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Volatile Fatty Acid Requirements of Cellulolytic Rumen Bacteria[1]

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A gas chromatographic method was developed which could separate the isomers isovaleric and 2-methylbutyric acid. Subsequent analyses revealed that most commercially available samples of these acids were cross-contaminated; however, one sample of each acid was found to be pure by this criterion. The growth response of seven strains of cellulolytic rumen bacteria (three strains of *Bacteroides succinogenes*, three strains of *Ruminococcus flavefaciens*, and one strain of *R. albus*) to additions of isobutyric, isovaleric, 2-methylbutyric, valeric, and combinations of valeric and a branched-chain acid was determined. Strains of *B. succinogenes* required a combination of valeric plus either isobutyric or 2-methylbutyric acid. Isovaleric acid was completely inactive. Either isobutyric or 2-methylbutyric acid was required for the growth of *R. albus* 7. Strain C-94 of *R. flavefaciens* grew slowly in the presence of any one of the three branched-chain acids, but a combination of isobutyric and 2-methylbutyric acids appeared to satisfy this organism's growth requirements. None of the individual acids or mixtures of straight- and branched-chain acids allowed growth of *R. flavefaciens* strain C1a which would approach the response obtained from the total mixture of acids. Further work indicated that all three branched-chain acids were required for optimal growth by this strain, although isovaleric acid only influenced the rate of maximal growth. Either 2-methylbutyric or isovaleric acid allowed growth of nearly the same magnitude as that of the positive control for *R. flavefaciens* B34b. The presence of acetic acid had little influence on the rate or extent of growth of any of the strains except *R. albus* 7, for which the extent of growth was markedly increased. Determination of the quantitative fatty acid requirements for the three *B. succinogenes* strains indicated that 0.1 μmole of valeric per ml and 0.05 μmole of 2-methylbutyric per ml permitted maximal growth. However, with isobutyric acid as the branched-chain component, strains A3c and B21a required 0.1 μmole/ml in contrast to S-85 which exhibited optimal growth at the 0.05 μmole/ml level. By use of mixtures of isobutyric and 2-methylbutyric acids, good growth of C-94 was obtained at concentrations of 0.1 and 0.01 μmole/ml, respectively. About 0.3 μmole/ml of each acid was required for satisfactory growth of C1a.

Bryant and Doetsch (4) found that the factors in rumen fluid required for the growth of *Bacteroides succinogenes* in a defined medium were a combination of a straight-chain volatile fatty acid, such as valeric or caproic, and a branched-chain acid, such as isobutyric, isovaleric, or 2-methylbutyric. Later studies by Allison, Bryant, and Doetsch (1) and Bryant and Robinson (5) indicated that the cellulolytic ruminococci also had a volatile fatty acid requirement, which could be satisfied only by a branched-chain acid such as isovalerate. However, subsequent investigations by Allison et al. (3) suggested that most commercial samples of isovalerate were contaminated with D-2-methylbutyrate, and that the growth response of *Ruminococcus albus* 7 was not proportional to isovaleric acid concentration, as it was with either isobutyric or 2-methylbutyric acid. In addition, this organism did not grow or incorporate radioactivity when the only source of volatile fatty acid was synthetic isovalerate-*3*-^{14}C. Although Bryant, Robinson, and Chu (6) and Dehority (8) found that a mixture of valeric and isovaleric acids satisfied the volatile fatty acid requirements of various strains of *B. succinogenes*, Wegner (Ph.D. Thesis,

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Univ. of Wisconsin, Madison, 1962), working with *B. succinogenes* S-85, was unable to obtain growth or incorporate the label with isovalerate-*1*-^{14}C. The object of the present study was twofold: first, to develop a method for separating isovaleric and 2-methylbutyric acids by use of a commercially obtained gas chromatographic apparatus, and, second, to determine the specific volatile fatty acid requirements for seven strains of cellulolytic rumen bacteria.

MATERIALS AND METHODS

Organisms and media. Strains A3c and B21a of *B. succinogenes* and strains B34b and C1a of *R. flavefaciens* were previously isolated and described in this laboratory (8). *B. succinogenes* S-85, *R. flavefaciens* C-94, and *R. albus* 7 were obtained from M. P. Bryant, Department of Dairy Science, University of Illinois, Urbana, and have been described in earlier publications (6, 7). The organisms were grown overnight in the complete medium of Scott and Dehority (10) and centrifuged, the pellet was washed, and the cells were then resuspended and diluted to an optical density (OD) of 0.2 with the complete medium minus volatile fatty acids. Each tube of test medium, containing 10 ml, was then inoculated with 0.2 ml of this suspension.

The basal test medium contained cellobiose, minerals, carbonate-bicarbonate buffer, B vitamins, casein hydrolysate, and cysteine and was identical to the complete medium of Scott and Dehority (10), with all volatile fatty acids except acetate removed. Whether the acids were added singly or in various combinations, the following concentrations (per milliliter) were used in all experiments except those on quantitative requirements: acetic, 1.33 mg (22.17 μmoles); isobutyric, 0.066 mg (0.75 μmole); and isovaleric, 2-methylbutyric, and valeric, 0.08 mg (0.78 μmole).

Except for the acetic acid, which was analytical reagent quality (J. T. Baker Chemical Co., North Phillipsburg, N.J.), the volatile fatty acids were obtained commercially from Eastman Chemical Products Inc., Kingsport, Tenn. and Matheson Co., Inc., East Rutherford, N.J. Several samples from different lots were obtained from each company.

Growth was estimated turbidimetrically at 600 mμ on a Bausch and Lomb Spectronic-20 colorimeter. Measurements were made at 0 hr, 12 hr, and then every 6 hr until the cultures had obtained their maximal growth. For the data reported in Table 1, two separate batches of each media were prepared, and two growth experiments were run in duplicate for each batch. Thus, each value represents the mean of eight tubes of medium. The only exception to this was in the case of C1a, for which only six values were obtained. In all other portions of the study, each combination was tested twice in duplicate.

Gas chromatography. An Aerograph Hy-Fy, model 600, equipped with a hydrogen flame ionization detector, was used throughout this study. Helium was used as a carrier gas. Columns were made of 8-ft (2.4-meter) lengths of one-eighth inch (0.3-cm) outer diameter Pyrex glass tubing and were packed with the solid support containing the adsorbed liquid phase.

Previous experimental work in this laboratory had indicated that it was possible to obtain a partial separation when mixtures of isovaleric and 2-methylbutyric acid were chromatographed on columns with stearic acid as the stationary phase. Thus, for purposes of this study, stearic acid was investigated as a possible stationary phase, and such factors as concentration, length of column, temperature, and carrier gas flow rate were studied. Satisfactory separation of these two acids was obtained on columns prepared by dissolving the required amount of stearic acid (to give a final concentration of 5 to 6% stearic acid in the stationary phase) in a minimal amount of ether containing phosphoric acid (added at a concentration of approximately 3% of the weight of stearic acid). The previously weighed solid support (Gas Chrom Z 80/100 mesh) was added to this mixture, and the slurry was stirred over a steam bath to evaporate the ether. The dried material was then packed into the column by means of vacuum and a hand vibrator. The column was conditioned in the chromatograph at 125 C with carrier gas passing through, until a steady base line resulted, usually at 20 to 24 hr. For these columns, optimal operating temperatures were in the range of 96 to 106 C at helium flow rates of 16.5 to 17.0 ml/min. Resolution was improved with longer columns and somewhat higher temperatures.

Gas chromatographic analysis of a number of different commercial samples of isovaleric and 2-methylbutyric acid revealed that most of them were contaminated with varying amounts of the other. However, one sample of each acid in which the presence of the other could not be detected by the techniques used was found. Figure 1 presents a composite photo of the gas chromatograms for all the acid samples used in the requirement portion of this study. The total amount of each acid injected was 30 μg or 6 μliters of a 0.5% solution. A series of studies on the minimal concentration of isovaleric or 2-methylbutyric acid which could be measured, revealed that total quantities below 0.5 μg were not detectable with the equipment used. Thus, any contamination below approximately 2% would not be discernible on the chromatograms shown in Fig. 1.

Figure 2 presents a gas chromatogram illustrating the separation of a mixture of the lower-chain volatile fatty acids which was obtained on a 12-ft (3.8-meter) column at 124 C and a helium flow rate of approximately 17 ml/min. Under these conditions, all the acids are completely resolved, except isovaleric and 2-methylbutyric, for which, however, the overlap of the two peaks is extremely small.

RESULTS

The growth response of the various strains of cellulolytic rumen bacteria to the volatile fatty acids is presented in Table 1. The three strains of *B. succinogenes* (A3c, S-85, and B21a) required a combination of isobutyric or 2-methylbutyric acid with valeric acid for growth. The rate and

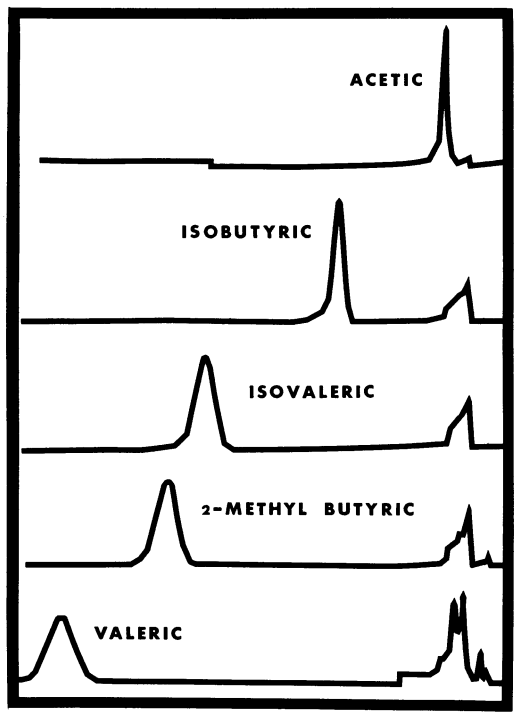

FIG. 1. *Gas chromatograms of the individual commercial acids used to study the volatile fatty acid requirements of rumen cellulolytic bacteria. These separations were obtained on an 8-ft (2.4-m), 5% stearic acid column at 105 C temperature and a helium flow rate of 17 ml/min.*

extent of growth with either of these combinations was almost identical to the response obtained with the control medium which contained all the acids. Except for a limited growth response by strains A3c and S-85 to isobutyric acid alone, none of the other acids or combinations would satisfy the volatile fatty acid requirement of these strains.

Response of the three *R. flavefaciens* strains (C-94, C1a, and B34b) to the different acids showed a great deal of variation. Results with strain C-94 indicated that this organism has an absolute requirement for volatile fatty acids. Although growth occurred in all media which contained the branched-chain acids, alone or in combination with valeric acid, both the rate and extent of growth were less than observed when all acids were present. The extent of growth in the presence of isobutyric acid approached the maximal growth obtained in the control medium, but the time required was slightly more than doubled. Isovaleric and 2-methylbutyric acids allowed partial growth, but only after a prolonged lag phase.

Strain C1a also showed an absolute requirement for volatile fatty acids; however, the only individual acid which appeared to have any activity was isobutyric. This response to isobutyric acid, alone or in combination with valeric acid, was very small and required an extended incubation period.

In a previous study (8), it was found that *R. flavefaciens* B34b was capable of some growth in a medium devoid of volatile fatty acids, providing that casein hydrolysate was present. However, the lag phase under these conditions was extremely long, between 48 and 72 hr. Inspection of the data in Table 1 for strain B34b reveals that growth occurred in all media, but the only acids which stimulated growth at a similar rate and extent to the control were 2-methylbutyric and isovaleric.

The response of *R. albus* 7 to the various volatile fatty acids appears to be quite uncomplicated. The presence of either isobutyric or 2-methylbutyric acid was required in the medium for growth comparable to that obtained in the control medium. Neither valeric nor isovaleric acids showed any ability to meet this organism's volatile fatty acid requirement.

Several earlier studies by Allison, Bryant, and Doetsch (1) and Bryant and Robinson (5) had indicated that with some strains of ruminococci the time required to reach maximal growth was extended considerably when acetic acid was not present in the medium. Since acetic acid was included in all of the media in the first phase of this study, an experiment was devised to evaluate this possible effect with the present strains. These results are presented in Table 2. For the three strains of *B. succinogenes*, the presence of acetic acid in the medium resulted in slightly higher optical densities, which reached their maximal values several hours earlier than those obtained in an identical medium without acetic acid. The magnitude of these differences, except for possibly A3c, was relatively small. *R. flavefaciens* C-94 showed a response to acetic acid similar to that observed

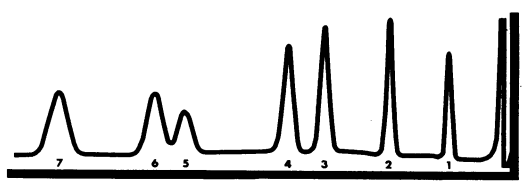

FIG. 2. *Separation of a known mixture of volatile fatty acids by gas chromatography. (1) Acetic acid, (2) propionic acid, (3) isobutyric acid, (4) butyric acid, (5) isovaleric acid, (6) 2-methylbutyric acid, and (7) valeric acid. Operating conditions for the 12-ft (3.8-m), 5% stearic acid column were 124 C and a helium flow rate of approximately 17 ml/min.*

with *B. succinogenes*, whereas the rate and extent of growth for strains C1a and B34b were not consistently influenced by the presence or absence of this acid. On the other hand, the extent of growth of *R. albus* 7 (as measured by an increase in OD) was markedly increased when acetic acid was included in the medium. The time required to reach maximal OD, however, was not changed.

Since none of the branched-chain acids alone, or in combination with valeric acid, supported growth of *R. flavefaciens* C1a comparable to that obtained from a mixture of all the acids, the possibility of a requirement for more than one of the branched-chain acids was suggested. Allison, Bryant, and Doetsch (2) have reported that optimal growth of *R. flavefaciens* C-94 could be obtained with a combination of isobutyric and isovaleric acids, but not with either acid alone. The data in Tables 1 and 2 support the observation that none of the branched-chain acids alone will

TABLE 1. *Growth response of seven strains of cellulolytic rumen bacteria to the addition of various volatile fatty acids*

Acids added[a]	Optical density						
	Bacteroides succinogenes			*Ruminococcus flavefaciens*			*R. albus* 7
	A3c	S-85	B21a	C-94	C1a	B34b	
None	0 (62)[b]	0 (62)	0 (62)	0 (62)	0 (67)	.27 (118)	0 (54)
All	1.53 (26)	1.40 (20)	1.37 (21)	.85 (26)	.68 (17)	.69 (28)	.75 (25)
Valeric	0 (62)	0.05 (40)	0 (60)	0 (64)	.03 (73)	.49 (92)	0 (54)
Isobutyric	0.74 (59)	0.60 (64)	0.06 (45)	.75 (63)	.27 (135)	.22 (67)	.72 (30)
2-Methylbutyric	0.04 (51)	0 (62)	0.03 (45)	.57 (150)	.04 (75)	.69 (31)	.78 (27)
Isovaleric	0 (62)	0 (62)	0 (60)	.54 (100)	.08 (103)	.62 (36)	0 (54)
Valeric + isobutyric	1.47 (26)	1.38 (19)	1.38 (24)	.76 (59)	.37 (91)	.38 (51)	.86 (22)
Valeric + 2-methylbutyric	1.50 (28)	1.38 (19)	1.34 (18)	.57 (151)	.05 (83)	.60 (35)	.82 (25)
Valeric + isovaleric	0 (62)	0.13 (47)	0 (60)	.54 (112)	.15 (115)	.62 (39)	0 (54)

[a] Acetic acid was added to all media at a concentration of 1.33 mg or 22.17 μmoles per ml. The volatile fatty acids under investigation, whether singly or in combination, were added at the following concentrations (per milliliter): isobutyric, 0.066 mg or 0.75 μmole; isovaleric, 2-methylbutyric, and valeric, 0.08 mg or 0.78 μmole.

[b] The figure in parentheses indicates the hours of incubation required.

TABLE 2. *Effect of acetic acid on the growth response of seven strains of cellulolytic rumen bacteria to volatile fatty acids*

Acids added[a]	Optical density						
	Bacteroides succinogenes			*Ruminococcus flavefaciens*			*R. albus* 7
	A3c	S-85	B21a	C-94	C1a	B34b	
Isobutyric				.76 (48)[b]	.24 (92)		
Acetic + isobutyric				.94 (52)	.21 (92)		
2-Methylbutyric						.66 (34)	.66 (20)
Acetic + 2-methylbutyric						.76 (30)	.98 (22)
Valeric + 2-methylbutyric	1.44 (22)	1.38 (21)	1.41 (24)				
Acetic + valeric + 2-methylbutyric	1.58 (20)	1.46 (16)	1.47 (18)				
All (isobutyric, 2-methylbutyric, valeric)	1.45 (26)	1.45 (21)	1.48 (24)	.85 (28)	.72 (18)	.72 (30)	.62 (21)
Acetic + all acids	1.68 (21)	1.56 (20)	1.57 (21)	.90 (22)	.67 (18)	.69 (30)	.90 (21)

[a] Acetic, isobutyric, 2-methylbutyric, isovaleric, and valeric acids were added at the same levels as given in Table 1, footnote *a*.

[b] The figures in parenthesis indicate the hours of incubation required.

allow optimal growth of this strain. Table 3 presents the growth responses of *R. flavefaciens* C1a and C-94 to all possible combinations of the three branched-chain acids.

For strain C1a, none of the branched-chain acids alone or in combinations of two supported the same growth rate as all three combined. However, the combination of isobutyric and 2-methylbutyric acids promoted growth to an equal OD after an additional 10 hr of incubation. Some slight response was observed with a mixture of isobutyric and isovaleric acids, whereas the combination of isovaleric and 2-methylbutyric was essentially inactive.

As mentioned previously, isobutyric acid promoted growth of strain C-94 to an OD comparable with that of the positive control, but required almost twice the incubation period. After an even more extended incubation, a partial response was obtained from each of the other acids. The response to a combination of isobutyric and 2-methylbutyric was similar to that obtained with all iso acids. The other two combinations were no better than the highest growth-promoting acid of each mixture alone.

These results suggest that strain C1a has a requirement for all three branched-chain acids. Based on the data obtained with combinations of two, it appears that the requirement for isobutyric acid can almost be considered as absolute. In the presence of isobutyrate, 2-methylbutyrate is highly stimulatory, whereas the response to isovalerate is of a much smaller magnitude and appears to be primarily concerned with rate. On the other hand, any of the three acids will support growth of strain C-94, although not at the rate or extent of all three combined. With this strain, however, a combination of isobutyric and 2-methylbutyric acids supported optimal growth, and no requirement was observed for isovaleric acid.

TABLE 3. *Growth response of Ruminococcus flavefaciens C1a and C-94 to branched-chain volatile fatty acids singly and combined*

Acids added[a]	Optical density	
	C1a	C-94
None	.03 (80)[b]	.05 (53)
All iso acids + valeric	.57 (16)	.77 (28)
All iso acids	.61 (14)	.89 (23)
Isobutyric	.22 (169)	.87 (48)
2-methylbutyric	.11 (95)	.60 (101)
Isovaleric	.09 (92)	.64 (95)
Isobutyric + 2-methylbutyric	.62 (25)	.91 (29)
Isobutyric + isovaleric	.38 (71)	.85 (42)
2-Methylbutyric + isovaleric	.17 (124)	.55 (88)

[a] Acetic acid was included in all media at a level of 1.33 mg or 22.17 μmoles per ml. The volatile fatty acids under investigation were added at the same levels as listed in Table 1, footnote *a*.

[b] The figure in parentheses indicates the hours of incubation required to reach maximal optical density.

Aside from the reports by Bryant and Doetsch (4) on *B. succinogenes* S-85, by Allison et al. (3) on *R. albus* 7, and by Allison, Bryant, and Doetsch (2) on *R. flavefaciens* C-94, no studies on quantitative volatile fatty acid requirements have been conducted. Since the qualitative requirements had been elucidated in the present work, it was desirable to quantitate these for several strains. Table 4 presents the quantitative requirements of strains A3c, B21a, and S-85 of *B. succinogenes* for valeric, isobutyric and 2-methylbutyric acids. Since two acids were required for growth, one acid was added to the basal medium at a constant level (0.5 μmole/ml), and the second acid was added at increasing concentrations from 0.0 to 0.5 μmole/ml. Optimal growth of all three strains occurred with valeric acid present in the medium at a concentration of 0.1 μmole/ml. Isobutyric acid was required at 0.1 μmole/ml for strains A3c and B21a; however, 0.05 μmole/ml appeared optimal for S-85. A concentration of 0.05 μmole/ml of 2-methylbutyric acid allowed maximal growth of all three strains.

Table 5 reports the results of quantitative requirement studies with *R. flavefaciens* C1a and C-94 for isobutyric and 2-methylbutyric acids. With these strains, a gradual increase in OD and decrease in incubation time was observed as acid concentration increased. Under these experimental conditions, with one acid present at a constant level (0.5 μmole/ml) and with increasing levels of the other acid, good growth of C-94 in a normal incubation time was achieved with 0.05 to 0.1 μmole of isobutyric acid per ml and 0.01 to 0.05 μmole of 2-methylbutyric acid per ml. On the other hand, about 0.3 μmole of each acid per ml was required for good growth of C1a.

DISCUSSION

Results of the present study substantiate the findings of Wegner (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1962) on the inability of isovaleric acid to satisfy the branched-chain portion of the fatty acid requirement of *B. succinogenes* S-85. Since identical results were obtained with two additional strains of *B. succinogenes*, these facts together suggest that isovaleric acid is probably inactive for all strains of this species. Based on the gas chromatographic analy-

TABLE 4. *Quantitative requirements of Bacteroides succinogenes strains A3c, B21a, and S-85 for isobutyric, 2-methylbutyric, and valeric acids*

Acids in basal medium[a]	Acid added	Concn (μmoles/ml) of added acid	Optical density		
			A3c	B21a	S-85
Isobutyric	Valeric	0	0.66 (66)[b]	0.14 (46)	0.68 (55)
		0.05	0.91 (19)	0.84 (17)	1.09 (17)
		0.1	1.36 (16)	1.38 (19)	1.42 (14)
		0.2	1.41 (14)	1.40 (14)	1.39 (15)
		0.3	1.45 (15)	1.42 (19)	1.40 (15)
		0.5	1.45 (15)	1.49 (17)	1.40 (14)
Valeric	Isobutyric	0	0.07 (9)	0 (25)	0.02 (15)
		0.005	0.05 (8)	0 (25)	0.16 (8)
		0.01	0.08 (8)	0 (25)	0.27 (8)
		0.05	0.76 (18)	0.23 (9)	1.39 (15)
		0.1	1.42 (18)	1.39 (19)	1.52 (17)
		0.3	1.55 (20)	1.46 (17)	1.51 (16)
		0.5	1.58 (19)	1.43 (15)	1.54 (16)
Valeric	2-Methylbutyric	0	0.05 (9)	0 (25)	0 (23)
		0.005	0.22 (12)	0.05 (10)	0.41 (8)
		0.01	0.39 (12)	0.34 (8)	0.66 (9)
		0.05	1.44 (15)	1.44 (15)	1.51 (17)
		0.1	1.56 (17)	1.46 (15)	1.54 (16)
		0.3	1.56 (18)	1.42 (17)	1.52 (15)
		0.5	1.59 (16)	1.49 (15)	1.50 (15)

[a] Acetic acid was included in all media at a concentration of 20 μmoles/ml. The level of isobutyric or valeric acid, when added to the basal medium, was 0.5 μmole/ml.

[b] The figure in parentheses indicates the hours of incubation required.

TABLE 5. *Quantitative requirements of Ruminococcus flavefaciens strains C1a and C-94 for isobutyric and 2-methylbutyric acids*

Acids in basal medium[a]	Acid added	Concn (μmoles/ml) of added acid	Optical density	
			C1a	C-94
2-Methylbutyric	Isobutyric	0	.05 (113)[b]	.04 (154)
		0.005	.22 (115)	.56 (151)
		0.01	.31 (99)	.49 (66)
		0.05	.51 (53)	.66 (28)
		0.1	.60 (42)	.79 (35)
		0.3	.71 (31)	.85 (32)
		0.5	.72 (31)	.91 (31)
Isobutyric	2-Methylbutyric	0	.42 (133)	.72 (133)
		0.005	.46 (84)	.79 (38)
		0.01	.48 (63)	.84 (34)
		0.05	.54 (53)	.87 (31)
		0.1	.66 (34)	.94 (30)
		0.3	.78 (30)	.97 (28)
		0.5	.83 (27)	.93 (27)

[a] Acetic acid was included in all media at a concentration of 20 μmoles/ml. The level of 2-methylbutyric or isobutyric acid, when added to the basal medium, was 0.5 μmole/ml.

[b] The figure in parentheses indicates the hours of incubation required.

sis of a number of commercial samples of isovaleric acid, it can be assumed that the growth-promoting effect of this compound previously reported is probably a result of 2-methylbutyric acid contamination.

Strains A3c and S-85 both showed a partial response to isobutyric acid alone (Tables 1 and 4) after a prolonged lag phase. The explanation for this is not immediately obvious. If the basal medium itself were contaminated with valeric or another straight-chain acid, some growth response should have occurred with 2-methylbutyric acid. On the other hand, if the isobutyric acid itself were contaminated, which was not detected by gas chromatographic analysis, then strain B21a should have shown similar growth. If the valeric acid requirement for B21a were different from A3c and S-85, which is not indicated in Table 4, and if the isobutyric acid were contaminated with valeric acid below the 2% lower limit of the gas chromatographic analysis, the maximal amount of valeric acid which could be present would be 0.01 μmole per ml of medium.

The minimal concentrations of valeric and isovaleric acids allowing good growth of *B. succin-*

ogenes S-85 for Bryant and Doetsch (4) were about 0.3 and 0.15 μmole/ml, respectively. The lower branched-chain acid requirement in the present study was expected, since their isovalerate activity was presumably due to contaminating 2-methylbutyrate; however, no obvious explanation is available for the lowered valeric acid requirement. The one basic difference between the two studies is that Bryant and Doetsch did not include acetic acid in their basal medium. Perhaps, acetate has some sparing effect on the *n*-valerate requirement, although it cannot entirely replace this acid.

Bryant, Robinson, and Chu (6) observed in 1959 that *B. succinogenes* S-85 had not lost its two component volatile fatty acid requirements after being maintained in the laboratory for over 5 years. It is of extreme interest that this requirement has remained consistent over an additional 7-year period of laboratory propagation.

Requirements of the ruminococci for volatile fatty acids differed quite markedly between the strains. These results would appear to parallel the findings of Bryant and Robinson (5), when they observed that the vitamin requirements of the different strains of ruminococci did not correlate well with the other variable characteristics within the genus. Although Allison, Bryant, and Doetsch (1) and Bryant and Robinson (5) were able to meet the volatile fatty acid requirements of nine different strains of ruminococci with a mixture of acetic acid and commercial isovaleric acid, the present study indicates that isovaleric acid is not effective for many strains. The probable reason for the marked responses obtained from commercial isovaleric acid have already been discussed; however, it should be noted that isovaleric acid itself did exhibit some ability to satisfy the growth requirements of *R. flavefaciens* C1a, C-94, and particularly B34b (Tables 1 and 3).

A direct comparison with the results of Allison, Bryant, and Doetsch (2) on the quantitative requirements of *R. flavefaciens* C-94 is difficult, since they tested increasing levels of isovalerate with a constant level of isobutyrate (0.1 μmole/ml) in the medium. With their isovalerate sample, a level of 0.1 to 0.2 μmole/ml of medium was required for maximal growth, which is markedly higher than the concentration required in the present work.

In general, the results of this and previous studies would indicate that the volatile fatty acid requirement of cellulolytic rumen bacteria can be divided into three main types: (i) All strains of *B. succinogenes* investigated have a two component requirement for volatile fatty acids, consisting of a straight-chain and a branched-chain acid. Valeric acid or a number of longer carbon straight-chain acids can satisfy the straight-chain requirement, and either isobutyric or 2-methylbutyric acid, but not isovaleric acid, can satisfy the branched-chain requirement. (ii) Several strains of *R. flavefaciens* appear to require two or more branched-chain acids for optimal growth. Of the three acids commonly used to satisfy this requirement, their level of activity, in descending order, would be isobutyric, 2-methylbutyric, and isovaleric. (iii) Some strains of *R. flavefaciens* and all strains of *R. albus* (3, 9) tested require only one branched-chain acid. In the case of *R. albus* 7, isobutyric or 2-methylbutyric acids are equally active, whereas isovaleric was completely inactive.

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