Ruminal Fermentation and Sugar Concentrations

A Model Experiment with *Selenomonas ruminantium*

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The maximal amounts of growth of *Selenomonas ruminantium* were examined in the media containing various amounts of glucose. The yields of cells per unit weight of glucose are linear functions to glucose concentrations in the ranges between zero to 0.005% and 0.005 to 0.7%. Cell yields per glucose are greater in the former range, indicating greater amounts of energy are available per glucose at lower concentrations. Growth responses in lactate media containing various amounts of glucose showed that the preincubation with larger amounts of glucose is inhibitory for the following growth and metabolism of lactate. The organism produces predominantly lactate in the glucose medium. However, volatile fatty acid productions increase when the initial concentrations of glucose become low. Isotopic studies showed that the lactate utilization yielding volatile fatty acids is inhibited by the preceding metabolism of high concentrations of glucose. These results were discussed with regard to normal and abnormal fermentations in the rumen.

Lactate has not been considered as an important intermediate in the ruminal fermentation and its concentration is usually kept low in the rumen¹⁾ However, lactate accumulates in the rumen when readily fermentable carbohydrates are fed in large amounts as rations²⁾. Since lactate is an unfavorable fermentation product, this is sometimes lethal to the animal³⁾. The increase of lactate is a very rapid process; it occurs within 30 min after feeding without significant bacterial multiplication⁴⁾. Thus, it is apparent that the normal fermentation, which yields volatile fatty acids, is shifted to the abnormal lactate fermentation under high concentrations of easily fermentable sugars. The shift occurs

in the absence of a significant change in bacterial flora, in which a number of lactate utilizers are present⁵⁾. Moreover, it has been known that many isolated rumen microorganisms produce a large amount of lactate in glucose media⁵⁾.

In order to understand the nature of normal and abnormal fermentations, a model experiment with an isolated sheep rumen bacterium, *Selenomonas ruminantium*, has been performed. The organism is selected because of its capacity to utilize either glucose or lactate as an energy source. The present paper deals with the effect of glucose concentrations on growth and fermentation products of this strain.

MATERIALS AND METHODS

Organism. *Selenomonas ruminantium* var. *lactilytica* strain B, isolated from the sheep rumen, is used in this study. This strain is called as such to distinguish

1) G. C. N. Jayasuriya and R. E. Hungate, *Arch. Biochem. Biophys.*, **82**, 274 (1959).

2) S. R. Elsdon, *J. Exptl. Biol.*, **22**, 51 (1945).

3) E. F. Annison and D. Lewis, "Metabolism in the Rumen", Methuen and Co., London, 1959, p. 167.

4) K. Nakamura and H. Takahashi, Unpublished observation.

5) R. E. Hungate, "The Rumen and Its Microbes", Academic Press, New York, 1966.

it from the strain S which was used in the previous publication⁶). The strain B shows the same nutritional requirements as the strain S but produces more volatile fatty acids from glucose than the strain S.

Media. Unless otherwise stated, cells were grown in a yeast extract-glucose medium or a yeast extract-lactate medium, both supplemented with *n*-valerate and biotin. The details of the compositions of these media have been described previously⁶).

Culture. The organism was cultured anaerobically under carbon dioxide atmosphere according to the procedure of Hungate⁷) with a slight modification. Growth of the organism was followed by measuring optical density with a Hitachi FPW 4 colorimeter equipped with a 66 filter (660 m μ). In some experiments, cell concentrations in dry weight basis were calculated from the data of optical density measurements with the aid of a standard curve.

Analysis of fermentation products and glucose consumption. A portion of the culture medium, which contained ¹⁴C-glucose or ¹⁴C-lactate as a carbon source, was withdrawn with a syringe and centrifuged at 4°C to remove cells. The supernatant solution was assayed for fermentation products and glucose consumption.

A definite amount of the supernatant was added authentic carrier organic acids (acetic, propionic and lactic acids, 25 μ moles, each as sodium salt) and then chromatographed by a silicic acid column according to the method of Belasco⁸). The eluates were titrated by N/200 potassium hydroxide in methanol. The fractions corresponding to each acid were collected and counted for radioactivity.

¹⁴C-Glucose remaining in the supernatant was separated by one dimensional paper chromatography using pyridine-*n*-butanol-water (3 : 2 : 1.5) solvent. The spot corresponding to glucose was located by autoradiography and was eluted with water. The radioactivity in the eluate was assayed. Radioactivity measurements were performed with a gas-flow windowless G-M counter (Irigaku Kenkyujo, Tokyo).

Unlabeled lactate was estimated by the method of Barker⁹). Unlabeled glucose was determined by the

method of Somogyi¹⁰). Before the assay, samples were deionized according to the method of Otaka and Yamauchi¹¹).

Chemicals. D-Glucose-U-¹⁴C was purchased from the Daiichi Seiyaku Co. Ltd. (Tokyo). DL-Lactate-U-¹⁴C was the fermentation product from sucrose-U-¹⁴C by *Lactobacillus plantarum*. Labeled lactate was purified by paper chromatography. The final product had the specific activity of 4000 cpm/m μ mole. Sucrose-U-¹⁴C was prepared from Canna leaves which assimilated ¹⁴CO₂.¹²) Other chemicals were from commercial sources.

RESULTS

Growth of the bacterium with glucose or lactate as energy source

The maximal amounts of cell growth obtained with media containing various amounts of glucose were plotted against glucose concentrations (Fig. 1a). The linear relationship exists between the amounts of growth and glucose concentrations up to 0.7% (37.8 μ moles/ml). When this relationship is examined more closely at lower concentrations of glucose, however, the amounts of the maximal growth no longer parallel to glucose concentrations

FIG. 1. Cell Yields per Unit Weight of Glucose.

Growth of each culture containing various amounts of glucose was followed for 24 hr. The maximal amounts of cell yields in each culture were plotted against initial concentrations of glucose added to the media.

6) S. Kanegasaki and H. Takahashi, *J. Bacteriol.*, **93**, 456 (1967).

7) R. E. Hungate, *Bact. Rev.*, **14**, 1 (1950).

8) I. J. Belasco, *J. Animal Sci.*, **13**, 748 (1954).

9) S. B. Barker, "Methods in Enzymology", Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1957, p. 241.

10) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).

11) Y. Otaka and A. Yamauchi, *Nippon Nogei-kagaku Kaishi*, **29**, 877 (1955).

12) M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert and P. E. Yankwich, "Isotopic Carbon", John Wiley and Sons, New York, 1949, p. 263.

as shown in Fig. 1b. Significantly larger amounts of the maximal growth per unit glucose were observed in the media containing glucose of 0.005% (2.7 μ moles/ml) or less.

Relationship between fermentation products and glucose concentrations

If one assume that glucose is metabolized via a strictly defined pathway to yield the same fermentation products irrespective of glucose concentrations, *i. e.*, the same amount of energy is available per unit glucose, the amounts of the maximal growth should then be proportional to glucose concentrations within the growth limiting range. The larger amounts of the maximal growth per unit glucose, therefore, imply a different metabolic pathway of glucose leading to greater yields of available energy and to different fermentation products as compared to the second linear portion of the response observed at higher glucose concentrations. In order to test this possibility, the fermentation products in the culture media, which contained three different levels of glucose, were examined. As shown

TABLE I. FERMENTATION PRODUCTS FROM 14 C-GLUCOSE

Glucose concentrations (%)	Propionate		Acetate formed		Lactate	
	cpm	molar %	cpm	molar %	cpm	molar %
0.5	52300	5.2	71200	10.5	854600	84.3
0.05	127200	12.1	122400	17.4	741600	70.5
0.005	256100	28.1	206500	34.0	344800	37.9

Three media, which contained three different levels of 14 C-glucose but approximately the same amounts of radioactivity, were inoculated and incubated for 10 hr. Culture fluids were analyzed for organic acids. The radioactivity in each acid was divided with its respective number of carbons, thus the value proportional to the amount of each acid in molar basis was obtained. The fraction of each acid in total acids formed on molar basis was calculated and was expressed as the molar percentage.

in Table I, the organism produced less lactic acid and more volatile fatty acids in the media which contained smaller amounts of glucose.

Utilization of lactate in the presence of glucose

One possible explanation of the above result might be the inhibitory effect of glucose on lactate metabolism which yield acetic and propionic acids. Growth of the organism in the lactate media supplemented with various amounts of glucose is shown in Fig. 2. When

FIG. 2. Growth of *S. ruminantium* in Lactate Media with Various Concentrations of Glucose.

Lactate media containing

●—●: 0.5% of glucose □—□: 0.1% glucose
 △—△: 0.005% glucose ○—○: no addition

the small amount (0.005%) of glucose was supplemented, a shorter lag period and a somewhat higher growth rate were observed compared to the growth in the plain lactate medium. Although the data have not been shown, very similar stimulatory effects were observed with glucose concentrations at 0.01 and 0.001%. On the contrary, when relatively large amount of glucose (0.1%) was added to the lactate medium, a typical diauxic growth curve was obtained. When the largest amount of glucose (0.5%) was included in the lactate medium, no diauxic growth was seen within the experimental period.

The results of similar experiments, in which the consumption of glucose and utilization of

TABLE II. CONSUMPTION OF LACTATE BY *S. ruminantium* IN LACTATE MEDIA CONTAINING VARIOUS AMOUNTS OF GLUCOSE

Time (hrs)	Glucose concentrations in lactate media (%)					
	0.5		0.1		0.005	
	Glucose	Lactate	Glucose	Lactate	Glucose	Lactate
	(mg/ml)					
0	4.61	18.0	0.95	19.0	0.03	18.8
6	3.87	19.8	0.04	19.4	0	18.9
9	0.01	19.4	0.02	18.0	—	17.7
16	0	17.7	0	13.0	—	10.8
24	—	—	—	2.7	—	4.1

The organism was cultured in lactate media (1.25%, as sodium lactate) containing 0.5, 0.1 or 0.005% of glucose. A portion of the culture was withdrawn at time intervals, and fluids were analyzed for glucose and lactate.

lactate were analyzed, are shown in Table II. When the smallest amount of glucose (0.005%) was added to the lactate medium, glucose was metabolized rapidly and the vigorous utilization of lactate followed. The medium amount of glucose addition (0.1%) resulted in the diauxic growth. In this case, glucose was almost completely metabolized within the first 6 hr and negligible utilization of lactate occurred until 9 hr. It is apparent that the organism consumes glucose first and after the lag period, lactate is metabolized. The lag period observed in the diauxic growth is attributable to the time which is needed for the transition of the energy source from glucose to lactate. When 0.5% of glucose was added to the lactate medium, glucose was metabolized rapidly as before, but the lactate utilization was inhibited to a remarkable extent. No vigorous metabolism of lactate occurred within the experimental period. The results suggest that glucose, when added in small amounts, stimulates the metabolism of lactate, but the preceding fermentation of excess glucose inhibits the utilization of lactate. This inhibitory effect is more pronounced when the higher concentrations of glucose are initially supplied to the organism.

The acid production from uniformly labeled ^{14}C -lactate was studied in the presence of two levels of glucose (0.5 and 0.005%). Lactate

was added to the media just before the onset of growth (3.5 hr). At late exponential growth phase (7 and 8 hr after the inoculation for 0.005 and 0.5% glucose media, respectively), culture fluids were analyzed for the distribution of radioactivity in the organic acids produced. Table III gives the counting data of

TABLE III. FATE OF ^{14}C -LACTATE ADDED IN GLUCOSE MEDIA

Glucose concentrations (%)	Propionate		Acetate		Lactate	
	cpm	molar %	cpm	molar %	cpm	molar %
0.5	6300	7.9	3900	7.4	67400	84.7
0.005	10600	17.6	10300	25.6	34200	56.8

The organism was cultured in the medium (10 ml) containing 0.5 or 0.005% of glucose. ^{14}C -Lactate (0.18 μmole) was added at 3.5 hr after the inoculation. After the culture, fluids were analyzed for radioactive organic acids. The molar percentages were calculated as described in the legend for Table I.

residual lactic acid and propionic and acetic acids formed. The data clearly indicate that lactate is metabolized in a much smaller extent in the medium containing 0.5% of glucose as compared to that in the 0.005% glucose medium.

Time course of acid production from 0.005% glucose

Figure 3 shows the time course of bacterial

FIG. 3. Growth and Consumption of Glucose by *S. ruminantium* in Medium Containing 0.005% Glucose.

○—○: Growth ●—●: Glucose

FIG. 4. Time Course of Volatile Fatty Acid Productions at the Later Period of Glucose Consumption.

growth as well as the glucose consumption in the medium containing 0.005% of ^{14}C -glucose. The amounts of acids formed at 5, 6 and 7 hr after inoculation were assayed (Fig. 4). As shown, lactic acid is predominantly formed until 5 hr when the concentration of glucose decreased to approximately 0.002%. However, vigorous production of volatile fatty acids

occurs in the following 1 hr without appreciable accumulation or metabolism of lactate. After 6 hr, when glucose was completely metabolized, volatile fatty acids were produced at the expense of lactate.

DISCUSSION

The data presented here demonstrate that the fermentation products are affected by initial concentrations of glucose added to the culture media. Low concentrations give relatively lower level of lactate and higher levels of volatile fatty acids. The excessive pre-fermentation of glucose appears to inhibit the subsequent metabolism of lactate as shown in Tables II and III. Although the present experiment is confined to *S. ruminantium*, this phenomenon appears to be responsible for the abnormal fermentation in the rumen which occurs by the excess feeding of carbohydrates to the animal³⁾. As shown in Table II, when excess glucose is supplied to *S. ruminantium*, the subsequent utilization of lactate is almost completely inhibited for 4 to 7 hr depending upon the concentrations of glucose initially supplied. However, the low level of glucose added to the lactate medium stimulated growth considerably (Fig. 2) and under such conditions lactate utilization was vigorous (Table II). Although no definite datum is available at the present time whether the production of volatile fatty acids in *S. ruminantium* proceeds with lactate as an obligate intermediate or the fermentation is shifted to the formation of volatile fatty acids from the lactate production under extremely low level of glucose, it may be concluded that the normal fermentation in the rumen, in which volatile fatty acids are the major products, proceeds under very low level of glucose. In accordance with the view, Hobson^{13,14)} showed that the production of volatile fatty acids by ruminal bacteria increases in a continuous culture when substrate concentrations are kept

13) P.N. Hobson, *J. Gen. Microbiol.*, **38**, 167 (1965).

14) P.N. Hobson, *ibid.*, **47**, 53 (1967).

low.

It has been reported from this laboratory that the level of easily fermentable sugars in the sheep rumen is extremely low as judged by the intracellular polysaccharide accumulation by whole rumen bacteria when the cells are incubated in a glucose medium¹⁵⁾ The

capacity of polysaccharide synthesis by these bacteria appears to contribute significantly for the maintenance of low level of easily fermentable sugars in the rumen.

15) R. Takahashi and H. Takahashi, *Agr. Biol. Chem.*, **31**, 1453 (1967).

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