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Specificity of the Heme Requirement for Growth of *Bacteroides ruminicola*[1]

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ABSTRACT

CALDWELL, D. R. (U.S. Department of Agriculture, Beltsville, Md.), D. C. WHITE, M. P. BRYANT, AND R. N. DOETSCH. Specificity of the heme requirement for growth of *Bacteroides ruminicola*. J. Bacteriol. **90**:1645–1654. 1965.—Previous studies suggested that most strains of *Bacteroides ruminicola* subsp. *ruminicola* require heme for growth. Present studies with heme-requiring strain 23 showed that protoheme was replaced by various porphyrins, uroporphyrinogen, coproporphyrinogen, certain iron-free metalloporphyrins, hemes, and certain heme-proteins containing readily removable hemes. Strain 23 utilized a wider range of tetrapyrroles than hemin-requiring bacteria previously studied. Inactive compounds included porphyrin biosynthesis intermediates preceding the tetrapyrrole stage and related compounds; uroporphyrin, chlorophyll, pheophytin, phycoerythrin, bilirubin, pyrrole, $FeSO_4$ with or without chelating agents; and representative ferrichrome compounds. Strain 23, two other strains representing predominant biotypes of *B. ruminicola* subsp. *ruminicola*, and one closely related strain grew in media containing heme-free protoporphyrin, mesoporphyrin, hematoporphyrin, or deuteroporphyrin, apparently inserting iron into several nonvinyl porphyrins. Porphobilinogen and porphyrin synthesis, apparently via the commonly known heme synthesis pathway, occurred during growth of heme-independent *B. ruminicola* subsp. *brevis* strain GA33 in a tetrapyrrole-free medium containing δ-aminolevulinic acid, but δ-aminolevulinic acid metabolism to porphobilinogen or porphyrins could not be detected in cells of heme-requiring strain 23 grown in the same medium with hemin added. Growth of strain 23 with uroporphyrinogen, coproporphyrinogen, or protoporphyrin IX replacing hemin suggests that part of the commonly known heme-biosynthesis pathway is present in this strain, but nutritional and metabolic evidence indicates that some or all of the enzymes synthesizing the tetrapyrrole nucleus from linear molecules are lacking or inactive.

Previous reports of heme growth factor requirements among bacteria are relatively rare, and the majority of reports have concerned aerobic and facultatively anaerobic species (White and Granick, 1963; Lascelles, 1961). Among the strict anaerobes, strains of *Bacteroides melaninogenicus* require hemin (Gibbons and Macdonald, 1960), and it has been shown previously that hemin replaces the rumen fluid growth requirement of the majority of strains of *B. ruminicola* subsp. *ruminicola* (Bryant and Robinson, 1962).

B. ruminicola appears to be one of the more important rumen microorganisms on the basis of its numbers in the rumen, its ability to ferment a wide variety of the carbohydrates of quantitative significance in ruminant rations (Bryant et al., 1958*a*), and its ability to deaminate certain amino acids (Bladen, Bryant, and Doetsch, 1961*a*) and to produce certain branched-chain volatile fatty acids (Bladen, Bryant, and Doetsch, 1961*b*) which are growth factors for many other functional rumen bacteria (Bryant and Robinson, 1962; Allison, Bryant, and Doetsch, 1962).

Since hemin replaces the rumen fluid requirement of the majority of strains of *B. ruminicola* subsp. *ruminicola*, and since hemin-requiring strains which were presumptively identified as *B. ruminicola* may comprise as much as 31% of the total strains nonselectively isolated from rumen contents (Bryant and Robinson, 1962), hemin appears to be an important metabolite for ruminal bacteria. A study of the specificity of the heme requirement of *B. ruminicola* subsp.

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ruminicola and of heme synthesis by *B. ruminicola* may contribute to an understanding of this organism's growth and metabolism in the rumen, and to knowledge concerning heme metabolism among saccharolytic anaerobes.

MATERIALS AND METHODS

Bacterial strains. The strains studied have been previously described by Bryant et al. (1958*a*, *b*). The majority of the work was conducted with heme-requiring type strain 23 of *B. ruminicola* subsp. *ruminicola*. Unless otherwise noted, the methods of strain maintenance, media preparation, and inoculation of test media were essentially those referred to by Bryant and Robinson (1962), except that 5-ml quantities of media were inoculated with 0.1-ml quantities of washed cells suspended at an optical density (OD) of approximately 0.1.

The medium used for growth of cells for inoculation was that described by Pittman and Bryant (1964)

Media. The composition of the basal medium used for testing the hemin-replacing activity of various crude extracts and of certain known compounds is given in Table 1. The ingredients of the medium, minus cysteine and sodium carbonate, were adjusted to *p*H 6.5 with 2.5 N NaOH and autoclaved. After cooling and addition of sterile CO_2-equilibrated sodium carbonate solution, the medium was tubed in 2.9-ml amounts in sterile, rubber-stoppered, Pyrex tubes (13 by 100 mm). Addition of 2-ml quantities of sterile, CO_2-equilibrated, test solutions and 0.1-ml quantities of sterile CO_2-equilibrated cysteine solution brought the tubed media to volume. The media were maintained anaerobically with a CO_2 gaseous phase at all times (Bryant and Robinson, 1962). In some experiments, sodium carbonate was incorporated into the medium with the test solutions. With the addition of hemin, this medium supported excellent growth of strain 23, whereas none was obtained in the medium without added hemin.

TABLE 1. *Composition of the basal medium used for the testing of suspected hemin-replacing compounds*

Component	Percentage
Glucose	0.3
Casein hydrolysate[a]	0.2
Mineral solution[b]	5.0
Volatile fatty acid solution[c]	2.0
B vitamin solution[d]	1.0
Resazurin	0.4
Cysteine $HCl \cdot H_2O$	0.05
Sodium carbonate	0.4

[a] This component was vitamin-free enzymatic hydrolysate from the Nutritional Biochemicals Corp. and was replaced in some experiments with Difco vitamin-free Casitone.

[b] KH_2PO_4, 6.6×10^{-3} M; NaCl, 1.5×10^{-2} M; $CaCl_2$, 1.8×10^{-4} M; $MgCl_2 \cdot 6H_2O$, 9.8×10^{-5} M; $MnCl_2 \cdot 4H_2O$, 5.1×10^{-5} M; $CoCl_2 \cdot 4H_2O$, 4.2×10^{-6} M; $(NH_4)_2SO_4$, 3.4×10^{-3} M; $FeSO_4 \cdot 7H_2O$, 3.6×10^{-6} M.

[c] Sodium acetate $\cdot 3H_2O$, 1.1×10^{-2} M; isobutyrate, DL-α-methylbutyrate, *n*-valerate, and isovalerate, 1.5×10^{-4} M of each.

[d] Thiamine $\cdot$ HCl, 5.9×10^{-6} M; pyridoxal $\cdot$ HCl, 9.8×10^{-6} M; nicotinamide, 1.6×10^{-5} M; riboflavine, 5.3×10^{-6} M; calcium-D-pantothenate, 7.8×10^{-6} M; *p*-aminobenzoic acid, 7.3×10^{-7} M; biotin, 2.0×10^{-7} M; folic acid, 1.1×10^{-7} M; cyanocobalamin, 1.5×10^{-8} M.

Materials tested for hemin-replacing activity. The sources and nature of the materials tested for hemin-replacing activity were as follows. Samples of bovine hemin, protoporphyrin IX, hematoporphyrin $\cdot$ 2HCl, deuteroporphyrin, δ-aminolevulinic acid, α-ketoglutaric acid, bilirubin, bovine hemoglobin, horseradish peroxidase, bovine liver catalase, and equine heart cytochrome *c* were obtained from Calbiochem. Samples of uroporphyrin, coproporphyrin, manganese protoheme, zinc protoheme, copper protoheme, mesoheme, deuteroheme, deuteroporphyrin, and porphobilinogen were obtained through the generosity of Sam Granick, Rockefeller Institute, New York, N.Y. Samples of the latter three compounds were also obtained from David Mauzerall, Rockefeller Institute. Both samples of porphobilinogen were gifts to Mauzerall and Granick from S. F. MacDonald, National Research Council, Ottawa, Canada. Coproporphyrin was prepared from diphtheria toxin broth as described by Sano and Granick (1961). The diphtheria toxin broth was a gift to Dr. Granick from Lederle Laboratories, Pearl River, N.Y. Mesoporphyrin and pyrrole were obtained from the Mann Research Corp., New York, N.Y. Sodium potassium chlorophyll and serine were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Pheophytin was obtained through the generosity of A. J. Corwin, Johns Hopkins University, Baltimore, Md. Phycoerythrin was a gift from H. W. Siegelman, Agricultural Research Service, Beltsville, Md. Ferrichrome was a gift from J. B. Neilands, University of California, Berkeley. Terregens factor was obtained through the generosity of A. G. Lochhead, Canadian Department of Agriculture, Ottawa, Canada. A sample of coprogen was generously supplied by B. L. Hutchings, Lederle Laboratories.

Preparation of stock solutions of test materials. The porphyrins and hemes were dissolved in either a 1:1 (v/v) mixture of ethyl alcohol and 0.2 M KOH, or in 5×10^{-3} M NaOH. The hemeproteins, δ-aminolevulinic acid, α-ketoglutaric acid, serine, ferrichrome, and coprogen were dissolved in distilled demineralized water. Pheophytin was dissolved in absolute ethyl alcohol. Sodium potassium chlorophyll and pyrrole were dissolved in 95% ethyl alcohol and diluted with water. Terregens factor was diluted from a 40%

ethyl alcohol solution. Bilirubin and phycoerythrin were dissolved in 5×10^{-3} M NaOH. Porphobilinogen was dissolved in sterile 1 M KOH. Porphobilinogen and pheophytin were incorporated into the medium by adding small volumes to each tube just prior to inoculation. The alkaline ethyl alcohol solutions of hemes and porphyrins and the solutions of pheophytin and porphobilinogen were considered sterile as prepared. With the exception of the solutions of bilirubin, ferrichrome, terregens factor, and pyrrole, the remaining solutions were sterilized by membrane filtration. Solutions of the latter compounds were sterilized by autoclaving.

Chemical and analytical methods. Porphyrinogens were prepared from aqueous solutions of coproporphyrin and uroporphyrin by reduction of these compounds under CO_2 with freshly ground sodium amalgam in dim light according to methods described by Mauzerall and Granick (1958) and Sano and Granick (1961). The use of sodium amalgam produced a *p*H of between 13 and 14, which was sufficient to prevent growth of any contaminants; aseptic, anaerobic addition of 0.1-ml samples of the reduced solutions per 5 ml of medium did not significantly alter the medium *p*H.

The heme was released from carefully weighed quantities of cytochrome *c* by treatment with 0.8% Ag_2SO_4 and acetic acid by the method of Paul (1950), and the released heme was extracted by the methods of Morrison and Stotz (1955). The final ether extracts were pooled and evaporated to dryness on a steam bath. The dry residues were dissolved in 5×10^{-3} M NaOH and sterilized by autoclaving. Aqueous dilutions of these solutions were tested for hemin-replacing activity, and the growth data were expressed as a function of the theoretically available heme. The results were compared, on the same basis, to those obtained with crystalline hemin subjected to identical procedures.

Broken-cell preparations were prepared by subjecting the bacteria, aerobically suspended in 50 mM phosphate buffer containing 10 mM $NaHCO_3$ at *p*H 7.0, to sonic vibration with a Branson sonic oscillator, probe type, for 1 min, keeping the temperature under 10 C. Microscopic examination showed that virtually complete rupture of cells resulted from this procedure. These preparations were incubated at 37 C with gentle shaking for 4 hr under an atmosphere of CO_2.

δ-Aminolevulinic acid and porphobilinogen were determined following the methods of Mauzerall and Granick (1956) involving the 2 N perchloric acid Ehrlich reaction. Porphobilinogen and δ-aminolevulinic acid were isolated after adding 2 ml of reaction mixture to 2 ml of 0.3 M trichloroacetic acid containing 0.01 M $HgCl_2$. This is essentially the method described by White and Granick (1963).

Coproporphyrin was isolated from the broken-cell incubation mixtures by the method described by White and Granick (1963). The isolation of porphyrins from cultures after growth was performed as follows. The media were made to *p*H 4.0 with 12 N HCl, and 2 volumes of ethyl acetate-acetic acid (3:1) were added. The mixture was shaken and the porphyrins were recovered by extraction into a small volume of 2 M HCl. Porphyrin was calculated as coproporphyrin ($E_{mM401} = 470$ in 1 M HCl). The porphyrinogens were allowed to become oxidized in the presence of dim light and air, as described by Sano and Granick (1961), prior to measurement of porphyrin. Hemin was isolated from the medium by the methyl ethyl ketone method of Falk (1964).

Uroporphyrin was extracted from rumen fluid by the cyclohexanone method of Falk (1964). Coproporphyrin was extracted from rumen fluid by the procedure described for isolation of porphyrins from cultures after growth, except that only 1 volume of ethyl acetate-acetic acid was used. Both uroporphyrin and coproporphyrin were removed from the organic solvents by extraction with 5-ml quantities of 2 M HCl. The HCl extracts were neutralized to *p*H 7.0 with 2.5 N NaOH, and 0.1-ml quantities were tested for growth-supporting activity with strain 23. Heme was extracted from rumen fluid by use of the methyl ethyl ketone procedure of Falk (1964). The organic layer was concentrated to a small volume (2 to 4 ml) by flash evaporation at 50 C, and the concentrate was treated with sufficient 0.1 M KOH-50% ethyl alcohol to make 10 ml; 0.1-ml quantities of this solution were tested for growth-supporting activity with strain 23.

The OD at 600 mμ, measured with a Spectronic-20 colorimeter (13-mm test tubes), was used as a measure of cell density. Except for media containing hematoporphyrin, copper protoheme, coproporphyrin, and cytochrome *c*, maximal OD of cultures was usually obtained after incubation for from 14 to 35 hr. Growth in media containing the latter compounds occurred only after prolonged incubation. Protein in cell suspensions was determined by measurement of absorbance at 540 mμ, by use of the biuret reagent (Gornall, Bardawill, and David, 1949) in the presence of 0.06% deoxycholate.

The growth rate of *B. ruminicola* subsp. *ruminicola* strain 23 as a function of hemin concentration was estimated by measurement of the average maximal OD changes observed in cultures during their most rapid phase of growth. Cultures were prepared in a common batch of basal medium supplemented with various concentrations of hemin. Duplicate cultures were used for each hemin concentration. OD measurements were made by use of uninoculated tubes of medium as standards. Estimates of the OD doubling time were based on the most rapid OD changes observed at short time intervals during an incubation period of 1.5 to 6 hr at 37 C.

Iron porphyrin contamination of deuteroporphyrin, protoporphyrin IX, hematoporphyrin IX, and mesoporphyrin, and the spontaneous formation of iron porphyrins in media incubated with porphyrin and $FeSO_4$, were checked by use

of the benzidine reagent and methods of Morrison and Stotz (1957).

RESULTS

Growth promotion by rumen fluid and rumen fluid extracts. Excellent growth of heme-requiring strain 23 was obtained (Table 2) in the basal medium supplemented with autoclaved rumen fluid obtained from mature cattle fed an alfalfa hay-grain ration and clarified by centrifugation, indicating that rumen contents from this source contain sufficient quantities of hemin-replacing factors to allow abundant growth of heme-requiring strains of *B. ruminicola.*

Table 3 shows the percentages of total growth factor activity for strain 23 in fractions obtained from extraction of replicate centrifuged samples of rumen fluid by various procedures. At least 90% of the total activity extracted was found in the fraction resulting from methyl ethyl ketone extraction, a procedure which has been used to extract protoheme from aqueous solution (Falk, 1964). The growth obtained from assay of this fraction was similar, on an equivalent rumen fluid basis, to that obtained in the basal medium supplemented with centrifuged whole rumen fluid. Very little growth factor activity (3 to 6%) was found in fractions obtained by procedures 1 and 2, which primarily extract porphyrins, and no improvement in the growth factor activity of these fractions was obtained after reduction of any porphyrins present in these extracts to porphyrinogens with sodium amalgam and subsequent determination of the activity of the reduced fractions with strain 23 in the dark. Neither the solvents employed nor the traces of sodium amalgam were inhibitory to the growth of strain 23 in the basal medium supplemented with extracts and hemin. No growth was obtained in the basal medium supplemented with solvents alone.

TABLE 2. *Growth of Bacteroides ruminicola 23 in the basal medium supplemented with centrifuged autoclaved rumen fluid collected from a mature cow 6 and 23 hr after feeding an alfalfa hay-grain ration*

Per cent rumen fluid in basal medium	OD × 100 of sample	
	6 hr	23 hr
40*	110	105
30	102	100
20	89	77
10	33	46
5	9	9

* Each value in the table is the average of two or four cultures. The data are taken from two experiments.

TABLE 3. *Growth of Bacteroides ruminicola subsp. ruminicola 23 in the basal medium supplemented with fractions obtained by extraction of centrifuged rumen fluid* with cyclohexanone (CH), ethyl acetate-acetic acid (EAA), or methyl ethyl ketone (MEK), and with the same fractions plus hemin*

Procedure	Rumen fluid equivalent supplementation	Maximal OD† obtained	Per cent of total activity extracted‡
	%		
1. CH without hemin	74	0.14	3
CH with hemin		1.21	
2. EAA without hemin	32	0.14	6
EAA with hemin		1.20	
3. MEK without hemin	5	0.32	92
MEK with hemin		1.13	
4. Hemin alone	0	1.14	

* Replicate 500-ml quantities of rumen fluid were used for each extraction.

† The optical densities shown are the averages of triplicate cultures.

‡ Calculated by adjusting the average optical densities observed to the optical densities expected for each extract at a level of extract supplementation equivalent to 100% rumen fluid. The portion of the total activity extracted which was present in a particular fraction was then determined.

Efforts to detect heme(s) in the methyl ethyl ketone extract were hampered by the presence of substances inhibitory to both colorimetric tests for hemes and to the spectrophotometric detection of these compounds or their pyridine hemochromogens.

Relationship between hemin concentration and growth. Figure 1 shows the relationship between hemin concentration and growth of strain 23. Although some variation occurred among experiments, maximal growth of strain 23 is a function of hemin concentration at concentrations between 10^{-8} and 10^{-7} M. Appreciable growth (OD > 0.10) was seldom obtained at or below a concentration of 5×10^{-9} M, and excellent growth (OD 1 to 1.2) was always obtained at a hemin concentration of 10^{-7} M. In addition to affecting the maximal growth obtained, hemin concentration, at low levels, has a marked effect upon the OD doubling time of strain 23 (Fig. 2).

Compounds which replace hemin as growth factors for strain 23. Table 4 gives the results of growth experiments with strain 23 in the basal medium supplemented with various compounds

which replace hemin as growth factors. All the active compounds are tetrapyrroles or heme-proteins from which the heme is readily removed. Growth in media containing hematoporphyrin was often somewhat slower, and often less growth was obtained than with other active compounds. Very little growth occurred in media containing coproporphyrin and copper protoheme and only after prolonged incubation.

Growth-supporting activity of the heme extracted from cytochrome c. Cytochrome *c*, a heme-protein in which the heme is covalently bound to the protein portion of the molecule, is quite inactive. However, the heme extracted from it supported growth similar to that obtained with hemin subjected to the same extraction (Fig. 3). It is evident that some growth-supporting activity was lost in the extraction procedure. The growth response to filter-sterilized cytochrome *c*, a low and delayed response at 6×10^{-6} M and none at 6×10^{-7} M, was much poorer than that obtained with autoclaved cytochrome *c* (Fig. 3), whereas the growth responses to filtered and autoclaved hemin were virtually identical (Fig. 4).

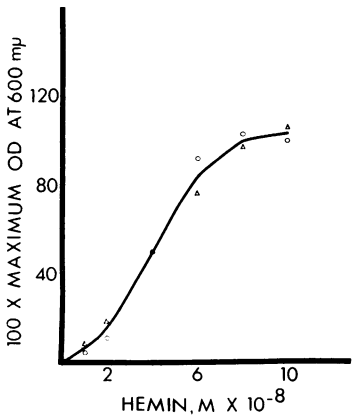

FIG. 1. *Maximal optical density of Bacteroides ruminicola subsp. ruminicola 23 obtained in the basal medium supplemented with various concentrations of hemin obtained from the California Corp. for Biochemical Research* (△) *and the Nutritional Biochemicals Corp.* (○).

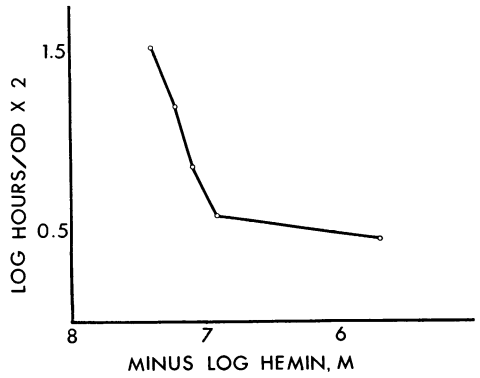

FIG. 2. *Effect of hemin concentration on the optical density doubling time of Bacteroides ruminicola subsp. ruminicola 23.*

TABLE 4. *Growth of Bacteroides ruminicola subsp. ruminicola 23 in the basal medium supplemented with various hemin-replacing compounds*

Compound	Concn	OD × 100*	Time to maximal OD
	M		*hr*
Protoporphyrin IX	2.0×10^{-8}	31	26
	7.0×10^{-8}	76	36
	1.5×10^{-7}	100	29
Mesoporphyrin	2.0×10^{-8}	54	39
	7.0×10^{-8}	101	29
	1.5×10^{-7}	108	27
Deuteroporphyrin	2.0×10^{-8}	67	40
	7.0×10^{-8}	102	34
	1.5×10^{-7}	109	28
Hematoporphyrin	2.0×10^{-8}	93	55
	7.0×10^{-8}	100	44
	1.5×10^{-7}	109	39
Coproporphyrinogen	6.0×10^{-6}	119	32
Coproporphyrin	6.0×10^{-6}	37	112
Uroporphyrinogen	3.8×10^{-6}	116	34
Deuteroheme	5.0×10^{-7}	117	32
	1.0×10^{-6}	104	32
Mesoheme	5.0×10^{-7}	117	32
	1.0×10^{-6}	119	32
Manganese protoheme	5.0×10^{-7}	117	32
	1.0×10^{-6}	115	32
Zinc protoheme	5.0×10^{-7}	109	34
	1.0×10^{-6}	127	34
Copper protoheme	5.0×10^{-7}	9	21
	1.0×10^{-6}	26	36
Hemoglobin	5.0×10^{-9}	42	49
	1.0×10^{-8}	72	32
	1.5×10^{-8}	77	41
	2.0×10^{-8}	102	28
Catalase	8.0×10^{-9}	4	19
	4.1×10^{-8}	81	29
Peroxidase	5.0×10^{-8}	28	98
	2.5×10^{-7}	63	75

* Data for the hemes, heme proteins, and porphyrinogens are average optical densities from duplicate tubes. The data for the porphyrins are averages from duplicate tubes of two or more replicate experiments.

Factors which are ineffective as hemin-replacing factors for strain 23. Many other compounds were tested and found incapable of replacing heme as

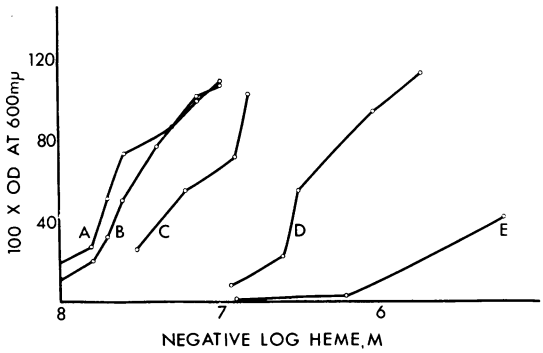

FIG. 3. *Maximal optical density of Bacteroides ruminicola subsp. ruminicola 23 obtained in the basal medium supplemented with autoclaved hemin (A), hemin extracted as described in the text and autoclaved (B), the heme specifically extracted from cytochrome c and subsequently autoclaved (C), autoclaved cytochrome c (D), and filter-sterilized cytochrome c (E).*

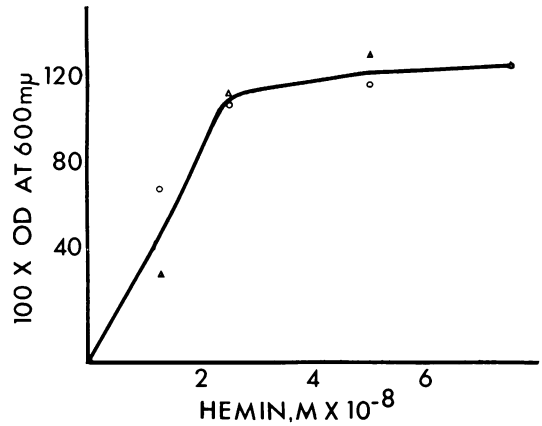

FIG. 4. *Growth of Bacteroides ruminicola subsp. ruminicola 23 in basal medium supplemented with various concentrations of hemin sterilized by filtration (△) and by autoclaving (○).*

a growth factor for strain 23. These included: (i) intermediates in the pathway of porphyrin biosynthesis in all other known heme-containing organisms and chemically related compounds included a mixture of 3.5×10^{-5} M α-ketoglutarate and 4.8×10^{-5} M serine, 1.5×10^{-5} M porphobilinogen, pyrrole in 10-fold dilutions from 1.5×10^{-3} to 1.5×10^{-6} M, and 2.5×10^{-6} M uroporphyrin; (ii) compounds chemically related to porphyrin, such as 2.6×10^{-6} M sodium potassium chlorophyll, pheophytin in 10-fold dilutions from 1.1×10^{-6} to 1.1×10^{-9} M, 3.5×10^{-6} M bilirubin and phycoerythrin in 10-fold levels from 2.3×10^{-6} to 2.3×10^{-10} M; and (iii) other inactive compounds, including 3.6×10^{-6} M $FeSO_4 \cdot 7H_2O$ with or without 10-fold levels of sodium citrate from 3.5×10^{-3} to 3.5×10^{-5} M, or sodium ethylenediaminetetraacetate at concentrations of 3.5×10^{-4} and 3.5×10^{-5} M, and 10-fold dilutions of the iron-binding agents ferrichrome (0.0001 to 1.0 μg/ml), coprogen (0.1 to 10 μg/ml), and terregens factor (0.01 to 10 μg/ml). With the exception of sodium ethylenediaminetetraacetate at or above a concentration of 3.6×10^{-4} M, none of the compounds tested was inhibitory to the growth of strain 23 in the basal medium with 3×10^{-6} M hemin added.

Growth on porphyrins with other heme-requiring strains of B. ruminicola and closely related organisms. Table 5 shows that hemin replaces the rumen fluid growth factor requirements of strains GA20 and $B_1$18 of *B. ruminicola* subsp. *ruminicola* and also of strain B127, a strain closely related to this species (Bryant et al., 1958*b*). Protoporphyrin IX, mesoporphyrin, hematoporphyrin, and deuteroporphyrin replace hemin as growth factors for all of these strains, but δ-aminolevulinic acid is inactive. No contamination of the porphyrins with hemes could be detected with benzidine reagent (Morrison and Stotz, 1957), and no spontaneous formation of iron porphyrin could be detected in uninoculated tubes of the $FeSO_4 \cdot 7H_2O$ containing basal medium supplemented with 3.5×10^{-5} M iron-free protoporphyrin IX, mesoporphyrin, deuteroporphyrin, or hematoporphyrin after incubation of the medium at 38 C for 1 week. A positive benzidine reaction was obtained in the same basal

TABLE 5. *Growth of certain rumen fluid factor-requiring strains of Bacteroides ruminicola and a closely related strain in the basal medium supplemented with hemin (H), protoporphyrin IX (P), mesoporphyrin (MP), deuteroporphyrin (DP), and hematoporphyrin (HP)**

Strain	Growth (OD × 100)†				
	H	P	MP	DP	HP
23	111	81	92	110	56
GA 20	123	98	104	121	72
$B_1$18	120	62	74	116	31
B127	100	99	88	107	76

* The concentration of porphyrins added was 1.2×10^{-7} M. The concentration of δ-aminolevulinic acid was 1.5×10^{-5} M. There was no growth in basal medium alone, or in basal medium supplemented with δ-aminolevulinic acid.

† Average optical densities from duplicate tubes of medium.

medium supplemented with hemin at a concentration as low as 4.0×10^{-8} M.

In contrast to the data in Table 5, *B. ruminicola* subsp. *ruminicola* strains B932-1, B888-1, and B747-1 do not require hemin, since they exhibited good growth (OD 0.5 to 1.2) in the basal medium (Table 1) without added hemin, and also grew well (OD > 0.6) in the defined medium of Pittman and Bryant (1964) with hemin deleted. These strains represent unusual biotypes of *B. ruminicola* subsp. *ruminicola*. Strains representing predominant biotypes required tetrapyrroles.

Porphyrin biosynthesis by B. ruminicola. Incubation of both hemin-requiring strain 23 and hemin-independent strain GA33 with δ-aminolevulinic acid and porphobilinogen indicates that the enzyme δ-aminolevulinic acid dehydrase is absent or inactive in the hemin-requiring strain when tested under conditions in which 21% of the added δ-aminolevulinic acid is converted to porphobilinogen by the heme-independent strain. Both strains utilize more δ-aminolevulinic acid than can be accounted for as porphobilinogen or porphyrin. In both cases, less than 0.03 moles of coproporphyrin was found per 30 mg of protein (Table 6). The inability to convert porphobilinogen to porphyrinogens when incubated under conditions in which other hemin-independent bacteria readily form coproporphyrinogen (White and Granick, 1963) remains a puzzle. During the growth cycle, however, δ-aminolevulinic acid was readily converted to porphobilinogen and coproporphyrinogen by strain GA33 (Table 7). The hemin-independent strain formed 8 times more porphyrinogen when grown in the presence of δ-aminolevulinic acid than when grown in the absence of this compound, and at least 500 times more porphyrinogen than the hemin-requiring strain. In the hemin-independent strain, 0.4% of the added δ-aminolevulinic acid appeared as porphobilinogen and 25% as coproporphyrinogen. Thus, the hemin-independent strain appears to utilize a porphyrin biosynthesis pathway involving δ-aminolevulinic acid and porphobilinogen. This pathway cannot be detected in the hemin-requiring strain under conditions in which this same pathway is readily demonstrable in the hemin-independent strain.

TABLE 6. *Synthesis of porphobilinogen (PBG) from δ-aminolevulinic acid (ALA) by anaerobic broken-cell suspensions* of Bacteroides ruminicola incubated for 4 hr at 37 C*

Strain	ALA†		PBG	
	0 hr	4 hr	0 hr	4 hr
Heme-requiring 23				
Control without added substrates	71	62	32	30
Control + ALA	980	462	61	63
Control + PBG	62	62	225	208
Heme-independent GA33				
Control without added substrates	66	64	31	29
Control + ALA	1,320	670	26	151
Control + PBG	67	64	350	420

* Suspensions were prepared in 50 μM phosphate buffer containing 10 μM $NaHCO_3$ (pH 7.6). The suspensions were equilibrated with oxygen-free CO_2, and were incubated in tightly stoppered 25-ml flasks in the dark.

† δ-Aminolevulinic acid and porphobilinogen were measured at the start and end of the incubation period. The results are expressed as millimicromoles per 30 mg of protein.

TABLE 7. *Synthesis of porphobilinogen (PBG) and coproporphyrinogen (CPG) by cells of heme-independent Bacteroides ruminicola GA33 during growth in a heme-free medium with and without δ-aminolevulinic acid* (ALA) and by heme-requiring strain 23 grown in the same medium with hemin added**

Strain	PBG†	CPG
Hemin-requiring 23		
Without ALA	0.02	0.02
With ALA	0.02	0.02
Hemin-independent GA33		
Without ALA	0.02	1.20
With ALA	0.76	9.40

* Cells (200 ml) were grown to OD 0.6 (18 hr) with and without the addition of 300 μmoles of ALA.

† Porphobilinogen was measured with the Ehrlich reagent. The values shown are the differences between 0- and 18-hr measurements. Coproporphyrinogen was determined after oxidation to coproporphyrin in dim light. The results are expressed as millimicromoles of substance produced per 200 ml of medium.

DISCUSSION

It was previously shown that most strains of *B. ruminicola* isolated from the rumen of adult cattle fed a variety of diets required rumen fluid for growth (Bryant et al., 1958*a*; Bladen et al., 1961*b*). Subsequent studies indicated that the rumen fluid requirement of most strains of *B. ruminicola* subsp. *ruminicola* could be replaced by hemin (Bryant and Robinson, 1962).

The present results show that most of the growth-promoting activity of rumen fluid for *B. ruminicola* 23 can be replaced by supplementa-

tion of the basal medium with a fraction of rumen fluid obtained by an extraction procedure previously shown to extract protoheme (Falk, 1964). Further study of the material in this extract would be necessary to determine the nature of the active factor(s) in rumen fluid. Extraction procedures 1 and 2 (Table 3) have been used to extract uroporphyrin and coproporphyrin (Falk, 1964). Since the porphyrinogens produced by reduction of pure samples of these porphyrins with sodium amalgam support excellent growth of strain 23, the failure of extracts prepared by procedures 1 and 2, when reduced with sodium amalgam, to support substantial growth suggests that uroporphyrinogen and coproporphyrinogen are of little importance as growth factors for rumen fluid-requiring strains of *B. ruminicola* in animals fed alfalfa hay-grain rations.

The present results show that strain 23 has a specific growth requirement for certain tetrapyrroles. The requirement is satisfied only by certain porphyrins, hemes, heme biosynthesis intermediates at or beyond the tetrapyrrole stage, metalloporphyrins, and certain hemeproteins from which the heme is readily removed. Cytochrome *c*, a heme-protein in which the heme is covalently bonded to the protein (Paul, 1960), is only slightly active and only after prolonged incubation unless treated with heat or procedures which specifically release its heme (Paul, 1950). Supplementation of the basal medium with extracts containing heme from cytochrome *c* allows growth similar to that expected from the theoretical heme content of this molecule. Strain 23 is thus very inefficient in removing the heme from cytochrome *c*.

Strain 23 is unable to grow when monopyrroles, heme biosynthesis intermediates preceding the tetrapyrrole stage, compounds containing the ring structure of chlorophyll, or linear tetrapyrroles are substituted for hemin. That the requirement is not simply an iron requirement is indicated by the failure of ferrous sulfate, in the presence or absence of chelating agents, to replace hemin, and by the failure of ferrichrome (Burnham and Neilands, 1961), terregens factor (Burton, Sowden, and Lochhead, 1954), and coprogen (Hesseltine et al., 1952), which appear to serve as iron-binding and transfer agents in other microorganisms (Neilands, 1957), to replace hemin as growth factors. Hemin can replace these compounds as growth factors for other microorganisms.

Although the growth requirement of strain 23 is specific for certain tetrapyrroles, this strain can utilize a wider variety of tetrapyrroles than hemin-requiring microorganisms previously studied (Lascelles, 1961; White and Granick, 1963). The present investigation shows that versatility in tetrapyrrole utilization is a common characteristic of tetrapyrrole-requiring strains of *B. ruminicola* subsp. *ruminicola*, since iron-free protoporphyrin IX, mesoporphyrin, hematoporphyrin, and deuteroporphyrin will replace hemin as growth factors for strain 23, and also for strains GA20, $B_1$18, and related strain B127. The present study further shows that, although a substantial portion of strains of *B. ruminicola* subsp. *ruminicola* require tetrapyrroles for growth, certain strains of this subspecies do not require these compounds. The tetrapyrrole-independent strains of this subspecies represented unusual biotypes. Strains representing more predominant biotypes required tetrapyrroles.

The growth of tetrapyrrole-requiring strains of *B. ruminicola* in media containing ferrous ion and iron-free porphyrins other than protoporphyrin IX is of interest, since White, Bryant, and Caldwell (1962) have shown that both heme-independent strain GA33 and heme-requiring strain 23 contain protoheme and cytochromes of the *b* type, identical in absorption spectrum. Presumably, other tetrapyrrole-requiring strains of *B. ruminicola* contain the same cytochrome. The hemin requirement of *Haemophilus influenzae* may be replaced by deuteroheme, mesoheme, and hematoheme, but, among the iron-free porphyrins, only protoporphyrin IX replaces the hemin requirement of this organism (Granick and Gilder, 1946). Gilder and Granick (1947) suggested that vinyl groups are essential for insertion of iron into porphyrins. The present results suggest that *B. ruminicola* can insert iron into a variety of porphyrins not containing vinyl. A direct demonstration of ferrochelatase activity (Labbe and Hubbard, 1960) in these bacteria would be of interest, since the present results do not preclude the nonenzymatic insertion of iron into porphyrin.

The growth of strain 23 in media containing hemes other than protoheme suggests the possibility that strain 23 can make cytochromes which contain hemes other than protoheme and that these compounds are active, as has been found with certain other heme proteins (Antonini and Gibson, 1960; Paul, Gewitz, and Volker, 1959; Smith and Gibson, 1959; Paul, 1959; Gibson, 1964). It is also possible that deuteroheme and mesoheme are converted to protoheme.

Certain facts indicate that the commonly known pathway of heme biosynthesis, involving δ-aminolevulinic acid and porphobilinogen, is present in heme-independent *B. ruminicola* subsp. *brevis*. These include the growth of strain GA33 in tetrapyrrole-free media, the production of porphobilinogen and porphyrins from δ-amino-

levulinic acid during growth of this strain, and the previous finding (White et al., 1962) that cells of this strain grown in a tetrapyrrole-free medium contain a cytochrome of the *b* type. A portion of the universal pathway is apparently present in heme-requiring strain 23, since this strain grows when uroporphyrinogen, coproporphyrinogen, and protoporphyrin IX, known tetrapyrrole intermediates in heme biosynthesis, are substituted for hemin. The failure of δ-aminolevulinic acid and porphobilinogen to replace hemin as growth factors for heme-requiring strain 23, and the apparent inability of this strain to metabolize δ-aminolevulinic acid to porphobilinogen or porphyrins under conditions in which the heme-independent strain readily forms these compounds, suggest that the heme requirement results from lack or inactivity of some or all of the enzymes involved in heme biosynthesis prior to those necessary for the utilization of uroporphyrinogen. At least one enzyme, δ-aminolevulinic dehydrase, is inactive or absent in the heme-requiring strain under conditions in which the reaction catalyzed by this enzyme may be readily shown in the heme-independent strain.

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