EFFECT OF SUCCINATE ON CELLULOSE DECOMPOSITION BY THE RUMINAL BACTERIA

III. STIMULATIVE EFFECT OF SUCCINATE ON CELLULOSE DECOMPOSITION IN REPRESENTATIVE STRAINS AND BACTERIAL FRACTIONS OF THE RUMEN

MASAKO HIGUCHI and HAJIME TAKAHASHI

*Department of Agricultural Chemistry, Faculty of Agriculture,
Tohoku University, Sendai*

Received October 18, 1965

In a previous paper of this series, HIGUCHI and UEMURA (1) showed that succinate stimulated cellulase formation in growing and resting cells of *Cellulomonas fimi* strain R2 isolated from sheep rumen fluid fed on hay and concentrates. This bacterium can grow either aerobically or anaerobically, if supplemented with proper nutrients, and is found at the order of 10^8 cells per g of rumen contents (2). Although no definite information is available at the present time which shows the role of this bacterium in cellulose decomposition in the rumen, it is generally believed that the digestion of cellulose is accomplished by a group of strict anaerobes such as *Ruminococcus flavefaciens*, *R. albus*, and *Bacteroides succinogenes* (3, 4).

The studies by SHIBATA and his coworkers (5), who first showed that the cellulolytic activities of rumen contents were significantly stimulated by the addition of succinate, led the authors to examine more precisely this effect in some representative cellulolytic anaerobic bacteria in the rumen. The results, described below, demonstrated that the stimulation of cellulase formation by succinate was a general phenomenon in the rumen bacteria.

MATERIALS AND METHODS

Pure cultures. The following three strains were selected as representative cellulolytic bacteria: *Ruminococcus flavefaciens* strain C-94, *R. albus* strain 7, and *Bacteroides succinogenes* strain S-85. All three strains ferment cellobiose and glucose except *R. flavefaciens* which does not ferment glucose. Succinic acid is produced from cellobiose in large quantities with *B. succinogenes*, and in a detectable amount with *R. flavefaciens*. No significant formation of succinate from cellobiose is observed with *R. albus*.

Culture conditions and analytical methods. Pure cultures were inoculated to 10 ml of TYR medium (6) containing 0.2% of cellobiose, grown approximately for 20 hr, and then transferred to 300 ml of TYR medium containing 0.2% cellulose powder (Toyo Roshi Co., Ltd. Tokyo). Incubation was continued for an additional 72 hr.

Utilization of cellulose in the culture medium was estimated by sub-

tracting the remaining cellulose from the initial amounts. The remaining cellulose was obtained by centrifugation, washed with 0.1% Tween 80 and then by water to avoid bacterial contamination, air-dried, and weighed. Reducing sugars accumulated in the cultures were separated by paper chromatography (7). Each spot was eluted with water and assayed by the method of SOMOGYI (8). Cellulase assays of the cells were done with sonic homogenates. Enzyme activity assays and the definition of the enzyme unit were as described previously (1). Protein was estimated by the FOLIN method (9).

Glucose grown cells were obtained by culturing the cells in TYR medium supplemented with 0.5% of glucose for 72 hr. Cells from 600 ml cultures were harvested and washed three times with salt solution containing $2.08 \times 10^{-3}M$ of cysteine and resuspended in 20 ml of the salt solution. Cell suspensions were incubated for 6 hr with either glucose ($5 \times 10^{-2}M$) or succinate at concentrations of 5×10^{-2} , 5×10^{-3} , or $5 \times 10^{-4}M$. Cellulase activity of the cells were assayed as mentioned above.

Bacterial fraction from the rumen was prepared as follows. Sheep rumen contents obtained before the morning diet was filtered through two sheets of gauze and freed from protozoa by centrifugation at $27 \times g$ for 5 min. The supernatant was recentrifuged at $10,000 \times g$ for 15 min. The precipitate, the bacterial fraction, was washed three times with salt solution containing cysteine ($2.08 \times 10^{-3}M$) and suspended in the same solution of the original volume. The suspension was diluted with TYR medium in dilutions ranging from 10^1 to 10^7 . Each suspension (10 ml) was placed in a test tube which contained a paper section (1.0×8.0 cm, Toyo Roshi, No. 2) with or without succinate ($5 \times 10^{-3}M$) and cultured for 14 days. The cellulase activity of the ruminal bacterial fraction was expressed with the reciprocal of the days needed to destroy the filter paper in the medium.

All incubations were at 37° under anaerobic conditions.

RESULTS

Effect of succinate on the cellulose decomposition by pure cultures of Ruminococcus

Growth, consumption of cellulose and accumulation of reducing sugars by *R. albus* and *R. flavefaciens* in cellulose-TYR medium with and without succinate are shown in Table 1. A stimulatory effect of succinate on the growth and cellulose decomposition was observed in both strains. In *R. albus*, stimulation was more remarkable at higher concentrations of succinate. In *R. flavefaciens*, stimulation by succinate was more pronounced at $5 \times 10^{-4}M$ than at $5 \times 10^{-3}M$.

Table 2 summarizes the cellulase activities of both strains. Cellulase activity was also stimulated by succinate approximately 2 to 3 times over the control both in specific and in total activities. The fact that the specific activities of cellulase in *R. albus* at both concentrations of succinate were almost identical while there was a marked difference in total activities

Table 1. Effect of succinate on the growth and the cellulose decomposition.

Succinate added (μ moles/ml)	Growth (mg of dry cells/ml)	Consumption of cellulose powder		Accumulation of reducing sugars	
		(mg/tube)	(%)	Glucose (mg/tube)	Cellobiose (mg/tube)
<i>R. albus</i>					
0	1.0	71.9	12.0	4.18	4.01
0.5	1.1	80.6	13.4	11.95	6.34
5.0	1.7	139.2	23.2	13.75	10.07
<i>R. flavefaciens</i>					
0	1.7	80.9	13.5	4.21	3.75
0.5	2.4	106.8	17.8	7.22	6.00
5.0	2.3	100.4	16.7	4.80	4.50

Table 2. Cellulase activity of the cells of *R. albus* and *R. flavefaciens*.

Succinate added (μ moles/ml)	Cellular protein (mg/tube)	Cellulase activity	
		Specific activity (units/mg protein)	Total activity (units/tube)
<i>R. albus</i>			
0	8.7	1500	13000
0.5	9.6	2900	27500
5.0	14.8	3200	47000
<i>R. flavefaciens</i>			
0	14.4	1000	15000
0.5	20.4	2100	43000
5.0	19.5	1800	35000

might be explained by a growth promoting effect of succinate at higher concentrations. The specific and total activities of cellulase in *R. flavefaciens* were greatest at lower concentrations of succinate. This might be explained by an inhibitory effect of this acid at higher concentrations.

Cellulase formation by glucose grown cells

R. albus and *B. succinogenes* grown in glucose-TYR medium showed no detectable cellulase activity. However, as shown in Figs. 1 and 2, when these cells were incubated in the presence of succinate, cellulase activity appeared. Small but detectable activity was also observed in the system where neither glucose nor succinate was added. In the presence of glucose, no significant activity was detected. Succinate was most effective at concentrations of 5×10^{-3} M in *R. albus*, but in *B. succinogenes*, the highest stimulation was at 5×10^{-4} M. The highest concentration of succinate ($5 \times$

Fig. 1. Cellulase formation of glucose grown cells of *R. albus*.

Glucose grown cells were incubated anaerobically in mineral medium containing glucose or succinate. The cellulase activity of the cells was assayed as described in the text. The activities were plotted versus incubation time. (○); No glucose or succinate. (●); $5 \times 10^{-2}M$ glucose. (△); $5 \times 10^{-2}M$ succinate. (▲); $5 \times 10^{-3}M$ succinate. (□); $5 \times 10^{-4}M$ succinate.

Fig. 2. Cellulase formation of glucose grown cells of *B. succinogenes*. Experimental conditions were the same as described in Fig. 1.

10^{-2}M) was still stimulatory for *R. albus* but was inhibitory for *B. succinogenes*.

The effect of succinate on cellulose decomposition by ruminal bacterial fraction

The stimulative effect of succinate on the decomposition of cellulose by ruminal bacterial fraction is shown in Fig. 3. An effect was observed

Fig. 3. Effect of succinate on the cellulose decomposition by rumen bacterial fraction.

Bacterial suspension, approximately 10^8 – 10^9 cellulolytic viable cells per ml as measured with roll tube method, was diluted from 10^1 to 10^7 with TYR medium. The suspension was incubated in tube containing a paper section with or without succinate ($5 \times 10^{-3}\text{M}$) until the paper was destroyed. The cellulase activity was expressed with the reciprocal of the days needed to destroy the filter paper.

at every dilution from 10^1 to 10^7 . Generally, the duration necessary to destroy the paper was shortened about 4 days in the presence of succinate.

DISCUSSION

Experiments with pure cultures of rumen bacteria showed a stimulative effect of succinate on the cellulose decomposition. A similar observation was previously reported with *Cellulomonas* (1, 10). Since the cultures used

were believed representative strains of ruminal cellulolytic bacteria and because the effect was also observed with the whole bacterial fraction from the rumen, it was concluded that this effect is a general phenomenon in rumen bacteria. Whether or not this is true for other cellulolytic microorganisms remains to be elucidated.

The concentrations needed for the maximum stimulation of cellulase formation by these bacteria appeared to have some relationship with the capacity of succinate formation from cellulose or cellobiose. *B. succinogenes*, which produced large amounts of succinate, was stimulated by the lowest concentration of this acid. The highest concentration was required for *R. albus* which did not produce succinate in detectable quantities. The weak succinate former, *R. flavefaciens*, was stimulated by intermediate concentrations of succinate. The range of succinate concentrations necessary to stimulate cellulase formation by these bacteria was from 5×10^{-3} to 5×10^{-4} M. This value coincided well with the reported level of succinate in sheep rumen fed on hay and concentrates (11). Therefore, the environment of the rumen is evidently favorable for cellulose digestion in regard to succinate concentrations.

Glucose grown cells of *R. albus* and *B. succinogenes* had no detectable cellulase activity. However, the enzyme was formed upon addition of succinate and thus the inducer-like effect of succinate reconfirmed. The fact that the cellulase was formed by incubation of glucose grown cells in carbon free medium indicated that the enzyme was constitutive rather than inducible. The nature of this succinate effect is under investigation.

SUMMARY

The stimulative effect of succinate on cellulose decomposition and cellulase formation was studied with *Ruminococcus flavefaciens* and *Ruminococcus albus*. The decomposition of cellulose by ruminal bacterial fraction was also stimulated by succinate. Glucose grown cells of *R. albus* and *Bacteroides succinogenes* which had no detectable cellulase activity formed the enzyme when the cells were incubated with succinate. These results suggest that the succinate effect is a general phenomenon in rumen bacteria.

The authors are grateful to Dr. M. P. Bryant of the United States Department of Agriculture for supplying the pure cultures of rumen bacteria used in this study. They are also indebted to Prof. T. Uemura of the Institute of Applied Microbiology, University of Tokyo, for his continuing interest and encouragement. This investigation was supported in part by a grant from the Ministry of Agriculture and Forestry.

REFERENCES

- (1) M. HIGUCHI and T. UEMURA: *J. Gen. Appl. Microbiol.*, **11**, 137, 145 (1965).
- (2) A. ENDO, T. OOKA and T. UEMURA: *Jap. J. Zootech. Sci.*, In Preparation.
- (3) M. P. BRYANT: *Bacteriol. Rev.*, **23**, 125 (1959).
- (4) E. T. HUNGATE: *Bacteriol. Rev.*, **14**, 1 (1950).
- (5) A. SHIBATA, K. OGIMOTO and C. FURUSAKA: cited from T. UEMURA, *Methods in Microbiology* (in Japanese), p. 160, Kyoritsu Shuppan Co., Tokyo, (1957).

- (6) M. HIGUCHI and T. UEMURA: *J. Agr. Chem. Soc. Japan*, **39**, 95 (1965).
- (7) I. SMITH: *Chromatographic Techniques*, p. 164, William Heinemann Medical Books, Ltd., London, (1958).
- (8) M. SOMOGYI: *J. Biol. Chem.*, **195**, 19 (1952).
- (9) O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL: *J. Biol. Chem.*, **193**, 265 (1951).
- (10) M. HIGUCHI, T. UEMURA and C. FURUSAKA: *J. Agr. Chem. Soc. Japan*, **36**, 455 (1962).
- (11) A. K. SIJPESTEIJN and S. R. ELSDEN: *Biochem. J.* **52**, 41 (1952).