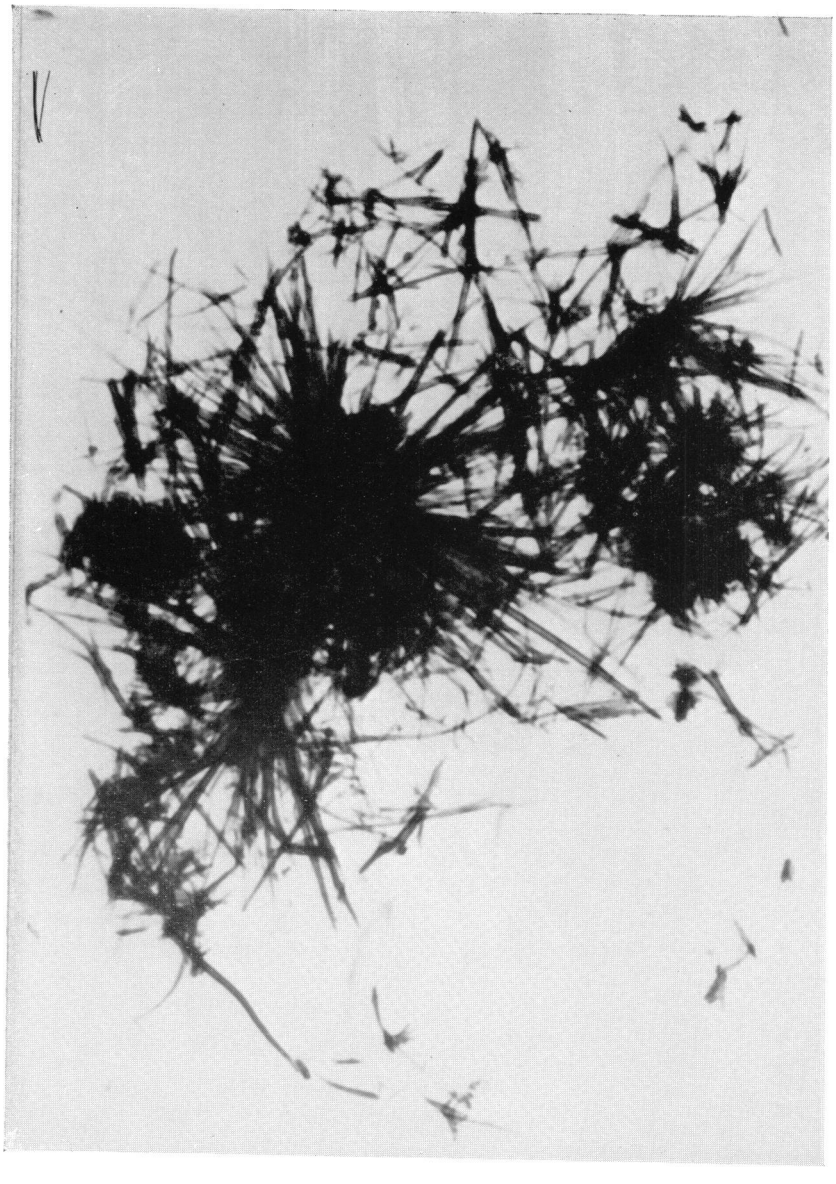
(I)

$R_1 = -CH_2\cdot CH_2\cdot CO_2H$ when
$R_2 = -CH:CH\cdot CO_2H$

and

$R_1 = -CH:CH\cdot CO_2H$ when
$R_2 = -CH_2\cdot CH_2\cdot CO_2H$

Porphyrinogens with vinyl or hydroxyethyl side chains combine readily with thiol compounds such as cysteine or cysteine-containing peptides (Sano, Nanzyo & Rimington, 1964) and one might envisage the coproporphyrinogenase reaction as proceeding via an initial desaturation of the 2- or 4-propionate side chains, then combination with thiol groups of the protein to form the thioether intermediate, from which CO_2 could readily be lost by decarboxyl-



EXPLANATION OF PLATE 1

S-411 porphyrin tetramethyl ester crystallized by cooling a hot chloroform–methanol (1:5, v/v) solution.

ation. This hypothesis is supported by the finding of Batlle, Benson & Rimington (1965) that sulphur-containing systems are almost certainly involved in the overall coproporphyrinogen–protoporphyrinogen reaction. Against the proposed reaction mechanism is, however, the fact that 2,4-*trans*-diacrylate deuteroporphyrinogen has been synthesized (Sparatore & Mauzerall, 1960) and shown not to behave as an intermediate in the enzyme-catalysed system *in vitro* (Sano & Granick, 1961). For this reason Batlle *et al.* (1965) suggested that it might be the 2,4-*cis* isomer that was involved. Sano (1966) has reported that synthetic 2,4-bis-(β-hydroxypropionate) deuteroporphyrinogen can be transformed enzymically into protoporphyrinogen anaerobically to the extent of 22%. This would suggest that the hydroxy compound is either a true intermediate or is easily converted into one, for example, into *cis*-2,4-diacrylate deuteroporphyrinogen by elimination of two molecules of water.

Because of the arguments set out above, it was decided to ascertain whether our monoacrylate porphyrin had a *cis* or *trans* configuration. N.m.r. studies indicated that it was predominantly *cis*, the lesser fraction separated by lower mobility on the alumina column being probably the *trans* isomer. A chemical artifact would be hardly likely to have *cis* configuration; some change from *cis* to *trans*, but not the reverse, could be expected to occur during manipulation, chromatography, etc. It would seem possible, therefore, that this meconium porphyrin may represent an intermediate in the coproporphyrinogen–protoporphyrinogen transformation in which only one propionate side-chain of coproporphyrinogen has been changed. If this supposition is correct one might expect to find 2,4-diacrylate deuteroporphyrin also in meconium. A search would be justified, although the fact that it would have an aetio-type spectrum would render detection less easy than that of porphyrin S-411 with its rhodo-type spectrum.

By countercurrent distribution studies and by comparison of the natural porphyrin with synthetic isomers Kennedy, Jackson, Kenner & Suckling (1970) found the tricarboxylate porphyrin from the Harderian glands of the rat to be formally derived from coproporphyrin III in having the 2-propionate group replaced by a vinyl group. These authors relate this observation to the presence of a formyl group in the 2- position of chlorocruoroporphyrin and the absence of a substituent in the 2- position of pemptoporphyrin, concluding that of the 2- and the 4-propionate groups of coproporphyrinogen III the former is the more susceptible to enzymic degradation. This conclusion is compatible with the finding of Granick & Levere (1964) that the rate of enzymic degradation of coproporphyrinogen IV, which has a methyl group at the 2-position, is only one-tenth that of coproporphyrinogen III. Although the present work does not justify a choice between the two isomers of structure I the above considerations suggest that in the S-411 porphyrin the acrylate group occupies the 2- rather than the 4-position.

While this present paper was being prepared for publication a report appeared (Smith, Belcher & Mahler, 1969) of the presence of the S-411 porphyrin in human meconium and bile and in bovine sternal bone marrow. On catalytic reduction it was transformed into coproporphyrin III. The finding of porphyrin S-411 is of special interest in view of its possible implication in the coproporphyrinogen–protoporphyrinogen transformation as discussed above.

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