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Production of Branched-Chain Volatile Fatty Acids by Certain Anaerobic Bacteria

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Net production of isobutyric acid, isovaleric acid, and 2-methylbutyric acid by cultures of *Bacteroides ruminicola* and *Megasphaera elsdenii* on media that contained Trypticase or casein hydrolysate continued (up to 5 days) after growth had ceased. Only trace quantities of these acids were produced in a medium that contained a mixture of amino acids that did not include the branched-chain amino acids. *M. elsdenii* produced increased quantities of the branched-chain fatty acids in a medium that contained Trypticase when glucose was reduced or eliminated from the culture medium. However, *B. ruminicola* produced increased quantities of branched-chain fatty acids and of phenylacetic acid from Trypticase when glucose was supplied at 3 mg/ml rather than at 1 mg/ml. Single strains of *Streptococcus bovis, Selenomonas ruminantium, Bacteroides amylophilus,* and *Butyrivibrio fibrisolvens* did not produce branched-chain fatty acids.

The presence of branched-chain 4- and 5-carbon acids in ruminal fluid became apparent when samples were first analyzed by gas-liquid chromatography (27). These acids have since been detected from the rumen and other anaerobic environments by many other workers, and the available evidence supports the early proposal (28) that these acids are produced from branched-chain amino acids. Isobutyric, isovaleric, and 2-methylbutyric acids in the rumen are produced by microbial attack on valine, leucine, and isoleucine, respectively (3, 5, 13, 21).

Data showing production of branched-chain acids by pure cultures isolated from the rumen and from other anaerobic environments has accumulated (15). Information is, however, minimal concerning production of branched-chain acids by these bacteria at different stages of growth or as influenced by composition of the culture medium. This study was conducted to supply such information.

MATERIALS AND METHODS

Organisms and culture conditions. The bacterial strains tested were isolates from the bovine rumen from the culture collection of M. P. Bryant and associates (6, 7). Strains were selected on the basis of previous evidence for branched-chain acid or ammonia (6) production or proteolytic activity, but no attempt was made to survey all ruminal species. All organisms were grown anaerobically under an atmosphere of CO_2; a modification of the methods developed by Hungate was used (8).

Test media listed in Table 1 were used. Separately sterilized anaerobic solutions of Na_2CO_3 and cysteine-hydrochloride were added to the other ingredients of each medium which had been sterilized under a CO_2 gas phase. Growth was measured as absorbance at 600 nm in tubes (18 by 150 mm). Bacterial strains were transferred one or more times in medium A before tests were made. All incubations were at 38°C.

Measurement of free volatile acids. Samples removed from cultures were treated with metaphosphoric acid (one part of 25% [wt/vol] solution of HPO_3 per five parts of sample), cooled to 4°C, and then centrifuged at 12,000 × *g* for 10 min. The supernatant fluid was removed and used for analysis.

Two methods were used to analyze free acids by gas-liquid chromatography. In method A we used a Teflon column (1.5 m by 2 mm) packed with SP-1200/H_3PO_4 (10/1%) on Chromosorb WAW, 80 to 100 mesh (Supelco, Inc., Bellefonte, Pa.). A hydrogen flame detector was used with helium as carrier, and the oven temperature was 115°C. Quantities of acids were estimated by measurement of areas under peaks (disc integrator) as compared with standard curves prepared with known quantities of acids. In method B we used a stainless steel column (2.16 mm by 2 m) packed with 0.3% SP-1000 + 0.3% H_3PO_4 on Carbopack A (Supelco, Inc.). Oven temperature was 150°C, and carrier (N_2) flow was 30 ml/min. Samples were injected with an automatic injection system, and an external standardization procedure based on digital integration of peak areas was used. This system permitted separation and measurement of the two branched-chain 5-carbon acids, 2-methylbutyric acid and isovaleric acid. The 5-carbon branched-chain acids used as standards (Eastman Organic Chemicals, Rochester, N.Y.) contained both isomers, with ratios of the acid expected to the contaminant of 9.77 for 2-methylbutyric acid and 8.88 for isovaleric acid.

Measurement of acids as butyl esters. Samples plus heptanoate (internal standard) were freeze-dried

TABLE 1. *Composition of media used to study branched-chain acid production*[a]

Ingredients	Medium[b]							
	A	B	C	D	E	F	G	H
Glucose	1	1	1	1	3	1	–	1
Cellobiose	1	1	1	1	–	–	–	–
Soluble starch	1	1	1	1	–	–	–	–
Trypticase[c]	15	–	–	–	15	15	15	5
Amino acid mixture[d]	–	+	–	–	–	–	–	–
Acid hydrolyzed casein[c]	–	–	10	–	–	–	–	–

[a] All media were adjusted to pH 6.8 and contained hemin (0.002 mg/ml) plus resazurin, vitamins, cysteine, carbonate, and minerals at the concentrations given by Bryant and Robinson (10); Na_2S was deleted.

[b] Quantities given are in milligrams per milliliter, except for the amino acid mixture.

[c] Trypticase was from Baltimore Biological Laboratories, and acid hydrolyzed casein was Hy-Case SF (Sheffield Chemical Co., Norwich, N.Y.).

[d] The amino acid mixture contained DL-alanine, DL-aspartic acid, L-arginine, L-glutamic acid, glycine, L-histidine, L-lysine-hydrochloride, DL-methionine, L-proline, L-serine, DL-threonine, and DL-tyrosine. All except tyrosine were present at a final concentration of 0.025 mg/ml. Tyrosine was present at 0.005 mg/ml.

at pH 10 and were butylated with butanol-hydrochloride. The chromatographic column, derivative preparation, operating conditions, and quantitation procedures were similar to those described by Salanitro and Muirhead (25). The butyl esters of 2-methylbutyric and isovaleric acids were separated from esters of straight-chain acids but not from each other.

The butyl ester of phenylacetic acid (Eastman Organic Chemicals) was eluted with a retention time of 1.29 relative to that of the internal standard, butyl heptanoate. Further studies are needed to prove conclusively that the substance that was prepared during butylation of culture supernatants in this study and that was eluted at exactly the same retention time as butyl-phenylacetate was actually butyl-phenylacetate. Other workers (18, 26) have shown that phenylacetate is the main product of phenylalanine metabolism in the rumen, and incubations of ruminal contents with 0.66 mM phenylalanine led to accumulation of increased quantities of this substance (at 1, 2, and 4 h) as compared with control incubations (M. J. Allison and H. M. Cook, unpublished data). It thus seems reasonable to assume that the substance we measured is phenylacetate.

RESULTS

Branched-acid production with different N sources. The quantities of branched-chain 5-carbon fatty acids produced by strain 23 or *Bacteroides ruminicola* and by *Megasphaera elsdenii* B159 in Trypticase medium (A) and in casein hydrolysate medium (C) are given in Fig. 1. Both organisms produced higher quantities of these acids in the Trypticase medium, and in both media the highest concentrations observed were in the last sample taken. This was long after growth, as measured by absorbance, had ceased.

Branched-chain 5-carbon acid production in the Trypticase medium (A) by *B. ruminicola* 23 and three additional strains of *B. ruminicola* is shown in Fig. 2. Strains 23, GA20, and $B_1$18 belong to the subspecies *ruminicola,* and GA33

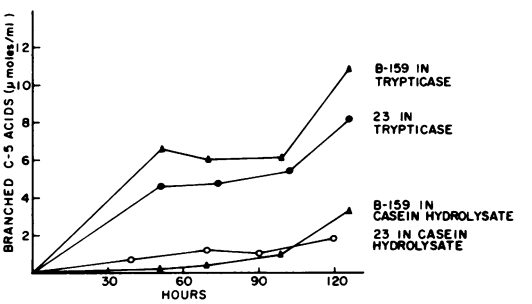

FIG. 1. *Concentrations of branched-chain 5-carbon acids produced by M. elsdenii B159 and by B. ruminicola 23 in media containing either Trypticase (medium A) or casein hydrolysate (medium C). Free acids were chromatographed by using method A.*

belongs to subspecies *brevis* (11). All four cultures appeared to have reached maximum growth (absorbance greater than 1.0) before samples were taken at 72 h, but production of the branched-chain acids continued after growth stopped.

The quantities of short-chain acids produced during incubation in media containing Trypticase (medium A), a mixture of amino acids that did not include the branched-chain amino acids (medium B), or casein hydrolysate (medium C) are shown in Fig. 3 and 4. Neither *M. elsdenii* B159 nor *B. ruminicola* 23 produced more than a trace of branched C_5 acids during growth on the amino acid medium (B). A peak with the same retention time as isobutyric acid was produced by *M. elsdenii.* Small quantities of isobutyric acid would probably not have been detected in the *B. ruminicola* culture because of the large propionic acid peak. Both of these cultures produced more branched-chain acids on the Trypticase medium than on the casein hydrolysate medium. This difference was probably due to the different quantities of the branched-

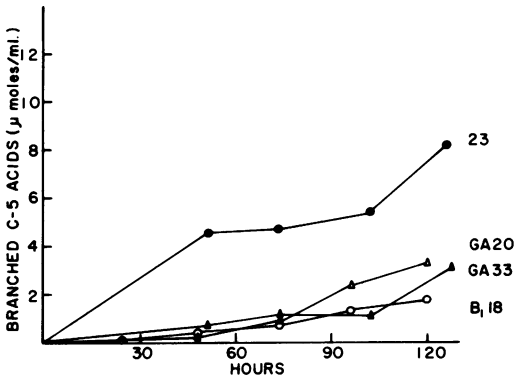

FIG. 2. *Concentrations of branched-chain 5-carbon acids produced by four strains of B. ruminicola during growth in the Trypticase medium (A). Free acids were chromatographed by using method A.*

chain amino acids in these media, or, in the case of strain 23, to more effective transport and metabolism of peptides as compared with free amino acids (22).

Strains FD-10 of *Streptococcus bovis*, HD-4 of *Selenomonas ruminatium*, 70 of *Bacteroides amylophilus*, and D-1 of *Butyrivibrio fibrisolvens* grew well on the Trypticase medium, but detectable quantities of branched-chain acids were not observed in samples assayed after 24 or 72 h.

Branched-chain acid production: effect of carbohydrate level in medium. The effect of glucose concentration on branched-chain acid production by *B. ruminicola* 23 and *M. elsdenii* B159 is shown in Tables 2 and 3. Growth of both organisms was greater with 3 mg of glucose per ml in the culture medium than with 0 or 1 mg/ml, and both organisms produced less branched-chain 5-carbon acid when the initial concentration of Trypticase was 5 rather than 15 mg/ml. Production of branched-chain 5-carbon acids by *B. ruminicola* was greater in the medium with an initial glucose concentration of 3 mg/ml than with a concentration of 1 mg/ml. Isobutyric acid was produced in measurable quantities only with glucose at 3 mg/ml. *B. ruminicola* did not grow when glucose was omitted from the culture medium.

M. elsdenii produced higher concentrations of isobutyric acid and branched-chain 5-carbon acids when the initial concentration of glucose was 1 rather than 3 mg/ml and still higher concentrations when glucose was deleted from the culture medium. This was true even though growth in the absence of glucose was greatly reduced (Table 3). The ratio of concentrations of branched-chain 5-carbon acids to isobutyrate decreased exponentially with time. The data was found to fit an equation of the general form: ratio = α exp $[B/(h + 1)]$. The α values that express the ratios in the asymptote of the curve (late in the fermentation) are given in Table 3.

Samples from these cultures taken at 145 h were also analyzed by chromatography of the free acids; method B, which permitted separation of the branched-chain, 5-carbon isomers, was used. The ratio of the concentrations of isovaleric acid (3-methylbutyric acid) to 2-methylbutyric acid produced by *B. ruminicola* was 1.11 ± 0.04 (n = 3), whereas with *M. elsdenii*, this ratio was 3.87 ± 0.51 (n = 4). The separation of these isomers is shown in Fig. 5. Agreement was good for measurements of the branched-chain 5-carbon acids collectively as butyl esters versus the sum of the measurements of isovaleric acid plus 2-methylbutyric acid made with method B. A regression of the former measurements versus the latter gave a slope of 1.0083, with a 95% confidence interval of 0.9556 to 1.0611.

Production of phenylacetate by *B. ruminicola* 23 continued after absorbance had reached a maximum in much the same way as did production of branched-chain 5-carbon acids (Table 2). Production of phenylacetate was also similarly

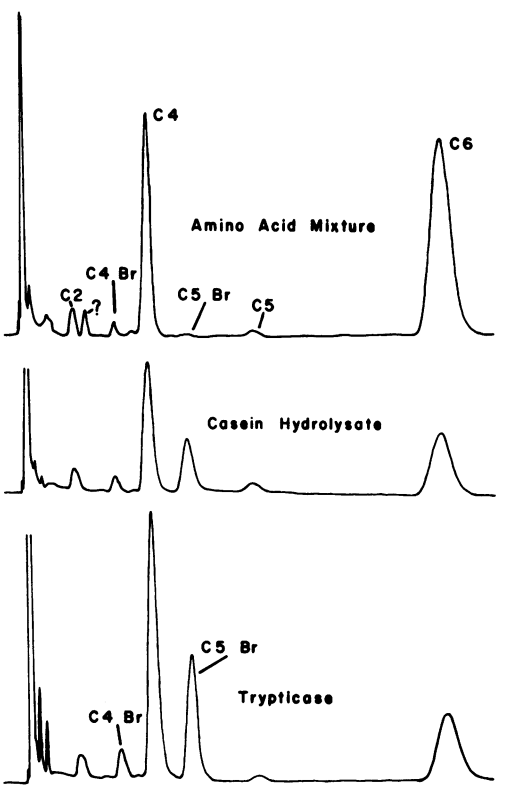

FIG. 3. *Chromatographic tracings of volatile fatty acids produced by M. elsdenii B159 after 121 h of incubation in three different media. Free acids were chromatographed by using method A.*

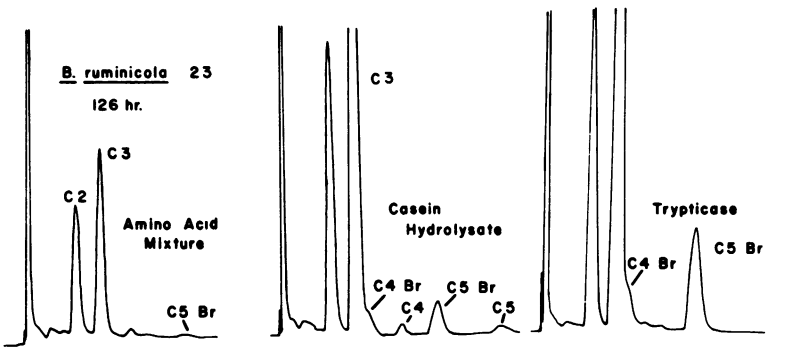

FIG. 4. *Tracings of chromatograms of volatile fatty acids produced by B. ruminicola after incubation for 126 h in three different media. Free acids were chromatographed by using method A.*

TABLE 2. *Production of branched-chain 5-carbon acids and phenylacetic acid by B. ruminicola 23*

Incubation time (h)	Medium[a]		
	E (3, 15)	F (1, 15)	H (1, 5)
21	(140) 1.66, 0.30[b]	(75) 0.77, 0.09[b]	(80) 0.42, 0.06[b]
35	(123) 2.17, 0.42	(67) 1.15, 0.16	(60) 0.66, 0.12
56	(116) 2.23, 0.48	(66) 1.46, 0.26	(54) 0.76, 0.15
81	(116) 2.21, 0.56	(56) 1.42, 0.30	(52) 0.91, 0.19
145	(116) 2.22, 0.51	(48) 1.45, 0.32	(44) 0.83, 0.18
Ratio[c]	3.93 (3.23–4.79)	4.08 (3.34–4.98)	4.24 (4.03–4.47)

[a] Culture media as given in Table 1. Values in parentheses in boxheads indicate initial concentrations (milligrams per milliliter) of glucose and Trypticase, respectively.

[b] The value in parentheses is an estimate of cell density as measured by absorbance ×100 at 600 nm. The next two values are measurements (micromoles per milliliter, measured as butyl esters) of the concentrations of branched-chain 5-carbon acids and phenylacetic acid, respectively.

[c] Asymptotic value (α) of ratio (branched-chain 5-carbon acids/phenylacetate) and 95% confidence interval of that value found by fitting data to the equation given in text.

TABLE 3. *Production of branched-chain 4 and 5-carbon acids by M. elsdenii B159*

Incubation time (h)	Medium[a]			
	E (3, 15)	F (1, 15)	G (0, 15)	H (1, 5)
21	(56) 0.50, 3.8[b]	(44) 0.72, 4.49[b]	(19) .52, 4.25[b]	(47) 0, 1.46[b]
35	(75) 0.61, 4.13	(49) 0.96, 5.18	(18) .94, 5.16	(46) 0.07, 1.29
50	(83) 0.68, 4.25	(46) 1.07, 5.36	(17) 1.22, 5.58	(37) 0.16, 1.62
81	(70) 0.82, 4.70	(38) 1.44, 5.41	(12) 1.39, 5.83	(34) 0.21, 1.64
145	(53) 0.87, 4.77	(22) 1.22, 5.39	(09) 1.48, 5.93	(23) 0.23, 1.68
Ratio[c]	5.24 (5.01–5.48)	4.21 (4.06–4.36)	3.33 (2.91–3.82)	4.76 (2.69–8.41)

[a] Culture media as given in Table 1. Values in parentheses in boxheads indicate initial concentrations (milligrams per milliliter) of glucose and Trypticase, respectively.

[b] The value in parentheses is an estimate of cell density as measured by absorbance ×100 at 600 nm. The next two values are measurements (micromoles per milliliter measured as butyl esters) of the concentrations of isobutyric and branched-chain 5-carbon acids, respectively.

[c] Asymptotic value (α) of ratio (branched-chain 5-carbon acids/isobutyrate) and 95% confidence interval of that value found by fitting data to the equation given in text.

influenced by the initial concentrations of glucose and Trypticase in culture media. The relationship between phenylacetate production and production of branched-chain 5-carbon acids by *B. ruminicola* could also be expressed by the equation given above to relate branched-chain 5-carbon acid production to isobutyrate production by *M. elsdenii*.

DISCUSSION

The branched-chain 4- and 5-carbon acids are incorporated by several bacterial species and are

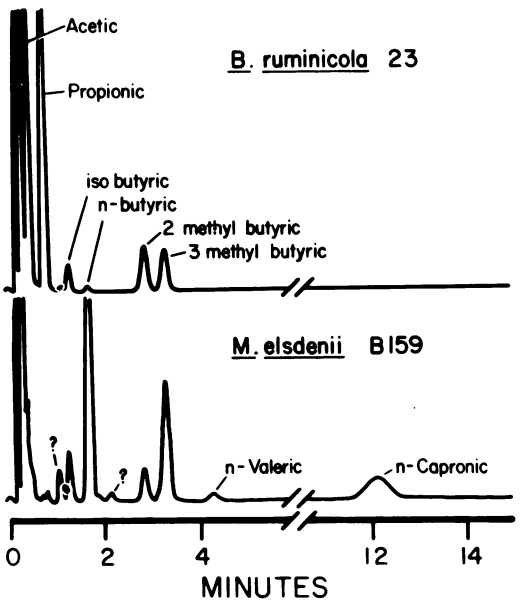

FIG. 5. *Measurements of acids produced (145 h) by B. ruminicola and M. elsdenii cultures grown in medium E containing glucose (3 mg/ml) and Trypticase (15 mg/ml). Chromatography was with SP-1000 + H_3PO_4 on Carbopack A (method B).*

used for resynthesis of the branched-chain amino acid from which they were derived or are used for synthesis of long-chain branched fatty acids or aldehydes (1). They are essential growth factors for several ruminal species and also for bacteria from other anaerobic environments (9, 29). Thus, the reactions which involve these acids are useful as models to illustrate microbial interactions in anaerobic ecosystems. It has already been shown that *Ruminococcus albus* and *B. ruminicola* will grow together on a casein-cellulose medium on which neither will grow alone. *R. albus* supplies *B. ruminicola* with cellulose degradation products needed as an energy source, and *B. ruminicola* supplies the casein degradation products, ammonia and branched-chain fatty acid, essential for growth of *R. albus* (12).

The ratio of leucine to isoleucine in Trypticase is 1.42 (14). This value is intermediate between the ratios of isovaleric to 2-methylbutyric acid present in *B. ruminicola* and *M. elsdenii* cultures at 145 h, being 1.11 and 3.87, respectively. Both of these organisms are able to use isobutyrate and both of the branched-chain 5-carbon acids in reductive carboxylation reactions to synthesize the carbon skeletons of valine, leucine, and isoleucine, respectively (2, 23). Because utilization and production reactions both likely occurred in these cultures, the measurements of acids should be considered as estimates of net rather than total production. *B. ruminicola* 23 produces phenylacetate and can also synthesize the carbon skeleton of phenylalanine by reductive carboxylation of phenylacetate; however, *M. elsdenii* B159, which does not have this biosynthetic capacity (1), did not produce measurable quantities of phenylacetate in these experiments.

The decrease with incubation time of the ratios of the concentrations of branched-chain 5-carbon acids to branched-chain 4-carbon acid in *M. elsdenii* cultures and of the ratio of branched-chain 5-carbon acids to phenylacetate in *B. ruminicola* cultures (Tables 2 and 3) was a consistent finding. The significance of these changes and of their description by the equation given is not now known.

M. elsdenii is known to ferment amino acids (19). Inhibition of amino acid degradation (as judged by production of branched-chain acids) by glucose is thus not surprising. Inhibition of the proteolytic activity of microbes by carbohydrates was described in earlier publications (4, 17). Except for *M. elsdenii,* carbohydrate was necessary for growth, and the levels used did not inhibit ammonia production by a number of ruminal organisms (6). A study of short-chain acid production by *Bacteroides fragilis* cultures (21) indicated that branched-chain 5-carbon acids were produced in greater quantities in a peptone yeast medium than in a peptone yeast plus glucose medium. Production of branched-chain 4- and 5-carbon acids by *B. fragilis* also increased with long incubation times.

Extrapolation from batch cultures of single organisms to open, continuous-flow systems such as the rumen is difficult. Thus, the study of factors influencing branched-chain acid production by these organisms in a continuous culture system would be of interest and no doubt more easily related to the in vivo situation than would data from batch cultures. However, in view of the evidence here for increased branched-chain acid production by *M. elsdenii* when glucose in the culture medium is limited, it is worth mentioning that concentrations of soluble carbohydrate in the rumen are usually low except during a short interval after feeding (16, 24). The increased ruminal concentrations of branched-chain 4- and 5-carbon fatty acids and of ammonia after fasting sheep 36 h (30) may be related to increased amino acid degradation rates and lower rates of assimilation of these fatty acids when carbohydrate is depleted.

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