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Vitamin Requirements of Several Cellulolytic Rumen Bacteria[1]

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ABSTRACT

SCOTT, H. W. (Ohio Agricultural Experiment Station, Wooster), AND B. A. DEHORITY. Vitamin requirements of several cellulolytic rumen bacteria. J. Bacteriol. **89**:1169–1175. 1965.—Four strains of cellulolytic bacteria recently isolated from in vitro rumen fermentations were used in this study. Nine water-soluble vitamins were tested in single-deletion and single-addition plus biotin experiments, each with and without charcoal-extracted casein hydrolysate. *Bacteroides succinogenes* A3C and B21a required only biotin under the above experimental conditions. *Ruminococcus flavefaciens* B34b showed an absolute requirement for biotin and was stimulated by *p*-aminobenzoic acid (PABA) in the single-deletion experiments. In the single-addition plus biotin experiments, PABA and, to a lesser extent, vitamin B_{12} appeared to be required for maximal growth. The presence or absence of casein hydrolysate did not affect the vitamin requirements for the aforementioned three strains. In the single-deletion experiments, *R. flavefaciens* C1a showed an absolute requirement for biotin and, when casein hydrolysate was omitted, for B_{12}. When casein hydrolysate was present, no requirement for B_{12} could be observed. In the single-addition experiments where the basal medium contained biotin and casein hydrolysate or B_{12}, PABA was required for maximal growth; however, the single deletion of PABA caused only slight retardation of growth. Investigation of the B_{12} or casein hydrolysate requirement of C1a revealed that a mixture of purified amino acids simulating casein hydrolysate satisfied this requirement. Subsequent work indicated that this requirement could be satisfied by the amino acid methionine.

The in vitro mixed culture technique has been widely used to study the rumen microflora. Primarily, these investigations have been conducted to determine the significance of the biochemical activities of the flora to the overall function of the rumen. Little concrete evidence is available to establish whether bacteria which proliferate in vitro are truly representative of the rumen microflora. Recently, Dehority (1963) isolated and characterized five strains of cellulolytic bacteria from 24-hr in vitro fermentations carried out with mixed cultures of rumen bacteria. Four of these strains, *Bacteroides succinogenes* A3c and B21a and *Ruminococcus flavefaciens* B34b and C1a, were selected for study of their vitamin requirements. These requirements were compared with the vitamin requirements found by Bryant, Robinson, and Chu (1959) and Bryant and Robinson (1961) for several strains of *B. succinogenes* and *R. flavefaciens* which had been isolated directly from the rumen of cattle.

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MATERIALS AND METHODS

The pure cultures used were carried on the rumen fluid-glucose-cellobiose-agar (RGCA) medium of Bryant and Burkey (1953). Anaerobiosis was maintained according to the techniques described by Hungate (1950). All gases were freed of oxygen by passing them over reduced hot copper turnings.

The complete medium was a slight modification of the defined medium used by Bryant and Robinson (1961), the major modifications being the deletion of Na_2S as a reducing agent and the addition of Zn^{++}, Fe^{++}, and vitamin B_{12}. Table 1 shows the composition of the complete medium from which vitamins were deleted as needed in studying the vitamin requirements. The complete medium was prepared by placing concentrated solutions of the minerals, volatile fatty acids, and B vitamins in a round-bottom 500-ml flask. Cellobiose, casein hydrolysate, distilled water, and resazurin, an indicator of anaerobiosis, were added, and the *p*H was adjusted to 6.7 with 2 N NaOH. The contents of the flask were boiled

TABLE 1. *Composition of the complete medium**

Compound	Amt (mg per 100 ml)	Compound	Amt (mg per 100 ml)
KH_2PO_4	90.0	Resazurin	0.1
NaCl	90.0	L-Cysteine·HCl·H_2O	100.0
$(NH_4)_2SO_4$	90.0	Cellobiose	400.0
$CaCl_2$	5.0	Casein hydrolysate	200.0
$MgSO_4$	5.0	Pyridoxine hydrochloride	0.2
$MnSO_4 \cdot H_2O$	2.0	Riboflavine	0.2
$FeSO_4 \cdot 7H_2O$	2.0	Thiamine hydrochloride	0.2
$ZnSO_4 \cdot 7H_2O$	2.0	Nicotinamide	0.2
$CoCl_2 \cdot 6H_2O$	0.2	Ca-D-pantothenate	0.2
Na_2CO_3	400.0	*p*-Aminobenzoic acid	0.01
Acetic acid	133.0	Folic acid	0.005
Isobutyric acid	6.6	Biotin	0.005
Isovaleric acid	8.0	B_{12}	0.0005
Valeric Acid	8.0		

* All minerals were of ACS reagent quality. The B vitamins were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

momentarily and then cooled while bubbling oxygen-free CO_2 through the medium. The flask was closed with a rubber stopper wired in place, and sterilized by autoclaving. After cooling, CO_2 was again aseptically passed into the medium, and previously sterilized cysteine hydrochloride and Na_2CO_3 were added. Gassing of the contents of the flask was continued until the medium was equilibrated with CO_2 and a *p*H of 6.6 to 6.7 was obtained. The medium was then tubed under CO_2 in 5-ml amounts in sterile rubber-stoppered culture tubes (16 × 150 mm).

The vitamin-free medium was identical to the complete medium, except for the omission of the vitamins. Acid-hydrolyzed casein hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio) was further purified by the method of Bryant and Robinson (1961). The vitamin-free media were prepared with and without charcoal-extracted casein hydrolysate (CECH) to study the effect of this constituent on the vitamin requirements of the pure cultures.

Stock solutions (0.1 ml) containing single vitamins or combinations of vitamins were placed, as required, in culture tubes and sterilized. The vitamin solutions were then diluted with vitamin-free basal medium (plus or minus CECH, as required) and inoculum to give a final volume of 5 ml. The final concentration of each vitamin was equal to that used in the complete medium.

Inoculum was prepared by transferring 0.1 ml of culture grown in the complete medium to the vitamin-free basal medium without CECH. After 12 to 20 hr of incubation, the cultures were transferred under anaerobic conditions to a sterile heavy-walled Pyrex centrifuge tube fitted with a rubber cap, and were centrifuged for 10 min at 16,000 × *g* in a SS-1 Superspeed Servall angle centrifuge. The cells were washed, suspended in the anaerobic dilution solution of Bryant and Burkey (1953), and diluted to an optical density (600 mμ) of 0.1 with the vitamin-free basal medium. The washed cells (0.1 ml) were used to inoculate 4.9 ml of medium.

Single-deletion and single-addition type experiments were conducted with and without CECH to determine the vitamin requirements of the four cellulolytic strains. The basal medium used in the single-addition experiments contained biotin, since this vitamin was found to be an absolute requirement for all strains.

Growth was measured turbidimetrically at 600 mμ on a Bausch and Lomb Spectronic-20 colorimeter. Measurements were made at 0 hr, 12 hr, and then every 4 hr until the cultures had attained their maximal growth. All fermentations were made in duplicate and replicated at least twice.

RESULTS

Preliminary studies revealed that all four strains would grow with ammonia as the sole source of added nitrogen. Therefore, all single deletion and addition experiments were run with and without the CECH to determine whether this had any effect on the vitamin requirements of the four strains. The single deletion of each of the nine B vitamins from the complete medium is shown in Table 2 for *B. succinogenes* A3c. Biotin was shown to be the only absolute vitamin requirement for strain A3c. The single addition of vitamins to the biotin-containing basal medium did not alter the growth response from that produced when biotin was the only vitamin present (Table 2). The data obtained for *B. succinogenes* B21a were identical to that presented for strain A3c. Although the addition of CECH shortened the lag phase and produced a slight increase in maximal optical density, its presence or absence had no effect on the vitamin requirements of the *B. succinogenes* strains.

TABLE 2. *Single vitamin deletion and single addition plus biotin experiments with Bacteroides succinogenes A3c in the presence and absence of CECH*

Vitamin	Growth (OD × 100)			
	Single vitamin deletion		Single vitamin addition + biotin	
	+CECH	−CECH	+CECH	−CECH
Pyridoxine	121 (24)*	108 (48)	122 (24)	114 (48)
Riboflavine	122 (24)	110 (45)	120 (24)	114 (45)
Thiamine	121 (24)	110 (42)	121 (24)	115 (42)
Nicotinamide	116 (24)	111 (42)	118 (24)	116 (42)
Pantothenate	122 (26)	112 (42)	115 (26)	114 (42)
PABA	122 (24)	112 (42)	120 (24)	114 (42)
Folic acid	121 (24)	111 (42)	119 (24)	115 (42)
Biotin	3 (24)	0 (42)	122 (24)	115 (42)
B_{12}	123 (24)	115 (42)	119 (24)	109 (42)
All vitamins			122 (32)	119 (48)

* Numbers in parentheses indicate the number of hours of incubation required to reach maximal optical density.

TABLE 3. *Single vitamin deletion and single addition plus biotin experiments with Ruminococcus flavefaciens B34b in the presence and absence of CECH*

Vitamin	Growth (OD × 100)			
	Single vitamin deletion		Single vitamin addition + biotin	
	+CECH	−CECH	+CECH	−CECH
Pyridoxine	87 (28)*	93 (32)	3 (100)	9 (96)
Riboflavine	84 (38)	91 (28)	3 (116)	1 (92)
Thiamine	82 (36)	93 (32)	0 (100)	3 (92)
Nicotinamide	85 (34)	89 (36)	0 (108)	11 (124)
Pantothenate	85 (32)	88 (36)	5 (116)	3 (124)
PABA	76 (46)	85 (36)	72 (42)	94 (38)
Folic acid	84 (32)	87 (36)	5 (92)	9 (84)
Biotin	0 (44)	0 (48)	7 (116)	7 (116)
B_{12}	87 (36)	93 (36)	18 (100)	21 (104)
All vitamins	87 (36)	93 (36)	90 (28)	89 (30)

* Numbers in parentheses indicate number of hours of incubation required to reach maximal optical density.

The vitamin requirements for *R. flavefaciens* B34b, studied under the same experimental conditions as the *B. succinogenes* strains, are shown in Table 3. Biotin was again the only absolute requirement in single-deletion experiments. The single-addition plus biotin experiments revealed that PABA was highly stimulatory and that B_{12} produced a slight growth response. Except for the single-addition plus biotin experiment with PABA, the presence or absence of CECH did not markedly affect the growth response of strain B34b.

Further studies on some of the vitamin interrelationships involving biotin, PABA, and B_{12}

TABLE 4. *Vitamin interrelationships for Ruminococcus flavefaciens B34b in the absence of CECH*

Vitamin added	Maximal growth (OD × 100)
None	7 (74)*
Biotin	12 (74)
Biotin + B_{12}	41 (74)
Biotin + PABA	90 (38)
Biotin + PABA + B_{12}	90 (28)
All vitamins	93 (30)

* Numbers in parentheses indicate number of hours of incubation required to reach maximal optical density.

TABLE 5. *Single vitamin deletion and single addition plus biotin experiments with Ruminococcus flavefaciens C1a in the presence and absence of CECH*

Vitamin	Growth (OD × 100)			
	Single vitamin deletion		Single vitamin addition + biotin	
	+CECH	−CECH	+CECH	−CECH
Pyridoxine	88 (28)*	97 (28)	9 (100)	2 (76)
Riboflavine	90 (28)	93 (28)	13 (112)	1 (88)
Thiamine	87 (24)	96 (28)	10 (108)	2 (96)
Nicotinamide	91 (28)	95 (28)	8 (112)	2 (88)
Pantothenate	89 (28)	94 (28)	12 (112)	2 (92)
PABA	83 (28)	88 (28)	60 (88)	2 (86)
Folic acid	90 (28)	94 (28)	11 (100)	2 (80)
Biotin	0 (48)	2 (88)	13 (108)	1 (80)
B_{12}	69 (76)	2 (74)	67 (54)	65 (50)
All vitamins			87 (24)	94 (28)

* Numbers in parentheses indicate number of hours of incubation required to reach maximal optical density.

for strain B34b are shown in Table 4. In the absence of all vitamins or in the presence of biotin alone, no appreciable growth occurred. The addition of biotin and PABA to the vitamin-free basal medium without CECH resulted in a marked growth stimulation, and biotin plus B_{12} allowed only partial growth after a prolonged lag phase. When biotin, PABA, and B_{12} were combined, these three vitamins gave the same growth response as the complete vitamin mixture.

Table 5 presents the data for the vitamin requirements of *R. flavefaciens* C1a in the presence and absence of CECH. Biotin and B_{12} were found to be absolute requirements for strain C1a in the absence of CECH. However, when CECH was added to the basal medium, B_{12} no longer appeared to be an absolute requirement in the single-deletion type experiments, although the time to reach maximal growth was markedly greater.

The single-addition plus biotin experiments indicated that other vitamins in addition to biotin and B_{12} are necessary for maximal growth. The presence of CECH in the biotin-containing basal medium resulted in a partial response to the singly added vitamins PABA and B_{12}. The response to B_{12} is apparently not influenced by the presence or absence of the CECH preparation. However, the single addition of PABA to the biotin-containing basal medium indicates that, in the presence of CECH, PABA was required to produce a response equivalent to that produced by the combination of biotin and B_{12}. Apparently, PABA is necessary for the replacement of B_{12} by CECH under the conditions of the experiment. To further study the possible replacement of B_{12} by CECH, graded levels of CECH were added to a medium which contained all B vitamins except B_{12} (Fig. 1). Under these conditions the CECH appeared to be required for strain C1a.

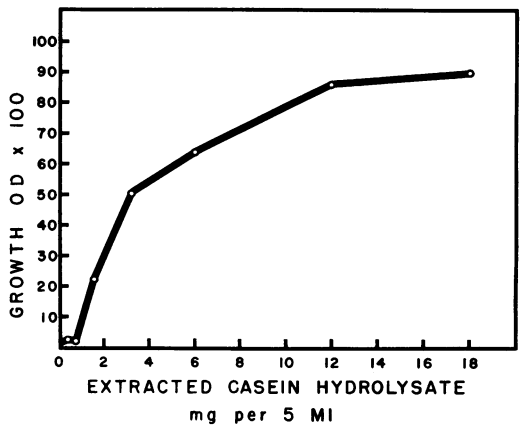

FIG. 1. *Response of Ruminococcus flavefaciens C1a to graded levels of CECH.*

A mixture of 19 amino acids (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared in concentrations simulating casein hydrolysate (Block and Bolling, 1945). By use of a basal medium deficient in B_{12}, the response produced by the simulated preparation was compared to that produced by CECH. An increase in optical density of 0.36 and 0.35, respectively, was obtained for the two preparations after 60 hr of incubation. Further investigations showed that methionine alone was capable of producing a

TABLE 6. *Effect of deleting PABA from a CECH-free medium on the replacement of vitamin B_{12} by L-methionine for Ruminococcus flavefaciens C1a**

Vitamin deleted	Maximal growth (OD × 100)
None	91 (24)†
B_{12}	95 (24)
B_{12}, PABA	27 (100)

* The basal medium contained the equivalent of 0.6 mg of L-methionine per 5 ml of medium.

† Numbers in parentheses indicate number of hours of incubation required to reach maximal optical density.

TABLE 7. *Effect of PABA on the replacement of vitamin B_{12} by L-methionine in a CECH-free medium for Ruminococcus flavefaciens C1a*

Vitamin added[a]	Growth (OD × 100)	
	L-Methionine[b]	B_{12}[c]
None	11 (76)[d]	45 (44)
PABA	79 (32)	86 (28)
All vitamins	91 (24)	93 (24)

[a] The vitamin biotin was included in the basal medium.

[b] The concentration of L-methionine was 0.6 mg per 5 ml of medium.

[c] Vitamin B_{12} was added to the basal medium at the same level as in the complete medium.

[d] Numbers in parentheses indicate number of hours of incubation required to reach maximal optical density.

growth response similar to that produced by CECH.

Simmonds (1948) found that some mutants of *Escherichia coli* K-12 would grow only if supplied with an exogenous source of cystine or cysteine. Since the cystine requirement was spared by methionine, homocysteine, or L-cystathionine, it was inferred that a portion of the required cystine is utilized in the biosynthesis of methionine. In the present study, L-cysteine was used as a reducing agent. In reducing the media, both cysteine and cystine should have been present. To determine if cystine was required for growth in addition to B_{12}, or whether the function of B_{12} was in the conversion of cystine to methionine, a medium without CECH was prepared by use of sodium sulfide as a reducing agent; 0.1 ml of 0.75% sodium sulfide solution prepared under nitrogen was used for each 5 ml of medium. Growth occurred in all tubes containing the complete vitamin mixture, which indicated that strain

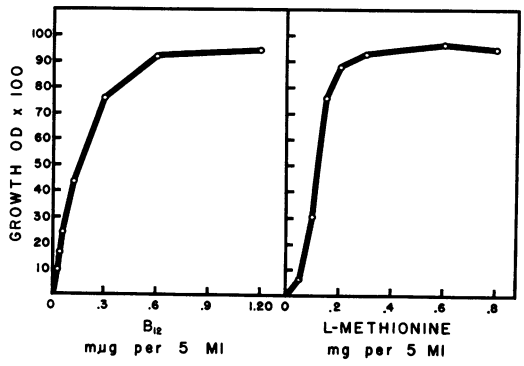

FIG. 2. *Response of Ruminococcus flavefaciens C1a to graded levels of vitamin B_{12} and L-methionine in a CECH-free medium.*

C1a would grow either in the presence or absence of an exogenous source of cystine. This was established by use of serial transfers through six tubes of cystine-free medium.

Additional vitamin interrelationships involving replacement of B_{12} by methionine are shown in Table 6. These data demonstrate the effect of deleting PABA from the CECH-free medium when L-methionine is used in place of B_{12}. When using the complete medium plus methionine, deletion of B_{12} had no effect on the growth of the organism. However, deletion of PABA resulted in a definite reduction of growth.

Further investigation of the above relationships carried out in the presence of B_{12} and L-methionine are shown in Table 7. In these experiments, PABA was added to the biotin-containing basal medium. The results indicated that the addition of PABA appeared to have a more pronounced effect in the presence of methionine than in the presence of B_{12}.

Figure 2 demonstrates the response of *R. flavefaciens* C1a to levels of B_{12} and methionine with all vitamins except B_{12} in the basal medium. The response curves produced by both compounds are quite similar; however, a small, but definite, lag was generally observed in the methionine curve. Maximal growth was obtained with approximately 0.6 mμg of B_{12} and between 0.3 and 0.6 mg of L-methionine per 5 ml of medium.

The level of biotin required to produce a maximal growth response was determined for all four organisms. All B vitamins, except biotin, were present in the basal medium. The required level appeared to be between 8 and 16 mμg of biotin per 5 ml of medium in all cases, and a typical growth response curve, obtained with strain C1a, is shown in Fig. 3.

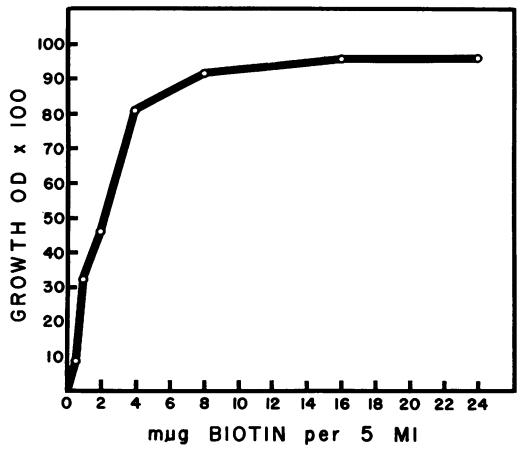

FIG. 3. *Response of Ruminococcus flavefaciens C1a to graded levels of biotin.*

DISCUSSION

In general, the vitamin requirements of the four strains of cellulolytic rumen bacteria isolated from in vitro fermentations were similar to those belonging to the same genera and species isolated from the rumen of cattle by Bryant et al. (1959) and Bryant and Robinson (1961). Although many of the strains of ruminococci isolated by these workers also required pyridoxine, our isolates did not. One possible reason for this difference may be that, in the original enrichment culture from which our microorganisms were isolated pyridoxine was not included in the basal medium (Dehority, 1963). Thus, a natural selection for ruminococci strains not requiring pyridoxine may have occurred. The most notable exception in the vitamin requirements of the present isolates was the absolute requirement for B_{12} by *R. flavefaciens* C1a. None of the previously isolated rumen bacteria has been shown to require B_{12}.

The two strains of *B. succinogenes* were found to require only biotin in a chemically defined medium. This agrees with the findings of Bryant et al. (1959) for *B. succinogenes* S85 and M19.

R. flavefaciens B34b showed an absolute requirement for biotin in the single-deletion experiments; however, PABA was required when the vitamins were added singly to the biotin-containing basal medium. Similar data were obtained by Bryant and Robinson (1961) for eight strains of ruminococci.

R. flavefaciens C1a exhibited an absolute requirement for biotin and B_{12} in single-deletion experiments, and growth was markedly stimulated by PABA in addition experiments. When casein hydrolysate or methionine was added to a medium containing all B vitamins except B_{12}, the requirement for B_{12} could no longer be demonstrated. When methionine is substituted for B_{12}, the requirement for PABA becomes almost absolute in both deletion and addition experiments.

Pittman, as reported by Bryant and Robinson (1962), found that methionine would replace the casein hydrolysate requirement of *B. ruminicola* GA33. However, B_{12} was included in their basal medium. In previous studies on the vitamin requirements of cellulolytic strains of rumen bacteria, the B_{12} requirement may have been satisfied by the inclusion of casein hydrolysate or similar components in the media (Bryant et al., 1959; Bryant and Robinson, 1961).

The possible interrelationships between PABA, B_{12}, and methionine in the nutrition of *R. flavefaciens* C1a are not clearly understood at this time. Studies with *E. coli* might suggest that PABA is required for the synthesis of folic acid or a folic acid-like compound, owing to an inability or limited ability of the organism to utilize an exogenous source of this vitamin (Gibson and Woods, 1960; Kisliuk and Woods, 1960). Folic acid and B_{12} could then be involved in a series of enzymatic reactions whereby methionine is synthesized by the transfer of a methyl group to homocysteine (Guest et al., 1960; Jones, Guest, and Woods, 1961; Guest, Friedman, and Foster, 1962). These reactions may be occurring in strain C1a; however, the marked response to PABA addition when methionine is present in the medium would suggest an additional function for this vitamin (Lampen, Jones, and Roepke, 1949). Although a longer lag phase was observed with methionine, this may only be an indication of the organisms' limited ability to utilize an exogenous source of the preformed amino acid. The observation that C1a does not require cysteine would not oppose the above suggested function, since the organism may be able to synthesize this amino acid and convert it into homocysteine. Several preliminary experiments were carried out in which attempts were made to replace methionine with betaine or choline. The complete lack of response to these compounds might suggest further that methionine does not function as a methyl-group donor.

Previous work by Johnson and Bently (1958) and Dryden et al. (1962) has shown that certain rumen bacteria can synthesize vitamin B_{12}, and that the concentration of this vitamin in rumen fluid is quite high. Thus, the establishment and presence of strains requiring B_{12} in the rumen would not be entirely unexpected.

The B_{12} requirement of strain C1a, in addition

to the other vitamin requirements of the four strains used in this study, confirm the results of studies on vitamins stimulatory to cellulose digestion by mixed ruminal cultures in vitro. Bentley et al. (1954, 1955) found that biotin, PABA, and, to a lesser extent, B_{12} increased digestion of cellulose by mixed cultures of cellulolytic bacteria. Hall, Cheng, and Burrows (1953) noted that, in addition to these three vitamins, folic acid, pyridoxine, and riboflavine were stimulatory to cellulose digestion by washed suspensions of rumen microorganisms. Dehority, el-Shazly, and Johnson (1960), using washed inoculum prepared from mixed cultures of cellulolytic rumen bacteria, obtained a response to different levels of biotin similar to that demonstrated by each of the four strains.

The establishment of the vitamin requirements of the four strains of cellulolytic bacteria isolated from in vitro fermentations provides further evidence that the bacteria which proliferate in vitro are true representatives of the rumen microflora. By determining the vitamin requirements of the four individual strains, it is possible to equate, in part, the qualitative and quantitative vitamin requirements of the mixed ruminal microflora with the individual species.

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