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Effect of Titanium(III) Citrate as Reducing Agent on Growth of Rumen Bacteria

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We compared the growth of 10 strains of rumen bacteria in an anaerobic medium reduced with cysteine hydrochloride, dithiothreitol, or titanium(III) citrate. The redox potential of medium reduced with cysteine hydrochloride was −167.8 mV; with dithiothreitol it was −175.8 mV; and with titanium(III) citrate it was −302.4 mV at a concentration of 5×10^{-4} M titanium and −403.9 mV at 2×10^{-3} M titanium. Maximum growth of the strains was generally lower with dithiothreitol or titanium(III) citrate than with cysteine hydrochloride, although growth was greater than in medium lacking an added reducing agent. Strains for which cysteine was required or markedly stimulatory grew only poorly with titanium(III) citrate. No strain grew in medium with sodium citrate as the energy source. Titanium(III) citrate could be used to reduce anaerobic media for some rumen bacteria if the exclusion of a sulfur-containing reducing agent is required.

Most functionally important rumen bacteria are obligately anaerobic and cannot initiate growth in culture unless the medium is poised at a sufficiently low redox potential (E_h) (2). Exclusion of oxygen from the medium, although essential to avoid oxygen toxicity (13), cannot alone achieve such low potentials (10). Therefore, a reducing agent is generally incorporated into the medium, and an appropriate redox indicator is added to register attainment of the desired E_h. Many reducing agents have been used to poise the E_h in media for obligate anaerobes, but cysteine hydrochloride, either alone or in combination with sodium sulfide (4) or hydrogen sulfide (10), has been used most extensively with rumen anaerobes. Exposure of cysteine-containing anaerobic media to atmospheric oxygen, however, may result in a bactericidal effect attributable to hydrogen peroxide formation (6). Sodium thioglycolate (16), ascorbic acid (7), sodium dithionite (17), glutathione (2), and dithiothreitol (8), have also been used. These reducing agents vary in the rate at which they react with oxygen, in toxicity, and in the contribution that they may make to bacterial nutrition. Dithiothreitol at low concentrations reacts rapidly with oxygen and has been used in combination with cysteine hydrochloride and sodium sulfide in media for the isolation of bovine rumen anaerobes (12), but its effect on the growth of pure cultures of rumen bacteria has not been studied extensively.

Recently, there has been renewed interest in reducing agents for the cultivation of obligate anaerobes. Brock and O'Dea (1) proposed the use of amorphous ferrous sulfide for enrichment of bacteria from habitats where organic reducing agents are undesirable or where soluble sulfide might be toxic. Zehnder and Wurhmann (20) tested titanium(III) citrate and showed that it was nontoxic to *Methanobacterium* strain AZ at concentrations below 0.6×10^{-3} M and to *Clostridium formoaceticum* and *Bifidobacterium bifidum* at concentrations below 2×10^{-3} M. However, titanium(III) citrate prevented anaerobic growth by two facultatively anaerobic strains. Both ferrous sulfide and titanium(III) citrate reacted faster than cysteine hydrochloride with oxygen, but the applicability of ferrous sulfide seems limited by its insolubility; titanium(III) complexed with citrate remains soluble within the usual range of culture pH values (20).

Titanium(III) citrate might offer some advantages over sulfur-containing reducing agents for the cultivation of rumen bacteria (for example, for the determination of sulfur requirements). Therefore, we compared the growth of several strains of anaerobic rumen bacteria and of a facultatively anaerobic strain in semidefined media reduced with cysteine hydrochloride, dithiothreitol, and titanium(III) citrate.

MATERIALS AND METHODS

Bacteria. Ten strains of bacteria were used. *Bacteroides amylophilus* 70 and *Megasphaera elsdenii* B159 were obtained from C. W. Forsberg, University of Guelph, Guelph, Ontario, Canada, and *Bacteroides ruminicola* subsp. *brevis* GA33 (ATCC 19188) was obtained from the American Type Culture Collection, Rockville, Md. *Coprococcus* sp. Pe_i5 and the faculta-

tively anaerobic strain *Streptococcus bovis* Pe18 were isolated in this laboratory (19). *Bacteroides ruminicola* subsp. *ruminicola* 23, *Butyrivibrio fibrisolvens* D1, *Eubacterium ruminantium* GA195, *Ruminococcus albus* 7, and *Selenomonas ruminantium* GA192 were obtained from M. P. Bryant, University of Illinois. Stock cultures of the bacteria were maintained on carbohydrate agar maintenance medium (5) at −70°C. Working stocks were maintained in the same medium and transferred biweekly.

Anaerobic technique. The anaerobic technique used was that of Hungate (10), as modified by Bryant and Burkey (3). Anaerobic conditions were maintained by displacing all air in the media and dilution fluids with CO_2 or N_2 made oxygen-free by passage over heated copper. Resazurin (0.0001%) was added as an indicator of E_h.

Titanium(III) citrate. Titanium(III) citrate was prepared as described by Zehnder and Wuhrmann (20), using $TiCl_3$ supplied as a nominal 20% solution by Fisher Scientific Co. Ltd., Edmonton, Alberta, Canada. The actual concentration of titanium in this solution was determined by atomic absorption spectrophotometry. The preparation procedure was carried out under N_2, and the titanium(III) citrate solution (pH 7) was sterilized by autoclaving anaerobically.

Medium. The basal medium (BM) used was that of Scott and Dehority (15), modified by omission of cellobiose and cysteine hydrochloride and addition of 0.0001% hemin. Maltose (12.5 mM) was normally added to BM for the growth of *B. amylophilus* 70, cellobiose (12.5 mM) was used for *R. albus* 7, and glucose (25 mM) was added for all other strains. In some experiments sodium citrate (5 mM) replaced these carbohydrates. The medium was reduced with L-cysteine hydrochloride monohydrate (final concentration, 0.05%; equivalent to 2.8×10^{-3} M) which had been prepared under N_2, with DL-dithiothreitol (final concentration, 0.01%; equivalent to 6.5×10^{-4} M), or with titanium(III) citrate added to provide a final concentration of 5×10^{-4}, 1×10^{-3}, or 2×10^{-3} M titanium. L-Cysteine hydrochloride was obtained from Fisher Scientific Co. Ltd., and DL-dithiothreitol was from Sigma Chemical Co., St. Louis, Mo. The BM, energy sources, and reducing agents (except dithiothreitol) were sterilized as separate solutions at 121°C for 15 min and after cooling to 30°C combined aseptically in the appropriate proportions. Dithiothreitol was sterilized by membrane filtration (pore size, 0.22 μm; Millipore Ltd., Mississauga, Ontario, Canada) before addition to the medium. The complete medium was dispensed under CO_2 in 3-ml portions into tubes (13 by 100 mm) which were closed with butyl rubber bungs. The final pH of the medium was 6.6 to 6.7.

Cultures. To prepare inocula, the strains were grown through three serial transfers in BM containing the appropriate carbohydrates and 0.05% cysteine hydrochloride; incubation was at 39°C. Cells in the late logarithmic phase were harvested aseptically by centrifugation under CO_2 at 10,000 × *g* for 10 min at 15°C and washed once with sterile unsupplemented BM. The cells were resuspended in BM and diluted to an optical density at 660 nm of 0.10 ± 0.01, as measured with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.), and 0.1-ml portions of the suspensions were used to inoculate tubes of test medium in triplicate. Cultures were incubated at 39°C, and the optical density at 660 nm was measured at 1-h intervals. To define maximum optical density at 660 nm, measurements were continued for 3 to 4 h beyond the time at which values ceased to increase. Incubation of cultures showing negligible growth was continued for 96 h before measurements were stopped. Maximum optical density values were corrected for zero time readings, and the net optical density values thus obtained were in turn corrected for deviation from Beer's Law by conversion to adjusted optical density (AOD) (18) values, using tables kindly supplied by C. W. Forsberg.

E_h. The E_h values of the media reduced with cysteine hydrochloride, dithiothreitol, and titanium(III) citrate were measured with a Radiometer PHM 64 pH meter and a PKS 75042 platinum/calomel electrode (Bach-Simpson Ltd., London, Ontario, Canada). The electrode was conditioned (14) and standardized with a saturated solution of quinhydrone in 0.01 M phosphate buffer (pH 6.6). All measurements were made under CO_2 at 25°C. To provide more rapid stabilization of readings with highly reduced samples, 1 drop of 0.01% safranin was added to each tube of medium before the readings were taken (14).

RESULTS AND DISCUSSION

The addition of titanium(III) citrate to BM after autoclaving and cooling to 30°C caused rapid (<10 s) disappearance of the pink color of resorufin, the transition reduction product of resazurin, even at the lowest titanium concentration used (5×10^{-4} M). The addition of dithiothreitol also resulted in rapid reduction of the medium, but the pink color did not disappear from BM for 15 to 20 min after the addition of cysteine hydrochloride. Thus, titanium(III) citrate and dithiothreitol reacted much more rapidly than cysteine hydrochloride with traces of oxygen remaining in the medium. No evidence of precipitation of $Ti(OH)_3$ in uninoculated medium was seen.

The E_h of BM containing 25 mM glucose but no added reducing agent was −127.5 mV (Table 1). This was 16.5 mV below the value (−111 mV) at which resorufin, the intermediate reduction product of resazurin, is 99% reduced to dihydroresorufin and therefore virtually colorless (11). The E_h of the medium was sufficiently low to permit initiation of growth by all of the bacterial strains tested which did not require cysteine for good growth (Table 2), but the medium tended to oxidize during handling. When cysteine hydrochloride was added to the medium, the E_h was poised at −167.8 mV, and lower E_h values were achieved with dithiothreitol and titanium(III) citrate (Table 1). With the latter reducing agent, E_h values were inversely propor-

tional to titanium concentration. Values achieved with 1×10^{-3} and 2×10^{-3} M titanium were low enough to support initiation of growth by methanogenic bacteria in a nutritionally adequate medium (10).

All of the bacterial strains tested grew well in BM containing a carbohydrate and 0.05% cysteine hydrochloride (Table 2). When the medium contained no reducing agent, however, the maximum AOD (AOD_{max}) often was lower or was reached after a longer incubation period than when cysteine hydrochloride was present. Either or both of these effects were observed with all of the strains tested. Cysteine was required by *B. ruminicola* 23, and it stimulated the growth of all other strains, particularly *B. ruminicola* GA33, *B. fibrisolvens* D1, *E. ruminantium* GA195, and *S. bovis* Pe_i8. *Coprococcus* sp. Pe_i5 and *S. ruminantium* GA192 grew well in medium reduced with dithiothreitol, but the AOD_{max} was lower or more slowly achieved or both with all of the other strains tested.

All of the strains except *B. ruminicola* 23 grew in the presence of titanium(III) citrate. Titanium concentration had little effect upon the magnitude or time of AOD_{max}, except with *B. fibrisolvens* D1 and *Coprococcus* sp. Pe_i5, in which the rate of growth decreased with increasing concentration. With *Coprococcus* sp. Pe_i5 and *M. elsdenii* B159, however, the AOD_{max} increased with increasing titanium concentration. The AOD_{max} of *B. amylophilus* 70 was greater with all concentrations of titanium tested than with cysteine hydrochloride, whereas that of all other strains was less. Nevertheless, it was higher than the AOD_{max} of all of these strains in the absence of a reducing agent, except *Coprococcus* sp. Pe_i5, *M. elsdenii* B159, and *R. albus* 7. It is unlikely that enhanced growth in the presence of titanium(III) citrate could be due to degradation of the titanium/citrate complex and utilization of the citrate for growth, since none of the strains grew within 4 weeks in BM supplemented with 5 mM sodium citrate and 0.05% cysteine hydrochloride. The possibility of growth inhibition by Ti^{4+}, which could be formed in the medium by oxidation of Ti^{3+}, was not investigated; Zehnder and Wuhrmann (20), however, concluded that titanium(IV) citrate was biologically inert for three anaerobic and two facultatively anaerobic bacterial strains.

TABLE 1. *E_h of modified Scott-Dehority medium reduced with cysteine hydrochloride, dithiothreitol, or titanium(III) citrate*[a]

Reducing agent	pH	E_h (mV)
None	6.61	−127.5
Cysteine hydrochloride (0.05%)	6.60	−167.8
Dithiothreitol (0.01%)	6.66	−175.8
Titanium(III) citrate (5×10^{-4} M)	6.60	−302.4[b]
Titanium(III) citrate (1×10^{-3} M)	6.63	−331.5[b]
Titanium(III) citrate (2×10^{-3} M)	6.61	−403.9[b]

[a] Scott-Dehority medium contained 25 mM glucose.
[b] E_h was measured in the presence of 1 drop of 0.05% safranin per 3 ml of medium.

Cysteine appeared to be required or stimulatory for the growth of *B. ruminicola* GA33, *B. ruminicola* 23, *B. fibrisolvens* D1, *E. ruminantium* GA195, and *S. bovis* Pe_i8 (Table 2). When these strains were grown in medium reduced with titanium(III) citrate (10^{-3} M titanium) and supplemented with cysteine hydrochloride, growth was generally proportional to the cysteine hydrochloride concentration, and the time required to achieve AOD_{max} was inversely proportional to the cysteine hydrochloride concentration, up to 100 μg/ml (Table 3). The growth

TABLE 2. *Growth of rumen bacteria in modified Scott-Dehority medium reduced with cysteine hydrochloride, dithiothreitol, or titanium(III) citrate*[a]

Organism	Growth (increase in AOD_{max}) with the following reducing agents:					
	None	Cysteine hydrochloride (0.05%)	Dithiothreitol (0.01%)	Titanium(III) citrate		
				5×10^{-4} M	1×10^{-3} M	3×10^{-3} M
B. amylophilus 70	0.63 (48)[b]	1.21 (8)	0.01 (24)	1.66 (10)	1.73 (9)	1.69 (9)
B. ruminicola GA33	0.10 (16)	2.38 (16)	0.83 (279)	0.17 (16)	0.30 (26)	0.20 (25)
B. ruminicola 23	0.01 (36)	2.42 (29)	0.03 (27)	0.08 (36)	0.08 (36)	0.03 (48)
B. fibrisolvens D1	0.09 (20)	1.82 (16)	0.35 (47)	0.16 (16)	0.19 (20)	0.19 (24)
Coprococcus sp. Pe_i5	1.73 (23)	2.04 (21)	2.36 (24)	1.47 (42)	1.58 (46)	1.85 (83)
E. ruminantium GA195	0.40 (31)	1.44 (14)	1.38 (86)	0.40 (18)	0.47 (19)	0.32 (19)
M. elsdenii B159	2.03 (32)	2.20 (34)	0.87 (135)	1.29 (51)	1.60 (46)	1.69 (46)
R. albus 7	0.74 (51)	1.76 (15)	0.80 (36)	1.19 (27)	1.20 (27)	1.16 (26)
S. ruminantium GA192	1.57 (9)	1.82 (9)	2.03 (10)	1.79 (10)	1.77 (10)	1.74 (10)
S. bovis Pe_i8	0.40 (10)	1.38 (5)	1.05 (7)	0.58 (9)	0.62 (8)	0.63 (7)

[a] Scott-Dehority medium contained 12.5 mM maltose for *B. amylophilus* 70, 12.5 mM cellobiose for *R. albus* 7, and 25 mM glucose for all other strains.
[b] The numbers in parentheses are numbers of hours of incubation required to reach AOD_{max}.

TABLE 3. *Effect of cysteine hydrochloride on growth of rumen bacteria in modified Scott-Dehority medium reduced with titanium(III) citrate*[a]

Organism	Growth (increase in AOD_{max}) with the following concn of cysteine hydrochloride (μg/ml):						
	0	5	10	25	50	100	200
B. ruminicola GA33	0.17 (15)[b]	1.12 (31)	>3.40 (22)	>3.40 (14)	>3.40 (13)	>3.40 (13)	>3.40 (13)
B. ruminicola 23	0.02 (29)	0.43 (207)	0.05 (157)	1.11 (109)	2.39 (44)	3.02 (36)	2.37 (43)
B. fibrisolvens D1	0.08 (18)	0.49 (18)	0.81 (16)	1.27 (16)	1.86 (17)	2.41 (16)	2.40 (16)
E. ruminantium GA195	0.01 (18)	0.30 (85)	0.50 (47)	1.04 (21)	1.89 (16)	2.03 (16)	1.90 (16)
S. bovis Pe_18	1.06 (7)	1.48 (7)	1.46 (6)	1.53 (6)	1.61 (6)	1.56 (6)	1.61 (6)

[a] Scott-Dehority medium contained 25 mM glucose and 10^{-3} M titanium.

[b] Numbers in parentheses are numbers of hours of incubation required to reach AOD_{max}.

of *B. ruminicola* GA33 and *S. bovis* Pe_18 was markedly stimulated by 5 μg of cysteine hydrochloride per ml, but 100 μg/ml was required for maximum growth of the remaining strains. The AOD_{max} values of *B. ruminicola* 23 and *E. ruminantium* GA195 were lower with 200 μg of cysteine hydrochloride per ml (equivalent to 0.02%) than with 100 μg/ml. This suggests that cysteine may become inhibitory to growth at concentrations exceeding those needed to satisfy nutritional requirements and could account for the lower AOD_{max} values found for all five strains in the presence of 0.05% cysteine hydrochloride alone (Table 2).

These results show that several representative rumen bacteria grew well in a medium reduced with titanium(III) citrate concentrations up to 2×10^{-3} M. Titanium(III) citrate could therefore be used simply and effectively in anaerobic media for some rumen bacteria for which the exclusion of a sulfur-containing reducing agent is required.

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