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Function of Growth Factors for Rumen Microorganisms

I. Nutritional Characteristics of *Selenomonas ruminantium*

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ABSTRACT

Nutritional characteristics of *Selenomonas ruminantium* var. *lactilytica* isolated from a sheep rumen were studied. The organism required for growth the addition of a clarified rumen fluid to a Trypticase-yeast extract medium with either lactate or glucose as an energy source. The requirement for rumen fluid was found to be satisfied by volatile fatty acids in glucose media and by biotin in lactate media. Straight-chain saturated fatty acids with C_3 to C_{10} carbon skeleton had been found to be effective. Among them, *n*-valerate was most effective at the lowest concentration. An abnormal morphology was observed with *n*-valerate-deficient glucose media. *n*-Valerate was essential in glucose media, and it was stimulatory in lactate media. Fermentation products from glucose were lactate, propionate, and acetate, and fermentation products from lactate were propionate and acetate. When cells were grown in a glucose medium containing *n*-valerate-C^{14}, the label was present in cell fractions. Almost all of the activity was found in lipid materials.

It is well known that some rumen bacteria require rumen fluid for growth, and that rumen fluid cannot entirely be replaced by yeast extract or Trypticase (7). Sometimes rumen fluid can be replaced by a mixture of volatile fatty acids. In most of the cases so far reported, replacement has been by branched-chain acids, sometimes in combination with straight-chain acids (1, 8). The requirement, however, has been mainly studied with cellulolytic rumen anaerobes, and there have been few reports dealing with noncellulolytic organisms.

Bryant and Robinson (3) reported that growth of several strains of *Selenomonas ruminantium* required or was stimulated by a mixture of volatile fatty acids which contained isobutyrate, *n*-valerate, isovalerate, and DL-α-methyl butyrate. Acetate was stimulatory only in the absence of casein hydrolysate in the culture medium. Hobson, Mann, and Smith (4) mentioned that acetate was stimulatory for growth of a strain of *S. ruminantium* even in the presence of casein hydrolysate. The addition of other volatile fatty acids including both straight- and branched-chain acids was not stimulatory, and was even inhibitory in some cases.

The present work was carried out to establish the nutritional characteristics of a freshly isolated strain of *S. ruminantium* var. *lactilytica*.

MATERIALS AND METHODS

Media. Three kinds of basal media were used unless otherwise stated: a Trypticase-yeast extract medium containing 0.2% each of Trypticase (BBL) and yeast extract (Daigo Eiyo Kagaku, Tokyo, Japan); a yeast extract medium containing 0.2% yeast extract; a casein hydrolysate-vitamins-valerate medium contained 0.25% vitamin-free casein acid hydrolysate (Nissui Seiyaku, Tokyo, Japan), a vitamin mixture, and 0.0019% *n*-valerate. As an energy source, 0.5% glucose, 2.5% (v/v) sodium DL-lactate (50%), or 1% (v/v) glycerol (85%) was added to the above media. The composition of the vitamin mixture (mg/100 ml) was as follows: thiamine-HCl, Ca pantothenate, nicotinamide, riboflavine, and pyridoxal-HCl, 0.2 each; *p*-aminobenzoic acid (PABA), 0.1; biotin, 0.01; folic and DL-thioctic acids, 0.005 each; and cobalamin 0.002 (final concentrations).

The medium used for the isolation of organisms was the lactate-Trypticase-yeast extract medium supplemented with 20% (v/v) clarified rumen fluid prepared as described by Allison et al. (1) and 1.2% agar.

Mineral salts added to the above media were essentially similar to those of Bryant and Robinson (3), except that 0.8% Na_2CO_3 was included instead of 0.4%. As a reducing agent, 0.075% cysteine-HCl was used.

Isolation of the organism. Rumen contents from which the organism was isolated were obtained via rumen fistula from a sheep which was fed once a day on hay and concentrates. The contents were collected before feeding, filtered through two sheets of cheese cloth, and centrifuged at 100 × *g* for 5 min to obtain the precipitate. The precipitate was washed twice with the mineral salts medium containing cysteine. Finally, the precipitate was diluted appropriately with the above medium, and *S. ruminantium* var. *lactilytica* was isolated by a roll-tube method. Almost all colonies which appeared in the roll tubes had the characteristic morphology of *S. ruminantium.*

Culture of the organism. The organism was cultured anaerobically according to the method of Hungate (6) in various media. All incubations were at 37 C and *p*H 6.8. The organism was precultured for 14 hr in a lactate-rumen fluid medium of essentially the same composition as the isolation medium except that the agar was omitted. Cells were harvested and washed twice with the salts solution. The optical density of the resulting suspension was adjusted to 0.2 at 660 mμ and 0.2 ml of the suspension was used as inoculum for 10 ml of medium. Bacterial growth was followed by measuring optical density in a Hitachi-FPW 4 colorimeter (Hitachi Seisakujo, Tokyo, Japan) equipped with a 66 filter (660 mμ).

Analysis of fermentation products. Analysis for organic acids produced was performed by silicic acid column chromatography according to the method of Belasco (2).

Isotopic experiment. n-Valerate-*1*-C^{14} (5 μc; final concentration = 0.0031%) was added to 100 ml of the glucose-yeast extract medium. After inoculation and incubation, cells were harvested, washed twice with water, and fractionated by the method of Wegner and Foster (8). A portion of the protein fraction was extracted twice with chloroform-methanol (1:3), at room temperature for 30 min and at 80 C for 1 hr. The solvent-soluble fraction was obtained after centrifugation. The other portion of the protein fraction was digested with pronase (200 μg/ml; Kaken Kagaku, Tokyo, Japan) for 24 hr at *p*H 8.0. The solubilized material was collected after centrifugation. Radioactivity of each fraction was counted with a gas-flow windowless counter (Erigaku Kenkyujo, Tokyo, Japan).

Chemicals. n-Valerate-*1*-C^{14} was purchased from Calbiochem. All other chemicals were commercial preparations of analytical grade.

Electron microscopy. Cells were harvested and washed twice with water. They were then placed on an collodion film, dried at room temperature, and shadowed with chromium. Electron micrographs of the cells were prepared with a Hitachi HW-1 electron microscope (Hitachi Seisakujo, Tokyo, Japan).

RESULTS

Growth response of S. ruminantium to n-valerate in glucose medium. S. ruminantium can utilize glucose, glycerol, or lactate as an energy source. The Trypticase-yeast extract medium supplemented with either glycerol or lactate did not support its rapid growth. However, abnormal type of growth was observed in the glucose medium (Fig. 1a). When 10 to 20% (v/v) of the clarified rumen fluid was added to the medium, normal growth was obtained with glucose, glycerol, or lactate. In the glucose medium, the clarified rumen fluid could be replaced by a mixture of volatile fatty acids consisting of acetate, isobutyrate, *n*-valerate, and isovalerate (Fig. 1a). Further experiments revealed that *n*-valerate was required for growth of this organism as shown in Fig. 1b.

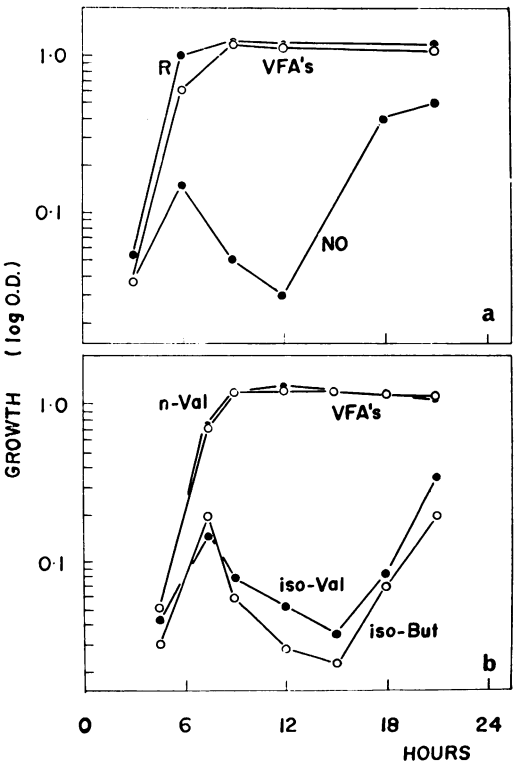

FIG. 1. *Growth response of Selenomonas ruminantium in the glucose medium. The following additions were made to the glucose-Trypticase-yeast extract medium: clarified rumen fluid (10%, v/v) (R), 0.306% Na acetate, 0.0017% Na isobutyrate, 0.0019% each of Na n-valerate and Na isovalerate (VFA's), and no addition (NO) for Fig. 1a. The volatile fatty acid mixture as described above but with acetate omitted (VFA's), 0.0017% Na isobutyrate (iso-But), 0.0019% each of Na n-valerate (n-Val) and Na isovalerate (iso-Val) for Fig. 1b.*

Vitamin requirements of S. ruminantium. Vitamin requirements were studied with casein hydrolysate as a nitrogen source. It was found that PABA was essential and biotin was stimulatory in the glucose medium (Fig. 2). The slower rate

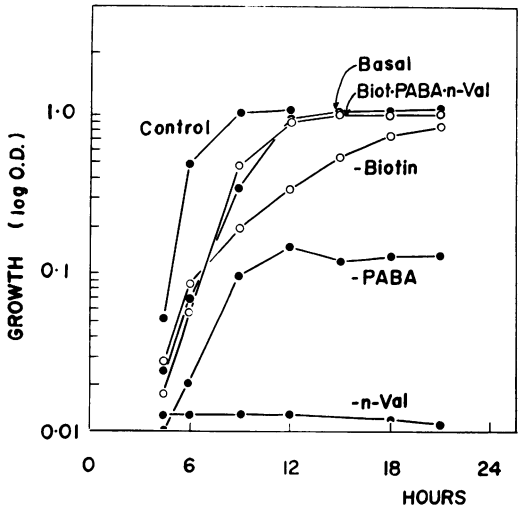

FIG. 2. *Vitamin requirements of Selenomonas ruminantium in the glucose medium. The basal medium (Basal) was the glucose-casein hydrolysate-vitamins-valerate medium. The following deletions were made from the basal medium: biotin (−Biotin), PABA (−PABA), n-valerate (−n-Val), and all vitamins except biotin and PABA (Biot·PABA·n-Val). The control medium (Control) was the glucose-yeast extract medium supplemented with 0.0019% n-valerate.*

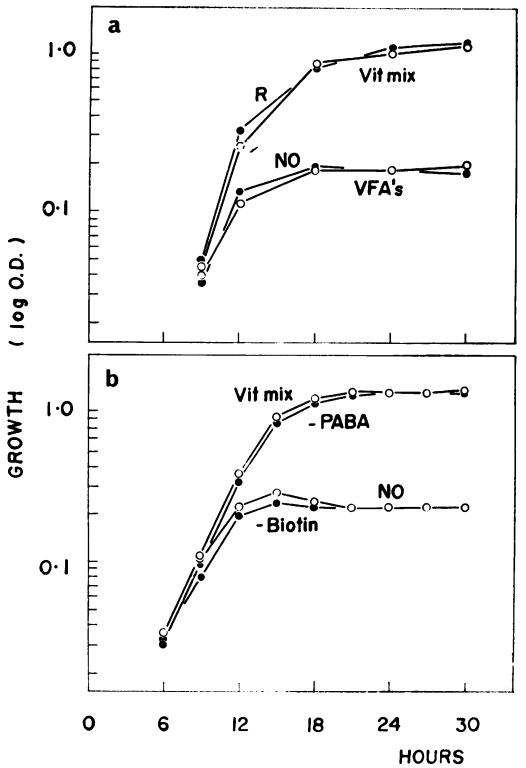

FIG. 3. *Growth response of Selenomonas ruminantium in the lactate medium. The basal medium was the lactate-Trypticase-yeast extract medium. The composition of the volatile fatty acid mixture was the same as that in Fig. 1a. The vitamin mixture had the same composition as described in Materials and Methods. The additions made to the basal medium were clarified rumen fluid (R), vitamin mixture (Vit mix), volatile fatty acids (VFA's), and no addition (NO) for Fig. 3a. For Fig. 3b, all experimental conditions were the same as above except that the following deletions were made from the vitamin mixture: PABA (−PABA) and biotin (−Biotin). The single deletion experiments except biotin gave essentially the same growth response as −PABA.*

of growth in the glucose-casein hydrolysate-vitamins-valerate medium (basal) as compared with the glucose-yeast extract medium supplemented with *n*-valerate (control) suggests an unidentified stimulatory factor(s) in yeast extract. The addition of L-tryptophan, adenine, guanine, uracil, and xanthine to the casein hydrolysate-vitamins-valerate medium, however, was without effect.

Figure 3a shows that rumen fluid could be replaced by the vitamin mixture but not with fatty acids in the lactate-Trypticase-yeast extract medium. Biotin was the sole effective vitamin in the mixture (Fig. 3b). As shown later in Fig. 4, biotin appears to be essential for growth of this organism in the lactate medium. The sluggish growth observed with the lactate-Trypticase-yeast extract medium, might be explained by a small amount of biotin contained in this medium. Probably the organism has a high requirement for biotin.

Stimulatory effect of n-valerate in lactate medium. The omission of Trypticase from the lactate-Trypticase-yeast extract medium resulted in a poor growth as shown in Fig. 4. The addition of *n*-valerate to the yeast extract medium, however, had a marked stimulatory effect. The growth was almost equal to that with the Trypticase-yeast extract medium (Fig. 4). This result suggests that Trypticase might contain *n*-valerate-like material. This is in accord with the observation that abnormal growth occurs either in the glucose-yeast extract medium with a low level of added *n*-valerate or in the glucose-Trypticase-yeast extract medium without added *n*-valerate (Fig. 1a).

Although biotin was essential for growth and no other vitamins tested were stimulatory (Fig. 4) in the lactate medium, growth in the semisynthetic medium (lactate-casein hydrolysate-vitamins-valerate medium) was poor as compared with that in the complex media (lactate-Trypticase-yeast extract medium supplemented with biotin or lactate-yeast extract medium supplemented with biotin and *n*-valerate; Fig. 4). Further

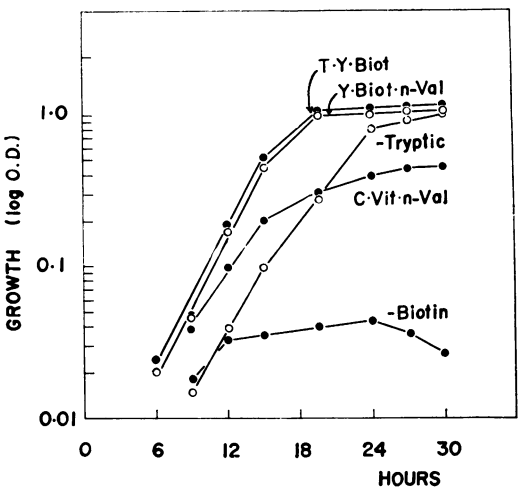

FIG. 4. *Stimulatory effect of n-valerate in the lactate medium. The experimental conditions were the same as in Fig. 3a and b. T·Y·Biot represents the growth in the lactate-Trypticase-yeast extract medium supplemented with biotin (0.01 mg/100 ml). The following deletions and additions were made: minus Trypticase (—Tryptic), minus Trypticase plus n-valerate (Y·Biot·n-Val), minus Trypticase and yeast extract plus casein hydrolysate, the vitamin mixture, and n-valerate, i.e., the casein hydrolysate-vitamins-valerate medium (C·Vit·n-Val), and minus biotin from the preceding medium (—Biotin).*

studies are needed to establish the requirements. Again, the addition of tryptophan and nucleic acid bases to the casein hydrolysate-vitamins-valerate medium had no growth-promoting effect. The requirement for growth factors in the glycerol medium was similar to that in the lactate medium. Every nutritional experiment presented here was performed at least three times. Consistent and reproducible results were obtained.

Relation between cell morphology and nutritional conditions. An electron micrograph (Fig. 5) illustrates the normal morphology of cells grown in the glucose-Trypticase-yeast extract medium supplemented with *n*-valerate (0.0019%). However, the omission of *n*-valerate resulted in abnormal growth as shown in Fig. 1a. Figures 7a and b show the appearance of the cells at 24 hr in a medium not supplemented with *n*-valerate. The cells were much longer and sometimes showed a ghostlike appearance. Although some normal cells were observed in these micrographs, almost all cells at the initial maximal growth at 6 hr showed elongated appearance. As shown in Fig. 6, cells grown in the lactate medium appeared to be smaller than those grown in the glucose medium. Thus far, no abnormal morphology has been found in lactate media.

Specificity of required fatty acids for growth. To know the specificity for the *n*-valerate requirement, various fatty acids were added individually and growth of the bacterium was recorded. It was found that any straight-chain saturated monocarboxylic acid with 3 to 10 carbons could support growth at certain concentrations in place of *n*-valerate in the glucose-yeast extract medium.

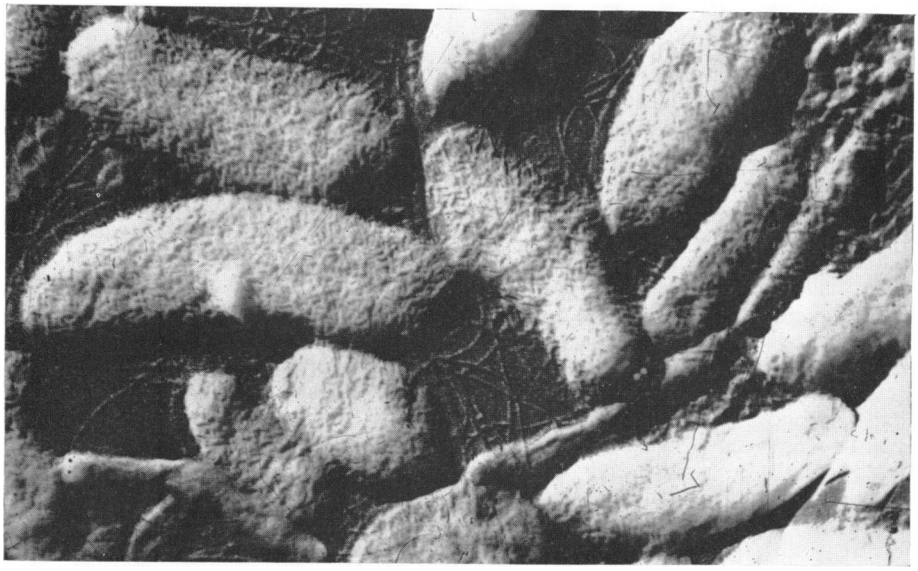

FIG. 5. *Electron micrograph of cells grown in the glucose-Trypticase-yeast extract medium supplemented with n-valerate (24-hr culture). × 12,500.*

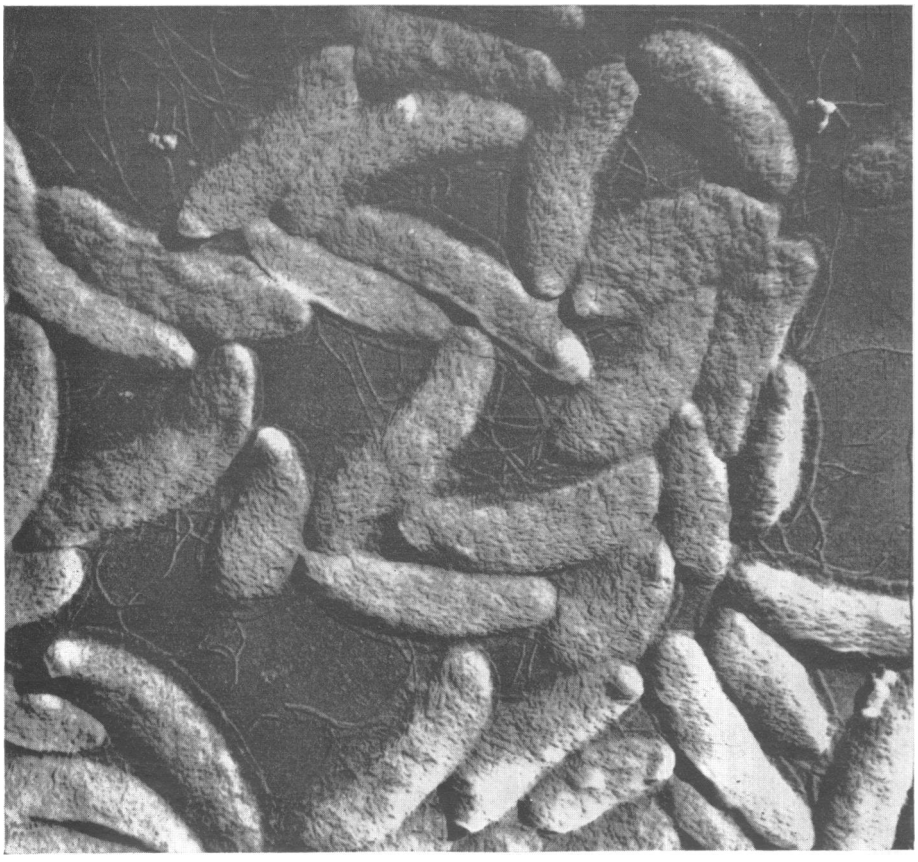

FIG. 6. *Cells grown in the lactate-Trypticase-yeast extract medium supplemented with biotin (24-hr culture).* × 12,500.

However, as can be seen in Fig. 8, *n*-valerate was most effective at the lowest concentration. Acetate was not effective at any concentration examined. Pyruvate, DL-lactate, DL-malate, fumarate, succinate, L-asparagine, L-glutamine, and putrescine, each at a concentration of 0.3%, and DL-mevalonic acid, at a concentration of 0.02%, were without effect. Pentadecanoic acid (C_{15}-saturated acid), which had been reported as a substitute for *n*-valerate in growth of *Bacteroides succinogenes* (8), was without effect for this bacterium at concentrations ranging from 0.0002 to 0.2%.

Incorporation of n-valerate-1-C^{14} into cell fractions. The distribution of radioactivity in cell fractions is shown in Table 1. Most of the assimilated C^{14} was located in protein and lipid fractions. The cold and hot acid-soluble fractions had little radioactivity. Approximately 80 to 90% of the radioactivity iu the protein fraction was extractable with chloroform-methanol dan was not digestible with pronase (Table 1). It is concluded, therefore, that most of the incorporated *n*-valerate existed as lipid materials within the cells.

Fermentation products from glucose and lactate. Amounts of 100 ml each of the glucose-Trypticase-yeast extract medium or the lactate-Trypticase-yeast extract medium supplemented with sufficient amounts of both *n*-valerate (0.0019%) and biotin (0.01 mg/100 ml) were inoculated and incubated. A portion of the culture (3 ml) was withdrawn at intervals with a syringe and was analyzed for fermentation products. The time course of the acid production is shown in Fig. 9. This organism produced lactate, propionate, and acetate from glucose and propionate and acetate from lactate.

DISCUSSION

The present data clearly demonstrated that the strain of *S. ruminantium* var. *lactilytica* repuired any of C_3 to C_{10} *n*-saturated fatty acids at certain concentrations when grown in a medium

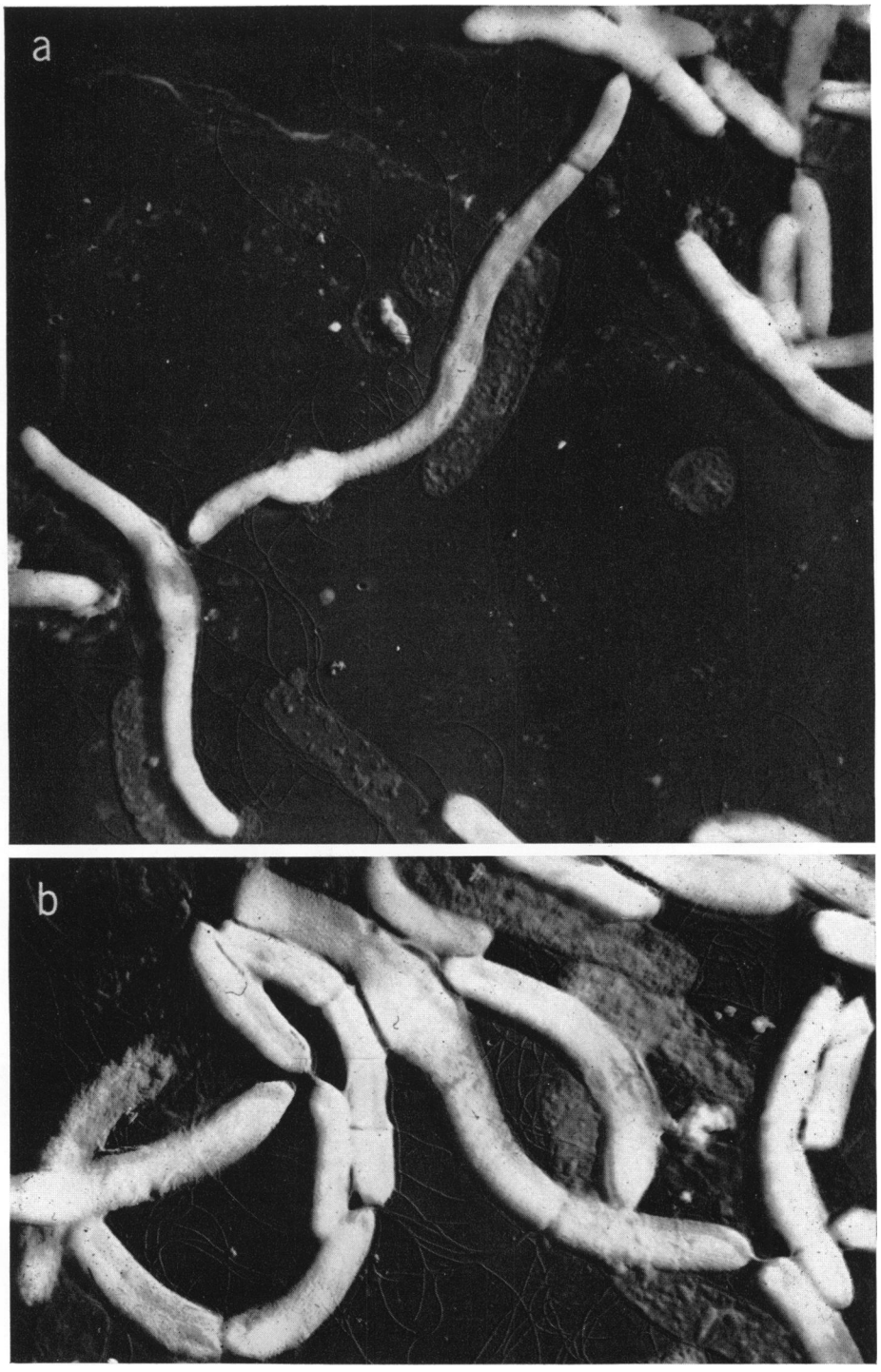

FIG. 7. *Cells grown in the glucose-Trypticase-yeast extract medium not supplemented with n-valerate (24-hr culture).* (a) × *5,000.* (b) × *7,500.*

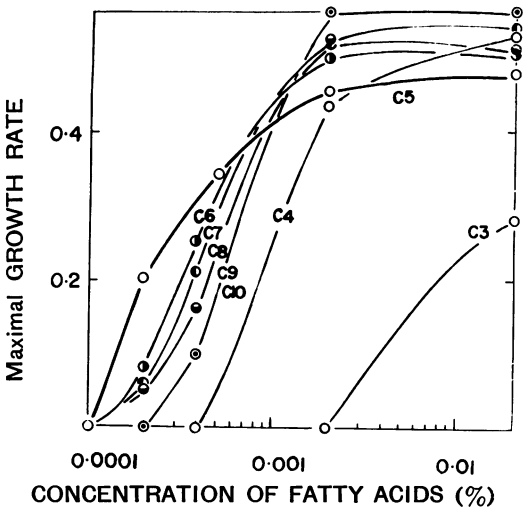

FIG. 8. *Growth of Selenomonas ruminantium in response to the concentration of n-saturated fatty acids. Various amounts of fatty acids as indicated were added to the glucose-yeast extract medium. The growth was followed at 3-hr intervals, and the maximal growth rates, i.e., the maximal increments in optical density per 3 hr, were plotted versus the concentrations of n-saturated fatty acids. No growth was observed in the medium supplemented with 0.2% propionate.*

containing glucose. Among them, *n*-valerate was most effective at the lowest concentration. Though *n*-valerate or *n*-caproate has been known as a growth factor of some strains of *B. succinogenes* and *Borrelia* sp. isolated from rumen contents, these organisms also required a branched-chain fatty acid in addition to *n*-valerate or *n*-caproate (8).

Bryant and Robinson (3) reported that several strains of *S. ruminantium* required variably a mixture of volatile fatty acids which contained *n*-valerate, although they did not identify which acid was essential for growth. According to Hobson et al. (4), a strain of *S. ruminantium* did not require any of volatile fatty acids except acetate. The discrepancy might be explained by the following observation. The organism grown in the glucose-Trypticase-yeast extract medium in the absence of added *n*-valerate, which produced the abnormal growth curve shown in Fig. 1, was transferred to the new glucose-Trypticase-yeast extract medium. After repeated transfers, the requirement for fatty acids was examined, and it was found that the organism thus selected no longer required added *n*-valerate. No such selection was observed when the organism was stored in the lactate-Trypticase-yeast extract medium supplemented with rumen fluid.

Of some interest is the finding that this organism produces propionic acid from lactate or glucose, and this acid is one of the effective acids in supporting growth in the glucose medium. *n*-Valerate, however, was essential for growth in the glucose medium but was stimulatory in the lactate medium. This might be due to the vigorous formation of propionate in the lactate medium, as was shown in Fig. 9.

A number of bacteria isolated from rumen contents require B vitamins, especially biotin and PABA (7). The strain studied here also showed the requirement for biotin and PABA. However, biotin was essential when the organism was cultured in the lactate medium, and was stimulatory when the glucose medium was used. PABA, on the contrary, was essential in the glucose medium, but was not effective in the lactate medium. The nutritional requirements of this organism in relation to the available energy sources are summarized in Table 2.

It has been reported by many workers that most of the ruminal bacteria require CO_2 for

TABLE 1. *Distribution of radioactivity in cell fractions of Selenomonas ruminantium grown in medium containing n-valerate-1-C^{14}*

Cell fractions	Total radioactivity		
	Counts per min	Per cent	
Whole cells	562,000	100	
Cold trichloroacetic acid-soluble	31,000	5.5	
Ethyl alcohol-ether soluble	310,000	55.1	
Hot trichloroacetic acid-soluble	1,000	0.0	
Residual (protein fraction)	200,000	35.6	100
Chloroform-methanol-soluble	155,000	27.6	77.5
Chloroform-methanol-insoluble	33,000	5.9	16.5
Solubilized by pronase	17,000	3.0	8.5
Not solubilized	183,000	32.6	91.5

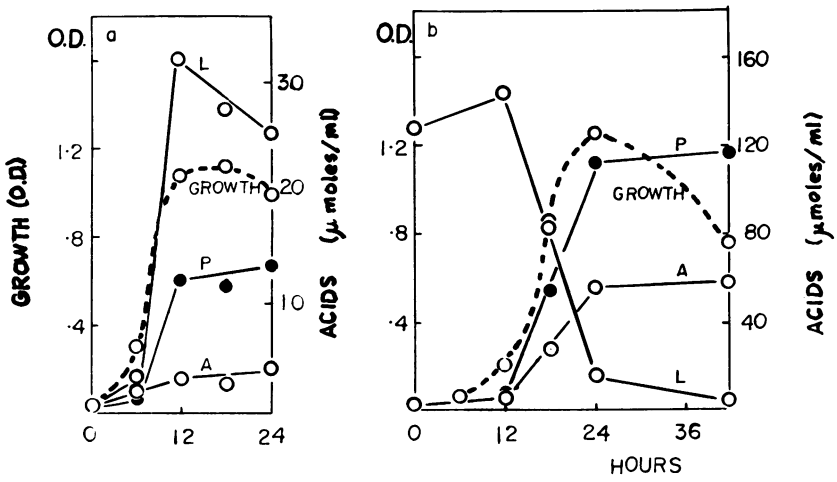

FIG. 9. *Growth and fermentation products of Selenomonas ruminantium in glucose (a) and in lactate (b) media. The following symbols were used: formation or utilization of lactate (L), formation of propionate (P), and acetate (A).*

TABLE 2. *Nutritional requirements of Selenomonas ruminantium as related to energy source*

Energy source	Essential requirement(s) for growth	Stimulatory for growth
Glucose	*n*-Valerate and PABA	Biotin
Lactate	Biotin	*n*-Valerate
Glycerol	Biotin	*n*-Valerate

growth (6). The *S. ruminantium* strain used in this study also required CO_2 for growth, both in glucose and in lactate media. In view of the reported mechanism of propionate formation in *S. ruminantium* (M. J. B. Paynter, M.S. Thesis, Sheffield Univ., Sheffield, England), which involves succinate as an intermediate, the fixation of CO_2 might play an important role in the metabolism of lactate or glycerol, and thus a larger amount of biotin might be needed under these conditions. Why PABA is essential in the glucose medium and not in the lactate medium is unresolved.

The isotopic studies presented here demonstrated that *n*-valerate was incorporated exclusively into lipid materials. Wegner and Foster (8) found that *n*-valerate was used mainly for the synthesis of n-C_{13} and C_{15} fatty acids and aldehydes, and they suggested that odd-numbered precursors yielded products with odd-numbered fatty acids, and that those with even numbers gave rise to even-numbered products. The synthesis of longer fatty acids was supposed to take place with precursors as starting blocks and subsequent additions of two carbon units to the carboxyl end of the molecules, as was described by Horning et al. (5). The analysis of labeled lipid materials with *n*-valerate is in progress.

ACKNOWLEDGMENTS

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