Effect of titanium (III) citrate as reducing agent on growth of rumen bacteria.

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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 $5 \times 10^{-4}$ M titanium and $-403.9$ mV at $2 \times 10^{-3}$ 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 Wurmann (20) tested titanium(III) citrate and showed that it was nontoxic to Methanobacterium strain AZ at concentrations below $0.5 \times 10^{-3}$ M and to Clostridium formocaceticum and Bifidobacterium bifidum at concentrations below $2 \times 10^{-3}$ 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;5 and the faculta-
TITANIUM(III) CITRATE AS REDUCING AGENT

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T. E. Chucks and Dehority (15), modified by omission of

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cellulbiose 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,

cellulbiose (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 concen-

tration, 0.05%; equivalent to 2.8 × 10⁻³ M) which had

been prepared under N₂ 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 dithio-

threitol) were sterilized as separate solutions at 121°C

for 15 min and after cooling to 30°C combined asep-

tically in the appropriate proportions. Dithiothreitol

was sterilized by membrane filtration (pore size, 0.22

μm; Millipore Ltd., Mississauga, Ontario, Canada) be-

fore addition to the medium. The complete medium

was dispensed under CO₂ in 3-ml portions into tubes

(13 by 100 mm) which were closed with butyl rubber

bungs. The final pH of the medium was 6.5 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 hy-

drochloride; incubation was at 39°C. Cells in the late

logarithmic phase were harvested aseptically by cen-

trifugation under CO₂ 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 me-

dium 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. Incuba-

tion of cultures showing negligible growth was con-

tinued for 96 h before measurements were stopped. Maximum

optical density values were corrected for zero time

readings, and the net optical density values thus ob-

tained were in turn corrected for deviation from Beer's

Law by conversion to adjusted optical density (AOD)

values, using tables kindly supplied by C. W. Forsberg.

Eₙ. The Eₙ values of the media reduced with cyste-

ine 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 phos-

phate buffer (pH 6.6). All measurements were made

under CO₂ 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 dithio-

threitol 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 un inoculated

medium was seen.

The Eₙ 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 dihydro-

resorufin and therefore virtually colorless (11). The Eₙ of the medium was sufficiently low to

permit initiation of growth by all of the bacterial strains tested which did not require cysteine

good growth (Table 2), but the medium tended to

oxidize during handling. When cysteine hydro-

chloride was added to the medium, the Eₙ

was poised at -167.8 mV, and lower Eₙ values

were achieved with dithiothreitol and

titanium(III) citrate (Table 1). With the latter

reducing agent, Eₙ values were inversely propor-
tional to titanium concentration. Values achieved with $1 \times 10^{-2}$ and $2 \times 10^{-3}$ 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$_{\text{max}}$) 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,5. Coprococcus sp. Pe,5 and $S$. ruminantium GA192 grew well in medium reduced with dithiothreitol, but the AOD$_{\text{max}}$ 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$_{\text{max}}$, except with $B$. fibrisolvens D1 and Coprococcus sp. Pe,5, in which the rate of growth decreased with increasing concentration. With Coprococcus sp. Pe,5 and $M$. elsdenii B159, however, the AOD$_{\text{max}}$ increased with increasing titanium concentration. The AOD$_{\text{max}}$ 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$_{\text{max}}$ of all of these strains in the absence of a reducing agent, except Coprococcus sp. Pe,5, $M$. 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 Wohrman (20), however, concluded that titanium(IV) citrate was biologically inert for three anaerobic and two facultatively anaerobic bacterial strains.

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,5 (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$_{\text{max}}$ was inversely proportional to the cysteine hydrochloride concentration, up to 100 mg/ml (Table 3). The growth

### Table 1. $E_{\text{a}}$ of modified Scott-Dehory medium reduced with cysteine hydrochloride, dithiothreitol, or titanium(III) citrate$^a$

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>pH</th>
<th>$E_{\text{a}}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.61</td>
<td>-127.5</td>
</tr>
<tr>
<td>Cysteine hydrochloride (0.05%)</td>
<td>6.60</td>
<td>-167.8</td>
</tr>
<tr>
<td>Dithiothreitol (0.01%)</td>
<td>6.66</td>
<td>-175.8</td>
</tr>
<tr>
<td>Titanium(III) citrate ($5 \times 10^{-4}$ M)</td>
<td>6.60</td>
<td>-302.4$^a$</td>
</tr>
<tr>
<td>Titanium(III) citrate ($1 \times 10^{-3}$ M)</td>
<td>6.63</td>
<td>-331.5$^a$</td>
</tr>
<tr>
<td>Titanium(III) citrate ($2 \times 10^{-3}$ M)</td>
<td>6.61</td>
<td>-403.9$^a$</td>
</tr>
</tbody>
</table>

$^a$ Scott-Dehory medium contained 25 mM glucose.

### Table 2. Growth of rumen bacteria in modified Scott-Dehory medium reduced with cysteine hydrochloride, dithiothreitol, or titanium(III) citrate$^a$

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth (increase in AOD$_{\text{max}}$, with the following reducing agents:</th>
<th>5 $\times 10^{-4}$ M</th>
<th>1 $\times 10^{-3}$ M</th>
<th>3 $\times 10^{-3}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B$. amylophilus 70</td>
<td>None (0.63 (48)$^b$)</td>
<td>1.21 (8)</td>
<td>0.01 (24)</td>
<td>1.66 (10)</td>
</tr>
<tr>
<td>$B$. ruminicola GA33</td>
<td>Cysteine hydrochloride (0.05%) (0.10 (16))</td>
<td>2.38 (16)</td>
<td>0.83 (279)</td>
<td>0.17 (16)</td>
</tr>
<tr>
<td>$B$. ruminicola 23</td>
<td>Dithiothreitol (0.01%) (0.01 (36))</td>
<td>2.42 (29)</td>
<td>0.03 (27)</td>
<td>0.08 (36)</td>
</tr>
<tr>
<td>$B$. fibrisolvens D1</td>
<td>Titanium(III) citrate ($5 \times 10^{-4}$ M) (0.09 (20))</td>
<td>1.82 (16)</td>
<td>0.35 (47)</td>
<td>0.16 (16)</td>
</tr>
<tr>
<td>Coprococcus sp. Pe,5</td>
<td>$E$. ruminantium GA195 (1.73 (23))</td>
<td>2.04 (21)</td>
<td>2.36 (24)</td>
<td>1.47 (42)</td>
</tr>
<tr>
<td>$M$. elsdenii B159</td>
<td>M. elsdenii B159 (1.40 (31))</td>
<td>1.44 (14)</td>
<td>1.38 (86)</td>
<td>0.40 (18)</td>
</tr>
<tr>
<td>$R$. albus 7</td>
<td>$S$. ruminantium GA192 (2.03 (32))</td>
<td>2.20 (34)</td>
<td>0.87 (135)</td>
<td>1.29 (51)</td>
</tr>
<tr>
<td>$S$. bovis Pe,5</td>
<td>$S$. bovis Pe,5 (1.57 (9))</td>
<td>1.82 (9)</td>
<td>2.03 (10)</td>
<td>1.79 (10)</td>
</tr>
</tbody>
</table>

$^a$ Scott-Dehory 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$_{\text{max}}$. 

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of *B. ruminicola* GA33 and *S. bouis* Pe₈ 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 AODmax 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 AODmax 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 $2 \times 10^{-3}$ 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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LITERATURE CITED


