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The Porphyrin Pigmentation of Subspecies of *Bacteroides melaninogenicus*

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Various subspecies of *Bacteroides melaninogenicus* differ in their pigmentation. Subsp. *asaccharolyticus* produces protohaem almost exclusively, subsp. *intermedius* both protohaem and a smaller proportion of protoporphyrin, and subsp. *melaninogenicus* mainly protoporphyrin with a trace of protohaem. As a consequence young colonies can be differentiated by their red fluorescence in u.v. light (365nm): subsp. *asaccharolyticus* does not fluoresce, subsp. *intermedius* shows a limited fluorescence, and subsp. *melaninogenicus* shows a bright fluorescence. The pigments were isolated as the dimethyl esters of protohaemin and of protoporphyrin and identified by electronic spectroscopy, mass spectrometry and comparisons by t.l.c. Incorporation of δ-aminolaevulinate into these pigments was not detected, nor was porphobilinogen formation observed. Subsp. *melaninogenicus* grown in the presence of [^{14}C]protohaemin formed [^{14}C]protoporphyrin. This appears to represent a novel biological demetallation.

Bacteroides melaninogenicus is an anaerobic microorganism which is found as part of the normal flora of the human mouth and intestine, and which has been isolated from many types of clinical infections (Lambe, 1974). The species has been divided into one asaccharolytic subspecies (*B. melaninogenicus* subsp. *asaccharolyticus*) and two saccharolytic subspecies (*B. melaninogenicus* subsp. *intermedius* and *B. melaninogenicus* subsp. *melaninogenicus*) on the basis of sugar fermentation (Holdeman & Moore, 1973). All three subspecies characteristically produce a dark pigmentation (brown to black) when grown on blood/agar. The closely related *Bacteroides oralis* does not produce such pigmentation, but otherwise appears to be indistinguishable from *B. melaninogenicus* subsp. *melaninogenicus* (Sutter *et al.*, 1972; Holdeman & Moore, 1973).

The black pigment of *B. melaninogenicus* grown on blood/agar plates was first described as melanin (Oliver & Wherry, 1921) because of its insolubility in several organic solvents. Shevsky *et al.* (1943) confirmed this insolubility in chloroform and acetone, but dissolved the pigment in weak alkali. Schwabacher *et al.* (1947) dissolved the black material in pyridine, and characterized it spectroscopically as haematin. This finding was contested by Tracy (1969), who found that strains of *Bacteroides fragilis*, *Bacteroides necrophorus* and *B. melaninogenicus* all formed black colloidal FeS when grown in liquid media containing cysteine and $FeSO_4$. This was confirmed for *B. melaninogenicus* and for certain isolates of *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhimurium* and *Clostridium welchii* (Duerden, 1975). However, the presence of FeS, which is presumably a consequence of H_2S formation in a medium containing ferrous iron, does not preclude the formation of a black pigment from haemoglobin, and this latter process was shown by Duerden (1975) to be a characteristic feature of *B. melaninogenicus*. The formation of these two different types of pigment was briefly studied by Reid *et al.* (1976).

The present paper describes an investigation of the formation of pigment from protohaem [a generic term used when it is not desirable or not possible to specify the oxidation state of the iron porphyrin; protohaemin and protoporphyrin are defined by structures (1) and (2) respectively] in strains of the three subspecies of *B. melaninogenicus*. The observations show that the rate of pigment formation and the depth of colour produced are subspecies-specific (Williams *et al.*, 1975; Shah *et al.*, 1976) and that red fluorescence under u.v. light (Myers *et al.*, 1969) is a characteristic of at least some strains of *B. melaninogenicus*.

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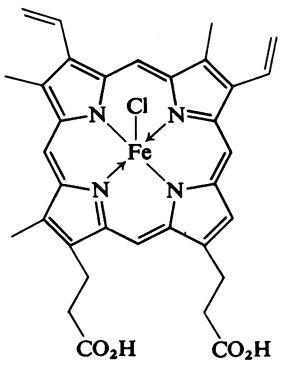
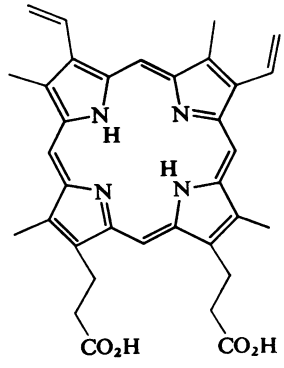


(1) Protohaemin



(2) Protoporphyrin

Materials and Methods

Spectra

Electronic spectra were obtained by using a Perkin–Elmer 402 (Beaconsfield, Bucks., U.K.), a Pye–Unicam SP.800B or SP.8000 (Cambridge, Cambs. U.K.), calibrated with holmium glass. Mass spectra were obtained with an AEI Ltd. MS902, by using direct insertion at the temperature stated, and an ionizing potential of 70eV. Fluorescence spectra were obtained with a Perkin–Elmer MPF3, by using solutions in dimethyl sulphoxide.

Chromatography

T.l.c. was on silica gel H (Merck, Darmstadt, Germany) irrigated with light petroleum (b.p. 60–80°C)/chloroform/methanol (9:9:1, by vol., system 1, or 140:100:3, by vol., system 2) or light petroleum (b.p. 60–80°C)/acetone (4:1, v/v, system 3). Fluorescence was examined qualitatively under a Gallenkamp medium-pressure mercury discharge lamp. High-pressure liquid chromatography was carried out on a Waters A202 chromatograph (Northwich, Cheshire, U.K.) with a μ-Porasil column (0.0305 m × 63 mm) eluted with heptane/methyl acetate (7:1, v/v) at 3 ml/min.

Bacteria

The subspecies of *B. melaninogenicus* used in this work are presented in Table 1. They have been previously described (Williams *et al.*, 1975; Shah *et al.*, 1976). *Haemophilus parainfluenzae*, type III, strains HK91 and HK96, were supplied by Dr. M. Kilian (Aarhus, Demmark).

Reference compounds

These were prepared from protohaemin isolated from ox blood. Protohaemin was converted into protoporphyrin dimethyl ester by Grinstein's demetallation/methylation procedure as described by Falk (1964), p. 132). Protohaemin dimethyl ester was prepared by metallation of protoporphyrin dimethyl ester (Martin, 1975).

Bacterial culture

Cells were grown on a protohaemin-enriched liquid medium BM (Shah *et al.*, 1976) or on blood/agar plates (Difco, E. Molesey, Surrey, U.K.) at 37°C in an O_2-free atmosphere ($N_2/H_2/CO_2$, 7:2:1). Both growth media contained 0.5% NaCl. Strains were maintained by weekly subculture on blood/agar plates, and checked for purity by morphological and physiological criteria (Shah *et al.*, 1976). Bacteria grown in liquid medium were centrifuged (5000*g*, 15 min), washed three times by centrifugation with distilled water, and freeze-dried. Cells grown on agar were carefully scraped off, dispersed in water to lyse any erythrocytes, and then centrifuged, washed and freeze-dried as above.

Extraction

A preliminary qualitative extraction of the porphyrin acids was made into pyridine (cf. Schwabacher *et al.*, 1947). Freeze-dried cells were ground in an agate mortar with a little pyridine; the liquid was centrifuged, the supernatant diluted with pyridine containing aqueous sodium dithionite and the electronic spectrum was recorded (Fig. 1). A similar extraction (of subsp. *melaninogenicus*) was made into dimethyl sulphoxide for spectrofluorimetry, the treatment with dithionite being omitted.

For the preparation of methyl esters, the freeze-dried cells (20 mg) were treated with 1 ml of BF_3/methanol (14%; BDH, Poole, Dorset, U.K.) for 3 h at 22°C. The solvent was removed under reduced pressure and the residue was extracted with chloroform (10 ml), and was then washed in turn with satd. aq. $NaHCO_3$ (2 × 10 ml) and water (2 × 10 ml). The extract was dried (over Na_2SO_4), concentrated under a stream of N_2, and submitted to preparative t.l.c. on 20 cm × 20 cm × 0.05 cm plates. The major bands were removed, extracted with chloroform, and identified by electronic spectroscopy and mixed t.l.c. with authentic esters. On 0.2 mm-thick Kieselgel 60F 254 plates (Merck, Darmstadt, Germany) protoporphyrin dimethyl ester had an R_F of 0.82 (system 1) and 0.27 (system 3), and protohaemin dimethyl ester had an R_F of 0.33 (system 1) and 0.07 (system 3). The quantity of the pigments was determined spectroscopically in chloroform by using $\varepsilon_{505} = 14150$ (Falk, 1964, p. 175) for the porphyrin ester and $\varepsilon_{512} = 10200$ (Martin, 1975) for the haemin ester. In control experiments freeze-dried horse blood (Difco) and protohaemin were submitted to the above extraction procedure.

The development of pigmentation was followed by examining the total growth from batches of six plates at daily intervals from days 3 to 10 (or day 7 for subsp. *melaninogenicus*). The cells were treated as described immediately above, and the results for strain T588 are presented in Table 3.

In order to obtain crystalline samples of the main pigments, bacteria were harvested from a large number of blood/agar plates (150 plates for strain T588, 50 for strain W83 and 30 for strain WAL2728) after 7 days' growth. The cells were washed, freeze-dried and treated with methanolic BF_3 as above, and the resulting pigment methyl esters were separated by t.l.c. on 40cm×40cm×0.2cm plates, by using system 1, or a double elution with system 3 and system 2 in that order. Each of the major components was extracted from the silica with chloroform, filtered, and crystallized from chloroform/methanol.

Biosynthetic experiments

(*a*) *Porphobilinogen biosynthesis.* Strains W83, T588 and WAL2728 were grown on blood/agar plates and *Haemophilus* strains HK91 and HK96 were grown on 'chocolate' blood/agar plates. After 48h the resulting cells were washed in 0.1M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH7.0, containing 1.0mM-dithiothreitol. The bacteria were harvested by centrifugation (5000*g*, 5min) and resuspended in batches (0.5ml) of 0.1M-Hepes buffer, pH7, containing 0.8mM-$MgSO_4$ to a cell density of 4mg dry wt. of cells/ml. Care was taken to ensure minimal exposure of *Bacteroides* strains to air: the vessels containing them were flushed for 30min with the gas mixture used for anaerobic culture. Cell samples obtained in this way were mixed with 0.5ml of 2mM-δ-aminolaevulinate (Sigma, Kingston upon Thames, Surrey, U.K.), a procedure similar to that described by Lascelles (1956) and Kilian (1974). Controls (cell suspensions without added δ-aminolaevulinate) were set up, and the preparations were incubated (up to 20h) under appropriate growth conditions (Kilian, 1974; Shah *et al.*, 1976). After incubation, the cell suspensions were centrifuged (10000*g*, 20min) and the supernatant was applied to a column (5cm×1cm) of Amberlite SRA68 anion-exchange resin (200 mesh; acetate form). Porphobiilinogen was eluted with 1M-acetic acid, and the eluate (2ml fractions) was treated with freshly prepared Ehrlich's reagent (Mauzerall & Granick, 1956). The A_{553} was measured (with acetic acid plus reagent as the reference) in a Gilford (Teddington, Middx., U.K.) model 240 spectrophotometer, and the amount of porphobilinogen was calculated by using $\varepsilon = 36000$ (Mauzerall & Granick, 1956).

(*b*) *Attempt to incorporate δ-amino[4-^{14}C]laevulinic acid into protoporphyrin.* Aqueous δ-amino[4-^{14}C]laevulinic acid (5ml, 2μCi/ml; The Radiochemical Centre, Amersham, Bucks., U.K.) was sterilized by filtration (0.22μm Millipore filter) and added to 90ml of BM liquid medium (Shah *et al.*, 1976) to which protohaemin had not been added. The broth was incubated at 37°C overnight under anaerobic conditions to check for sterility, and was then inoculated with 5ml of a 'protohaem-starved' culture of strain T588 produced by a 16h subculture in media without added protohaemin. Cells were harvested after 48h, checked for purity, washed, freeze-dried and treated with methanolic BF_3 as above. The chloroform extract of methyl esters was submitted to t.l.c. and the region of the plate corresponding to protoporphyrin dimethyl ester was extracted and assayed radiometrically.

(*c*) *Incorporation of [^{14}C]protohaemin into protoporphyrin.* [^{14}C]Protohaemin was prepared by incubating δ-amino[4-^{14}C]laevulinic acid with fresh chick erythrocytes (Custer *et al.*, 1964). The specific radioactivity (27200c.p.m./mg) was measured by treating the protohaemin (1mg) in chloroform (1ml) with benzoyl peroxide (0.5mg) and bleaching under a Gallenkamp medium-pressure mercury lamp for 16h. The colourless solution was mixed with NE250 scintillation fluid (10ml; BDH) and counted for radioactivity in a Packard (Caversham, Berks., U.K.) Tri-Carb liquid-scintillation counter, model 3000.

A solution of [^{14}C]protohaemin (1mg) in 1M-NaOH (0.02ml) and water (2.5ml) was sterilized by filtration (0.22μm Millipore filter) and rinsed through the filter with 2.5ml of water. The protohaemin solution was added to 90ml of BM broth, and incubated overnight as above. The broth was then inoculated with 5ml of a protohaem-starved culture of strain T588 and incubated anaerobically (37°C, 48h). The cells were harvested, washed, freeze-dried, treated with methanolic BF_3 and the pigments were chromatographed as described above. Both fluorescent and non-fluorescent components were extracted and were measured spectrophotometrically. The minor fluorescent component (in chloroform) was mixed with NE250 scintillation fluid and the radioactivity was measured directly. The non-fluorescent pigment was bleached (as described for protohaemin) before scintillation counting.

Results and Discussion

The bacteria studied here (Table 1) are all non-motile Gram-negative coccobacilli which differ in appearance on blood/agar plates. Subspecies *asaccharolyticus* (W83) produced black smooth shiny convex non-haemolytic colonies within 8–10 days, but at no stage were the colonies (or those of any other strain of subsp. *asaccharolyticus* examined) visibly fluorescent in u.v. light. Colonies of subsp.

Table 1. *Subspecies of Bacteroides melaninogenicus grown on blood/agar*

Subspecies	Strain no.	Haemolysis	Apparent development of pigmentation	Fluorescence after 2 days' growth
asaccharolyticus	W83	Non-haemolytic	Slow (black)	None
intermedius	T588	β-Haemolytic	Rapid (black)	Weak, at centre of colony
melaninogenicus	WAL2728	Non-haemolytic	Less rapid (brown)	Brilliant
	VPI9085	Haemolytic	Less rapid (brown)	Brilliant

intermedius (T588) became jet black within 3–4 days, but were smaller, rough and β-haemolytic. Colonies of subsp. *melaninogenicus* (VPI9085 and WAL2728) showed a vivid red fluorescence in u.v. light after 1–2 days' growth on blood/agar. This brilliant red fluorescence, and the associated development of a less-intense pigmentation (light to dark brown, not black) appears to offer a presumptive identification of the subsp. *melaninogenicus* grown on blood/agar. In contrast subsp. *intermedius* (T588) showed only weak (though definite) red fluorescence in the pale central parts of young colonies. Other strains of subsp. *intermedius* behaved similarly. It is presumed that the quenching of fluorescence in older colonies is due to increasing deposition of pigment (concentration quenching) and possibly also to metallation as the colony develops. At any event, the inverse relationship between fluorescence and the rate of pigmentation development provides a valuable method of detecting strains of subsp. *melaninogenicus*, which, because of their pale colour after short periods of incubation, may otherwise be overlooked.

When treated with sodium dithionite, pyridine extracts of all of the subspecies of *B. melaninogenicus* showed a prominent band at about 558 nm ascribed to the pyridine haemochrome. Indeed, with subsp. *asaccharolyticus* (W83) the extract showed a spectrum ($\lambda_{max.}$ 528, 558 nm) very similar to that of pyridine protohaemochrome. For subsp. *intermedius* (T588) and, particularly, for subsp. *melaninogenicus* (VPI 9085), additional maxima were present at 518, 593 and 638 nm, consistent with the presence of a metal-free porphyrin (Fig. 1). Moreover, these two extracts

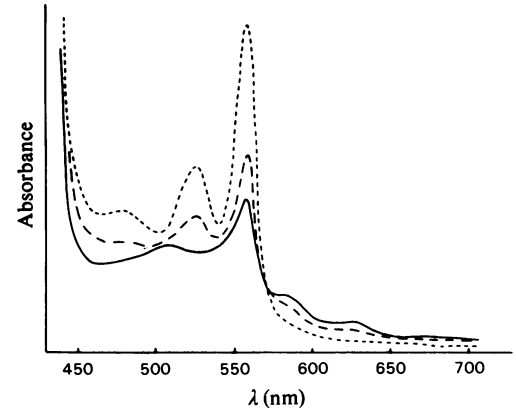


Fig. 1. *Absorption spectra of pyridine extracts of subspecies of B. melaninogenicus in the presence of aqueous sodium dithionite*

See the Materials and Methods section for experimental details. ----, Subsp. *asaccharolyticus* (W83); – – – –, subsp. *intermedius* (T588); ——, subsp. *melaninogenicus* (VPI9085).

Table 2. *Pigments from B. melaninogenicus subspecies after methylation*

Subspecies	Relative amount	Colour on the t.l.c. plate	Fluorescence on the t.l.c. plate under 365 nm radiation	Identification (as dimethyl ester)	Number of other minor/trace components on preparative plate excluding material at the origin
asaccharolyticus	Merest trace	—	Red	Protoporphyrin?	3
(W83)	Major	Brown	—	Protohaemin	
intermedius	Minor	Red	Red	Protoporphyrin	5
(T588)	Major	Brown	—	Protohaemin	
melaninogenicus	Major	Red	Red	Protoporphyrin	2
(WAL2728)	Trace	Brown	—	Protohaemin*	

* Identified by t.l.c. only.

fluoresced in u.v. light, whereas that from subsp. *asaccharolyticus* did not.

The pigments were methylated and separated by preparative t.l.c. The results are presented in Table 2. Minor components, including blue pigments, were noted. Occasionally the blue pigments were produced in larger amounts; possibly in these cases small amounts of oxygen were present, and the blue pigments are regarded as biliverdins arising by coupled oxidation (Bonnett & McDonagh, 1973). For subsp. *asaccharolyticus* (W83) only protohaem was detected as a significant component. After 4 days' growth strain W83 contained 10mg of protohaem/g of dry cells which rose to 26mg/g of dry cells at 5 days and to 35.5mg/g of dry cells at 9 days. A trace of protoporphyrin was probably present, but it was not in sufficient quantity to show up as a coloured spot, although a red fluorescence was observed at the correct R_F value.

For subsp. *melaninogenicus* (VPI9085 and WAL 2728) protohaem was formed in only minor amounts. Protoporphyrin increased in the colonies on blood/agar plates from 2.6–3.0mg/g of dry cells at 3 days to a maximum of 6.8–7.3mg/g of dry cells at 7 days.

For subsp. *intermedius* (T588) both protohaem and protoporphyrin were detected, the former predominating. The development of pigmentation here was followed for 10 days (Table 3). During this time the haem/porphyrin ratio fell from 25 (day 3) to 7.7 (day 10).

The identification of protoporphyrin dimethyl ester and protohaemin dimethyl ester was established beyond doubt in large-scale isolation experiments in which the crystalline products were obtained from cells grown for 7 days on blood/agar plates. Subspecies *asaccharolyticus* strain W83 yielded 18mg of protohaemin dimethyl ester identified by electronic spectrum, t.l.c. and high-pressure liquid chromatography. From subsp. *intermedius* strain T588 both protoporphyrin dimethyl ester (8mg, 0.5% yield on a dry-cell-weight basis) and protohaemin dimethyl ester (72mg, 4.2% yield on a dry-cell-weight basis) were obtained. The protoporphyrin dimethyl ester was indistinguishable from an authentic sample with respect to its electronic spectrum and its chromatographic behaviour (t.l.c. and high-pressure liquid chromatography). The accurately measured molecular ion appeared at *m/e* 590.288 in accord with the value of 590.289 calculated for $C_{36}H_{38}N_4O_4$. The mass spectrum of the protohaemin dimethyl ester sample isolated from strain T588 had peaks (relative abundances in parentheses) at *m/e* 680 (*M*, 100%), 645 (*M*−Cl, 30), 613 (6), 472 (13) and 499 (17). A similar mass spectrum was recorded for the sample of the haemin ester from subsp. *asaccharolyticus*. Cells of subsp. *melaninogenicus* strain WAL2728 gave 4mg of protoporphyrin dimethyl ester, identified by chromatographic comparisons, and by its mass spectrum, which had peaks (relative abundances in parentheses) at 590 (*M*, 100%), 559 (*M*−MeO, 8), 531 (*M*−CO_2Me, 6) and 517 (*M*−CH_2CO_2Me, 20).

It is concluded that the colour of subsp. *asaccharolyticus* is due to iron protoporphyrin, whereas subsp. *intermedius* is pigmented both with this and with protoporphyrin, the former being dominant. The oxidation state of iron *in vivo* is not known with certainty, but the iron complex is isolated as the chloroiron(III) derivative and may well occur as protohaemin in the cell. Only traces of iron protoporphyrin are detected in subsp. *melaninogenicus* grown on blood/agar, the main pigment being protoporphyrin.

Earlier reports of porphyrins from *B. melaninogenicus* have been meagre. Myers *et al.* (1969) observed fluorescence, but did not identify the fluorescent species. Protoporphyrin has been detected in strain CR2A of *B. melaninogenicus*, but its occurrence was attributed (Rizza *et al.*, 1968) to demetallation caused by the extraction procedure (acidified acetone). Although a weak linkage to cell constituents (such as protein) is not excluded, the following observations clearly demonstrate that metal-free protoporphyrin is present as such in the cells of subsp. *intermedius* and *melaninogenicus*.

(i) An extract of subsp. *melaninogenicus* with dimethyl sulphoxide fluoresces with maxima at 592 and 634nm, the second emission being virtually coincident with that (635nm) of protoporphyrin in the same solvent. (The 592nm maximum is not

Table 3. *Pigment formation in B. melaninogenicus subsp. intermedius (strain T588) grown on blood/agar (six plates)*

Length of incubation (days)	Dry weight of cells (mg)	Protoporphyrin concentration (mg/g of dry cells)	Protohaemin concentration (mg/g of dry cells)	Protohaemin / Protoporphyrin
3	24.7	1.68	41.7	24.8
4	25.3	2.14	40.8	19.0
5	47.1	1.59	24.0	15.1
6	49.3	2.19	77.0	35.0
7	58.5	4.27	60.2	14.1
8	54.9	4.55	55.4	12.2
9	50.1	6.99	—	—
10	49.1	7.40	57.0	7.7

assigned at present but presumably belongs to one of the minor compounds; Table 2.)

(ii) The extraction procedure used here produced practically no protoporphyrin from cells of subsp. *asaccharolyticus*, nor from haemoglobin, nor from protohaemin, i.e. it does not cause removal of iron from the protohaem system.

Preliminary biosynthetic experiments were carried out to examine the origin of the protoporphyrin. With a liquid medium containing δ-aminolaevulinic acid the two *Haemophilus* strains HK91 and HK96 produced porphobilinogen, in confirmation of earlier observations (Kilian, 1974), but the *B. melaninogenicus* subspecies (strains W83, T588 and WAL 2728) did not generate detectable quantities of this compound. δ-Amino[4-^{14}C]laevulinic acid was not incorporated into the protoporphyrin fraction obtained from subsp. *intermedius* (T588) grown in liquid culture (under conditions of haem depletion in order to make any incorporation of the precursor the more evident). However, [^{14}C]protohaemin was incorporated in a similar culture; after chromatographic purification, 40% of the applied radioactivity was recovered, 36% being present in the protohaemin dimethyl ester, and 3.6% in the protoporphyrin dimethyl ester. This result implies that the protoporphyrin arises in some way by the biological demetallation of protohaem. This is not, as far as we are aware, a known process in haem metabolism. Indeed, it is unexpected, since the iron–porphyrin complex is a robust species (unless reduced to the porphyrinogen level), and the normal biological route (haem catabolism) by which the metal is extricated involves rupture of the porphyrin ring (Lathe, 1972). McCall & Caldwell (1977) have reported spectroscopic results which were interpreted in terms of the demetallation of (added) manganese protoporphyrin, with incorporation of iron, in cultures of the related anaerobe *Bacteroides ruminicola* subsp. *ruminicola* (strain 23), although the free porphyrin was apparently not detected. The unambiguous identification in the present work of protoporphyrin as a pigment of a haem-dependent anaerobe, together with the preliminary labelling studies, and the time course of pigment formation where both haem and porphyrin pigments are formed in appreciable amounts (Table 3), suggests that in these anaerobes protohaem is the precursor of protoporphyrin. It should prove possible to test this postulate with the help of biosynthetic experiments designed to distinguish between intact incorporation and fragmentation–recombination pathways.

We are grateful to Dr. M. Kilian (Aarhus) for supplying the two *Haemophilus* strains, to Mr. D. S. Price for preparing the [^{14}C]protohaemin, and to the British Council for a research scholarship (to B. M.).

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