REDUCTION OF INORGANIC COMPOUNDS WITH MOLECULAR HYDROGEN BY MICROCOCCUS LACTILYTICUS

I. STOICHIOMETRY WITH COMPOUNDS OF ARSENIC, SELENIUM, TELLURIUM, TRANSITION AND OTHER ELEMENTS

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ABSTRACT

WOOLFOLK, C. A. (University of Washington, Seattle) AND H. R. WHITELEY. Reduction of inorganic compounds with molecular hydrogen by Micrococcus lactilyticus. I. Stoichiometry with compounds of arsenic, selenium, tellurium, lead, thallium, vanadium, manganese, iron, copper, molybdenum, tungsten, osmium, ruthenium, gold, silver, and uranium, as well as molecular oxygen. Chemical and manometric data indicate that the following reductions are essentially quantitative: arsenate to arsenite, pentavalent and trivalent bismuth to the free element, selenite via elemental selenium to selenide, tellurate and tellurite to tellurium, lead dioxide and manganese dioxide to the divalent state, ferric to ferrous iron, osmium tetroxide to osmate ion, osmium dioxide and trivalent osmium to the metal, uranyl uranium to the tetravalent state, vanadate to the level of vanadyl, and polyvalent molybdate ions to molybdenum blues with an average valence for molybdenum of +5. The results of a study of certain other hydrogenase-containing bacteria with respect to their ability to carry out some of the same reactions are also presented.

The reduction of inorganic compounds in an atmosphere of hydrogen by cell suspensions and by extracts containing hydrogenase has been reported for many bacteria. Thus, the hydrogenase-coupled reductions of inorganic sulfur compounds by Desulfovibrio (Postgate, 1959; Peck, 1959) and of inorganic nitrogen compounds by a variety of bacteria (Nason and Takahashi, 1958; Gest, 1954) have been thoroughly investigated. The reduction of oxygen at the expense of molecular hydrogen ("Knallgas reaction") as a source of energy for the autotrophic growth of members of the genus Hydrogenomonas has been studied in detail (Packer, 1958). The latter reaction is also known to occur in a variety of other bacteria (Gest, 1954), including the obligate anaerobe, Desulfovibrio (Postgate, 1956).

Evidence has also been presented for the biological reduction of a number of other inorganic compounds, although molecular hydrogen has not been shown to serve as the electron donor. For example, the production of elemental selenium (Levine, 1925; Zalokar, 1953) and tellurium (Corper, 1915; Morton and Anderson, 1941), of molybdenum blue (Jan, 1939; Marchal and Girard, 1948), of arsenite (Green, 1918), and of phosphite and hypophosphite (Barrenscheen and Beck-Widmanstetter, 1923; Rudakov, 1927) by microorganisms growing in media containing selenate, tellurate, molybdate, arsenate, or phosphate, respectively, has been reported. Reduced methylated derivatives of arsenate, arsenite, tellurite, selenite, and selenite can be produced by various molds during growth (Challenger, 1951). In addition, the reduction of manganese dioxide (Mann and Quastel, 1946) and ferric iron (Bromfield, 1954) by soil bacteria has been studied.

The reductions of tellurite (Terai, Kamahora, and Yamamura, 1958) and selenite (Falcone and Nickerson, 1960) have been observed in cell-free preparations of Mycobacterium avium and Saccharomyces cerevisiae, respectively, and some of the properties of the enzymes mediating these reactions have been investigated. It has also been shown that vanadate, selenite, and phospho-
molybdate can oxidize reduced cytochrome c$_{55}$ obtained from Desulfovibrio (Ishimoto et al., 1957).

A systematic survey of inorganic compounds has disclosed that cell-free extracts of Micrococcus lactilyticus (Foubert and Douglas, 1948; classified as Veillonella alcalescens Prévot in Bergey's Manual, 7th ed., 1957) are capable of utilizing molecular hydrogen for the reduction of a wide variety of inorganic compounds (Woolfolk, Ordal, and Whiteley, 1959; Woolfolk, 1960; Woolfolk and Whiteley, 1961). This paper presents data on the stoichiometry of reduction of certain of these compounds, on the ionic species used as substrates, and on the rates of these reductions. Certain other bacteria containing hydrogenase are compared with respect to their ability to reduce some of these same compounds.

**MATERIALS AND METHODS**

*Cultivation of bacteria and preparation of extracts.* Bacteria were grown in the following media: *M. lactilyticus*, in a complex medium containing lactate (Whiteley and Douglas, 1951); *Micrococcus aerogenes* (Peptococcus aerogenes), in a peptone-yeast extract medium with glutamate (Whiteley, 1957); *Escherichia coli* strain B on the basal medium of McNall and Atkinson (1956) containing 5 × 10^{-8} M (NH$_4$)$_2$SO$_4$; Azotobacter vinelandii, in Burk's medium as used by Newton, Wilson, and Burris (1953); Desulfovibrio desulfuricans, in a medium similar to that of Postgate (1951) to which 0.4% sodium lactate was added; Clostridium pasteurianum, in the medium of Carnahan and Castle (1958) using NH$_4$Cl as a nitrogen source; and Rhodobacter vannielii, in the medium of Murray and Douglas (1950) under illumination.

Cells were harvested by centrifugation, washed once or twice with distilled water, suspended in one to two volumes of water, and exposed to sonic oscillation for 20 to 45 min in a Raytheon 10-kc sonic oscillator. The resulting preparation was centrifuged in the cold at 25,000 × g for 20 min and the supernatant fraction was used as a "crude extract." Protein was determined by the method of Lowry (Layne, 1957).

*Preparation of substrates.* Sodium phosphate was obtained by the hydrolysis of phosphorus trichloride in water followed by neutralization of the solution with sodium hydroxide. Sodium hypophosphate (Na$_3$H$_2$P$_2$O$_7$) was prepared by the method of Leininsen and Chulaki (1953).

Potassium tellurate (chromatographically free of tellurite) was prepared by treatment of potassium tellurate with hydrogen peroxide. Ferric hydroxide and cupric hydroxide suspensions were obtained by neutralization of the appropriate chlorides. Commercial manganese dioxide and lead dioxide were reduced by extracts of *M. lactilyticus* under hydrogen, but higher specific activities could be obtained with suspensions containing highly dispersed particles of these substances. Manganese dioxide and lead dioxide suspensions were obtained by treatment of potassium permanganate or lead acetate with sodium sulfite or sodium hypochlorite solutions, respectively. The suspensions were purified by repeated centrifugation and resuspension. Colloidal elemental selenium was prepared by the action of hydroxylamine hydrochloride on sodium selenite solutions in the presence of 5% gum arabic, and was purified by repeated centrifugation. Bismuth hydroxide [Bi(OH)$_3$] and bismuthyl hydroxide [Bi(OH)]$_2$ were obtained by addition of excess sodium hydroxide to bismuth subcarbonate suspensions at room temperature and 100 C, respectively (Charlot, 1954). Other compounds tested as substrates were obtained from commercial sources.

*Manometric procedures.* The reduction of inorganic compounds was followed by measuring the uptake of hydrogen in a conventional Warburg apparatus at 30 C. The vessels were flushed with hydrogen from which oxygen had been removed by passage through a hot copper column. Reaction mixtures having a total volume of 2.2 ml routinely contained from 5 to 50 μmoles of substrate in one side arm, and 150 μmoles of tris(hydroxymethyl)aminomethane buffer (pH 7.0) and 1 ml of extract containing approximately 30 mg of protein in the main compartment. In the experiments where stoichiometric relationships were determined, the substrate was incubated with the reaction mixture until the uptake of hydrogen gas had ceased, and the vessel contents were then analyzed for the end products.

When molecular oxygen was used as the substrate, one side arm gas vent was replaced with a serum stopper to permit the introduction of oxygen into the vessel by means of a hypodermic syringe. The stoichiometry of oxygen uptake during the utilization of both oxygen and hy-
drogen was determined by the method of Schatz and Bovell (1952).

Analytical methods. For the determination of arsenite, proteins were removed from the reaction mixture by precipitation with trichloroacetic acid, and the supernatant fraction was neutralized with sodium bicarbonate and titrated with iodine in excess bicarbonate. Total arsenate plus arsenite was estimated colorimetrically by the molybdenum blue method of Sandell (1944), with hydrazine as the reducing agent. Arsine was determined by flushing a reaction vessel with hydrogen and trapping the flushed material in an absorption vessel as described by Sandell (1944). The contents of the absorption vessel were then analyzed by the molybdenum blue method.

Solutions of selenite and tellurite and suspensions of lead dioxide were determined quantitatively by titration with standard permanganate (Charlot and Bezier, 1957). Osmium trichloride solutions were similarly estimated, on the assumption that the osmium was oxidized to the tetroxide.

Elemental selenium and tellurium were determined colorimetrically after dissolution in concentrated sulfuric acid (Wiberley et al., 1953), using selenite and tellurite solutions (determined as above) as standards. Tellurate could also be determined by this method after reduction to the element with acidified stannous chloride. Hydrogen selenide produced by the reduction of selenite and selenium was absorbed by addition of cadmium chloride to the center well of the Warburg vessel as described by Postgate (1951) for hydrogen sulfide. After the enzymatic reaction with selenium or selenite had gone to completion, 0.5 ml of 5 N H₂SO₄ was added to the reaction mixture from one of the side arms to decompose metal selenides that might have precipitated in the reaction mixture, and the vessel was incubated for an additional 30 min. The contents of the center well were then oxidized with excess permanganate in 6 N HCl. After 10 min, excess acidified stannous chloride was added, and elemental selenium, and hence the selenide formed during the reduction, was then determined colorimetrically as above.

To measure vanadyl vanadium, excess iodine and sodium bicarbonate were added to samples of reaction mixtures, which were then assayed with standard arsenite solution. Hydroxide produced during the enzymatic reduction of molybdate and phoshomolybdate was measured by adding sufficient standard acid to achieve the original pH value of the reaction mixture. Molybdenum blues formed by the reduction of phosphomolybdate complexes can be distinguished spectrophotometrically from those formed by reduction of molybdate per se. This method was used for the characterization of the product formed by M. lactilyticus from molybdate.

Arsenate and arsenite were separated by chromatography of reaction mixtures on paper, using the solvent system of Yamaguchi (1953). Ammoniacal silver nitrate was added as a spray to locate these compounds as well as phosphate. Phosphate, phosphite, and hypophosphate were separated and detected by paper chromatography, as described by Bonnin and Sue (1952). Vanadyl vanadium was identified as the product of vanadate reduction by the chromatographic method of Stevens (1956), using 8-hydroxyquinoline in 20% citric acid to develop the chromatograms. Paper chromatography was also used to determine that tellurate preparations were free of tellurite (Ghosh Mazumdar and Lederer, 1957).

RESULTS

Reduction of compounds of the nitrogen family of elements. Extracts of M. lactilyticus are capable of reducing nitrate, nitrite, hydroxylamine, and hydrazine to ammonia at the expense of molecular hydrogen (Woolfolk et al., 1959). In view of these reactions, it was considered of interest to test the ability of this organism to reduce compounds of arsenic, phosphorus, antimony, and bismuth, all members of the nitrogen family of elements: the results of these investigations are discussed here. Information on the stoichiometry and pathway of reduction of the nitrogen compounds will be presented in a separate communication.

Arsenate, but not p-arsanilic acid or arsenite, can be reduced in a hydrogen atmosphere by extracts of M. lactilyticus. One mole of arsenite (identified by paper chromatography) was produced per mole of hydrogen taken up (Table 1); the total concentration of arsenate plus arsenite remained constant during the reduction, and arsine could not be detected as an end product.

Sodium bismuthate was reduced with an uptake of hydrogen (Table 1), and a dark brown
suspended material, probably elemental bismuth, accumulated. A similar reaction was observed when a suspension of bismuthyl chloride was added as a substrate. Preparations of white bismuth hydroxide [Bi(OH)₃] and yellow bismuthyl hydroxide [Bi₂O(OH)] were also reduced to elemental bismuth; however, they could not be used for quantitative determinations because of the variable composition of the bismuth hydroxide preparations. Although the initial rate of bismuthate reduction was in excess of that observed with bismuth hydroxide (Table 5), the rate of the reduction of the latter compound was consistent with its formation as an intermediate in the conversion of bismuthate to bismuth. Bismuthyl hydroxide, bismuthyl chloride, and bismuth subcarbonate (bismuthyl carbonate), on the other hand, are reduced much more slowly, probably owing to the limited solubilities of these compounds or the state of their dispersion. Antimonate and antimonyl compounds could not serve as substrates for reduction by \textit{M. lactilyticus}.

There have been several reports (Barrenscheen and Beck-Widmanstetter, 1923; Rudakov, 1927) indicating the reduction of inorganic phosphorus compounds by bacteria. Orthophosphate, pyrophosphate, tetrametaphosphate, insoluble metaphosphate, phosphite, hypophosphate, and hypophosphate (\(\text{P}_3\text{O}_{10}^-\)) were tested as substrates with hydrogen and extracts of \textit{M. lactilyticus}. Utilization of hydrogen was not observed with these compounds, and examination of reaction mixtures by paper chromatography did not give any evidence of reduction. It should be noted that, except for hypophosphate, the reduction of these phosphorus compounds would not be expected on the basis of thermodynamic considerations.

\section*{Reduction of compounds of the oxygen family of elements.} The introduction of oxygen into reaction vessels containing hydrogen and \textit{M. lactilyticus} extract resulted in a rapid uptake of gas (Table 2). No oxygen uptake was observed when nitrogen was substituted for hydrogen.

Selenite was reduced in a two-step reaction (Fig. 1). In the first step, there was a rapid uptake of sufficient hydrogen for the production of elemental selenium, and the reaction mixtures became bright orange in color as a result of the production of colloidal selenium. After this, there was a slower uptake of hydrogen, owing to the reduction of the colloidal selenium to selenide. Colloidal selenium suspensions were similarly

\begin{table}[h]
\centering
\caption{Reduction of compounds of arsenic and bismuth by \textit{Micrococcus lactilyticus}}
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Substrate} & \textbf{Amount added} & \textbf{Hydrogen uptake} & \textbf{Arsenate} \\
& \textbf{\(\mu\)moles} & \textbf{\(\mu\)moles} & \textbf{\(\mu\)moles} \\
\hline
Arsenate & 10.0 & 10.2 & 9.62 \\
Bismuthate & 10.0 & 25.4 & - \\
Bismuthyl & 10.0 & 16.7 & - \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Reduction of molecular oxygen and compounds of selenium and tellurium by \textit{Micrococcus lactilyticus}}
\begin{tabular}{|l|c|c|c|c|c|}
\hline
\textbf{Substrate} & \textbf{Amount added} & \textbf{\(\text{H}_2\) uptake} & \textbf{Selenium} & \textbf{Tellurium} \\
& \textbf{\(\mu\)moles} & \textbf{\(\mu\)moles} & \textbf{\(\mu\)moles} & \textbf{\(\mu\)moles} \\
\hline
Oxygen & 6.5 & 12.8 & - & - \\
Selenite & 7.8 & 14.8 & 7.6 & - \\
Selenite & 7.8 & 23.8 & - & 7.6 \\
Selenium & 8.6 & 8.5 & - & 8.6 \\
Tellurate & 2.5 & 7.3 & - & 2.6 \\
Tellurite & 6.5 & 13.4 & - & 6.7 \\
\hline
\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Reduction of selenite, selenium, tellurite, and uranyl acetate in an atmosphere of hydrogen by \textit{Micrococcus lactilyticus}. Each vessel contained approximately 30 mg of extract protein, the indicated substrate, and buffer in a total volume of 2.2 ml; 1,000 \(\mu\)moles of phosphate buffer (pH 7.0) were used for selenite, selenium, and tellurite; 800 \(\mu\)moles of acetate buffer (at a final pH of 5.8) were used for uranyl acetate; 0.3 ml of 20\% KOH was added to the center well for reactions with tellurite and uranyl acetate, and 0.4 ml of 10\% CdCl₂ was present for reactions with selenite and selenium. \(\bullet\), 7.8 \(\mu\)moles of sodium selenite; \(\Delta\), 24.8 \(\mu\)moles of selenium; \(\Delta\), 7.8 \(\mu\)moles of tellurite; \(\bigcirc\), 10 \(\mu\)moles of uranyl acetate.}
\end{figure}
reduced to selenide (Fig. 1). The amounts of selenium formed during the first step of this reaction, and the amounts of selenide ultimately produced from selenite and colloidal selenium, are shown in Table 2. Selenate was not reduced. A number of sulfur compounds are reduced by extracts of *M. lactilyticus*; these data are presented in the accompanying paper (Woolfolk, 1962).

Tellurite (Fig. 1) and tellururate were converted to dark brown colloidal tellurium, which was not reduced further. The quantities of hydrogen utilized and the amounts of tellurium formed with these substrates are shown in Table 2. A comparison of the specific activities with tellurate and tellurite (Table 5) suggests that tellurate is reduced to tellurium via the rate determining formation of tellurate.

**Reduction of compounds of the transition elements.** Vanadate may also serve as a substrate for reduction; the amount of hydrogen taken up (Table 3) was sufficient to account for the production of vanadyl compounds. A substance having the same mobility as vanadyl chloride was detected as a product of the reduction, and titrimetric analysis indicated a quantitative reduction of vanadate to vanadyl (Table 3). The reaction mixtures became light blue-green during the reduction, probably because of the formation of vanadyl hydroxide.

When molybdate was added as a substrate in reaction mixtures containing phosphate buffer (pH 5.5), there was a rapid uptake of sufficient molecular hydrogen (Table 3) for reduction of molybdate to the pentavalent state. This reaction was accompanied by the formation of a blue color in the reaction mixtures, suggesting that molybdate was reduced to a molybdenum blue compound. In general, the molybdenum in

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount added</th>
<th>H₂ uptake</th>
<th>Vanadyl Hydroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanadate</td>
<td>42.8</td>
<td>23.4</td>
<td>44.0</td>
</tr>
<tr>
<td>Molybdate</td>
<td>20.0</td>
<td>10.6</td>
<td>—</td>
</tr>
<tr>
<td>Phosphotungstic acid</td>
<td>10.0</td>
<td>2.6</td>
<td>19.8</td>
</tr>
</tbody>
</table>

*Expressed as MoO₄²⁻.

**TABLE 4. Reduction of compounds of osmium, lead, manganese, iron, and uranium by *Micrococcus lactilyticus***

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount added</th>
<th>H₂ uptake</th>
<th>µmoles</th>
<th>µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmium tetroxide</td>
<td>15.6</td>
<td>12.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmium dioxide</td>
<td>12.7</td>
<td>25.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmium (+3) chloride</td>
<td>7.3</td>
<td>10.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lead dioxide</td>
<td>6.25</td>
<td>6.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manganese dioxide</td>
<td>10.0</td>
<td>10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferric hydroxide</td>
<td>10.0</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>10.0</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uranil hydroxide</td>
<td>10.0</td>
<td>9.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

these uncharged complex oxides (molybdenum blues) has an average valence between +5 and +6, although several compounds of this type are known with an average valence for molybdenum of exactly +5 (Glemser and Lutz, 1947). If a less acid pH is used (i.e., 6.5), reaction mixtures turn from blue to red brown, probably because of the decomposition of molybdenum blue to molybdenyl hydroxide (Glemser and Lutz, 1951).

The formation of molybdenum blue was probably due to the reduction of molybdate or isopolymolybdate ions, and not to the reduction of phosphomolybdate complexes. This conclusion receives support from the fact that phosphomolybdate complexes are stable only at more acid pH values than those used in the enzymatic experiments. Furthermore, the spectra of molybdenum blues formed from molybdate proper and from phosphomolybdate are known to differ (Charlot and Bézier, 1957), and the spectrum of the molybdenum blue formed by *M. lactilyticus* resembles the former.

Phosphotungstic acid was reduced by extracts of *M. lactilyticus*, although only a small amount of hydrogen was utilized (Table 3). This reduction was also accompanied by the formation of a blue color (probably a tungsten blue) but the reaction was not investigated further.

Osmium tetroxide could also serve as a substrate for reduction (Table 4). The absence of the black color characteristic of osmium dioxide indicates that this compound was not formed as the end product, and the quantity of hydrogen taken up suggests that osmate ion was produced. Furthermore, osmium dioxide (added as a
suspension) was reduced to elemental osmium, as was trivalent osmium chloride.

Ferric hydroxide in suspension was converted to ferrous hydroxide with an uptake of hydrogen, and ferricyanide was similarly reduced to ferrocyanide (Table 4). Suspensions of manganese dioxide were reduced with the uptake of a stoichiometric amount of hydrogen for the production of divalent manganese.

Small uptakes of hydrogen were noted when silver phosphate, ruthenium hydroxide (+3), or auric gold were the substrates; orange-colored colloidal silver, divalent ruthenium, and colloidal gold, respectively, were formed. Similarly, cupric hydroxide was reduced to cuprous hydroxide (hydrogen uptake only 70% of theoretical).

**FIG. 2.** Effect of pH on the rate of reduction of vanadate, molybdate, selenite, tellurite, and arsenate by extracts of *Micrococcus lactilyticus.* Each vessel contained 1,000 μmoles of phosphate buffer, extract, and substrate, in a total volume of 2.2 ml at the indicated final pH. ○, 40 μmoles of vanadate, 13.4 mg protein; ●, 20 μmoles of molybdate, 17 mg of protein; △, 10 μmoles of selenite, 44.5 mg of protein; ▲, 10 μmoles of tellurite, 44.5 mg of protein; □, 10 μmoles of arsenate, 58 mg of protein. To permit the inclusion of all five curves in the same figure, the specific activity for each curve was multiplied by a factor. The value of this factor was 0.81 for arsenate, 0.17 for tellurite, 0.18 for molybdate, 0.09 for selenite, and 0.08 for vanadate.

**FIG. 3.** Effect of increasing concentration on the rate of reduction of vanadate and molybdate. Each vessel contained 1,000 μmoles of phosphate and 42 mg of protein in a total volume of 2.2 ml. ○, vanadate at a pH of 8.5; ●, molybdate at a pH of 5.8.

Although a limited hydrogen uptake was observed, suggesting that these reactions can be coupled to hydrogenase, a significant portion of the substrate was reduced nonenzymatically by the extract.

**Reduction of compounds of other elements.** Extracts of *M. lactilyticus* are capable of converting lead dioxide to divalent lead (Table 4). There was no evolution of oxygen from reaction mixtures during this reaction, indicating that the lead dioxide was not decomposed. When thallous hydroxide was used as a substrate, thallous hydroxide was produced, although only a fraction of the expected hydrogen uptake was observed. Since heat-denatured extracts caused the reduction of this compound (detected by the bleaching of the dark brown thallic hydroxide), it may be concluded that the reaction observed with *M. lactilyticus* was largely nonenzymatic.

Hydrogen was rapidly oxidized by extracts of *M. lactilyticus* in the presence of uranyl compounds, with the formation of tetravalent uranium (Table 4). Initially, a rapid, almost linear, uptake of 0.5 mole of hydrogen was observed, followed by a long second phase in which the rate of hydrogen uptake progressively diminished. It is possible that the first phase was due to the reduction of uranyl (hexavalent uranium) to pentavalent uranium. It is known that uranium at the latter valence is unstable and undergoes dismutation to uranyl and tetravalent...
uranium. The second step of hydrogen uptake may be the result of the reduction of uranyl produced by this dismutation.

**Kinetics of the reduction of a number of inorganic ions.** The effect of pH on the rate of reduction of arsenate, selenite, tellurite, vanadate, and molybdate is shown in Fig. 2. It is of interest that the reactions with arsenate, selenite, and tellurite had pH optima of approximately 6.5 to 7.0, and declined in rate to negligible values when the pH value approached 8.0. These ions have $pK_a$ values of approximately 6.8 to 7.7, corresponding to the formation of the divalent ions from the monovalent ions. It is possible that the actual ionic species reduced are the monovalent ions, and that the activities decline with increasing pH as a result of the diminishing concentration of this form of the substrate. On the other hand, vanadate, which does not have a $pK$ in this region, was reduced readily at pH 8.0, indicating that the decline in activities with the other substrates was not due to inactivation of hydrogenase or possible intermediate electron carriers. The moderate rates observed for arsenate, selenite, and tellurite at pH 5.5, in contrast to the negligible rate observed at pH 8.0, are in accord with the hypothesis that the monovalent forms of these ions are reduced.

It is well known that the dissociation of molybdate and vanadate in solution is characterized by the formation of numerous polyanions, particularly under acidic conditions. The enzymatic reduction of molybdate was negligible at pH 7.0, but reached an optimum at approximately pH 5.5. The predominant ion in molybdate solutions at the latter pH is Mo$_7$O$_{21}^{4-}$ (Saxena and Saxena, 1961), and the increase in enzymatic activity noted at this pH may be due to a requirement for this form of molybdate. At the more alkaline pH values, Mo$_7$O$_{21}^{4-}$ would be in equilibrium with less polymerized ions, for example, Mo$_5$O$_{16}^{4-}$. At a more alkaline pH, therefore, the concentration of Mo$_5$O$_{16}^{4-}$ would be expected to increase exponentially with increasing molybdate concentration. As shown in Fig. 3, the rate of molybdate reduction increased almost exponentially with increasing total molybdate concentration, whereas vanadate reduction (discussed below) increased linearly. These results suggest that Mo$_7$O$_{21}^{4-}$ is the actual ionic species reduced in the reaction catalyzed by *M. lactilyticus* extracts. As shown in Table 3, titrimetric determinations indicate the production of 2 $\mu$moles of base per $\mu$mole of hydrogen uptake in molybdate reduction, as required by the equation for Mo$_7$O$_{21}^{4-}$ reduction (shown in Table 5). In solutions having pH values between 5 and 9, vanadate is predominantly in the ionic form, H$_2$VO$_4^-$, which is in equilibrium with divanadate and tetravanadate polyanions (Charlot, 1954; Filipovic et al., 1954). The linear increase in the rate of vanadate reduction by *M. lactilyticus* (Fig. 3) with increasing substrate concentration suggests that the H$_2$VO$_4^-$ ion may be the form of vanadate required in the reduction.

When solutions of uranyl compounds are buffered at neutrality, uranium is almost quantitatively precipitated in the form of uranyl hydroxide [UO$_2$(OH)$_2$] (Charlot, 1954). The relatively high rate of reduction of uranyl by extracts of *M. lactilyticus* at pH 5.2 (Fig. 1) suggests the possibility that uranyl ion is the actual species reduced. In this connection, it is of interest that the reduction was even more rapid when citrate buffers were used. Citrate is known to stabilize uranyl ion by the formation of a complex.

**Rates of reduction.** The specific activities of most of the reductions catalyzed by *M. lactilyticus* are shown in Table 5. These values were obtained with crude extracts without the addition of electron carriers, although compounds such as benzyl viologen considerably enhanced the rates of reduction of several of the compounds (Woolfolk et al., 1959). Cell suspensions were also capable of reducing most of the compounds listed in Table 5; however, the relative rates of the various reductions were not the same as with extracts (Woolfolk et al., 1959).

**Inorganic reductions with other hydrogen donors and other bacteria containing hydrogenase.** The reduction of inorganic compounds may be coupled not only to hydrogenase, but also to fermentations which yield hydrogen. Thus, when pyruvate (Whiteley and Ordal, 1957) or hypoxanthine (Whiteley and Ordal, 1956) are fermented in the presence of inorganic compounds, the production of hydrogen is partially or completely inhibited and the inorganic compound is reduced. Not all of the compounds listed in Table 5 have been tested, but many of the sulfur and nitrogen compounds, as well as arsenate, selenite, tellurite, and ferricyanide, are reduced as a result of these fermentations. Di- and triphosphopyridine
nucleotides could not serve as hydrogen donors for these reductions.

Certain other hydrogenase-containing bacteria were examined with respect to their ability to reduce some of the above inorganic compounds. *C. pasteuri*um and *D. desulfuricans* reduced many of the compounds rapidly (Table 6) but did not reduce nitrate or arsenate. In addition to the compounds listed in Table 6, *Desulfovibrio* was also capable of reducing osmium tetroxide, bismuthate, ferricyanide, manganese dioxide, molybdate, and uranyl hydroxide. Extracts of *E. coli* catalyzed the reduction of hydroxylamine, nitrate, and oxygen, as reported by McNall and Atkinson (1957) and Lascelles and Still (1946), but none of the other inorganic compounds tested. Extracts prepared from *A. vinelandii* reduced only oxygen (noted previously by Wilson, Lee, and Wilson, 1942), whereas extracts of *R. vannei*lii, which have a hydrogenase capable of reducing methylene blue, did not reduce any of the inorganic compounds tested. Suspensions of young cells (approximately 10 hr of growth) of *M. aerogenes* reduced most of the inorganic compounds reduced by *M. lactilyticus*, but the specific activities were low. These reactions were not

### Table 5. Specific activities and \( E_\theta \) values of the inorganic reductions catalyzed by extracts of *Micrococcus lactilyticus*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction catalyzed by extracts of <em>M. lactilyticus</em></th>
<th>Specific activity</th>
<th>( E_\theta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenium</td>
<td>( \text{Se} + \text{H}_2 \rightarrow \text{HSe}^- + \text{H}^+ )</td>
<td>6.1</td>
<td>-0.66</td>
</tr>
<tr>
<td>Metabisulfite</td>
<td>( \text{S}_2\text{O}_5^- + \text{H}_2 \rightarrow \text{S}_2\text{O}_4^- + \text{H}_2\text{O} )</td>
<td>83.0</td>
<td>-0.48</td>
</tr>
<tr>
<td>Benzyl viologen</td>
<td>( \text{BV}_{ox} + \frac{1}{2} \text{H}<em>2 \rightarrow \text{BV}</em>{red} )</td>
<td>440</td>
<td>-0.36</td>
</tr>
<tr>
<td>Molybdate</td>
<td>( \text{MoO}_4^{2-} + 3 \text{H}_2 \rightarrow 6 \text{MoO}_3 + 6 \text{OH}^- )</td>
<td>58.0</td>
<td>-0.32</td>
</tr>
<tr>
<td>Phosphotungstate</td>
<td></td>
<td>23.0</td>
<td>-</td>
</tr>
<tr>
<td>Uranyl hydroxide</td>
<td>( \text{UO}_2(\text{OH})_2 + \text{H}_2 \rightarrow \text{U(OH)}_4 )</td>
<td>18.5</td>
<td>-0.26</td>
</tr>
<tr>
<td>Sulfur</td>
<td>( \text{S} + \text{H}_2 \rightarrow \text{H}_2\text{S} )</td>
<td>32.6</td>
<td>-0.25</td>
</tr>
<tr>
<td>Bismuth hydroxide</td>
<td>( \text{Bi(OH)}_3 + \frac{3}{2} \text{H}_2 \rightarrow \text{Bi} + 3 \text{H}_2\text{O} )</td>
<td>3.4</td>
<td>-0.15</td>
</tr>
<tr>
<td>Ferric hydroxide</td>
<td>( \text{Fe(OH)}_3 + \frac{3}{2} \text{H}_2 \rightarrow \text{Fe(OH)}_2 + \text{H}_2\text{O} )</td>
<td>24.1</td>
<td>-0.12</td>
</tr>
<tr>
<td>Tetrathionate</td>
<td>( \text{S}_2\text{O}_5^- + \text{H}_2 \rightarrow 2 \text{S}_2\text{O}_4^- + 2 \text{H}^+ )</td>
<td>1.4</td>
<td>-0.08</td>
</tr>
<tr>
<td>Tellurite</td>
<td>( \text{HTeO}_2^- + 2 \text{H}_2 \rightarrow \text{Te} + 2 \text{H}_2\text{O} + \text{OH}^- )</td>
<td>15.1</td>
<td>-0.07</td>
</tr>
<tr>
<td>Arsenate</td>
<td>( \text{H}_3\text{AsO}_4 + \text{H}_2 \rightarrow \text{HAsO}_3 + \text{OH}^- + \text{H}_2\text{O} )</td>
<td>2.7</td>
<td>0.00</td>
</tr>
<tr>
<td>Vanadate</td>
<td>( \text{H}_2\text{VO}_4 + \frac{3}{2} \text{H}_2 \rightarrow \text{VO(OH)}_3 + \text{OH}^- )</td>
<td>59.0</td>
<td>+0.19</td>
</tr>
<tr>
<td>Selenite</td>
<td>( \text{HSeO}_3^- + \text{H}_2 \rightarrow \text{Se} + 2 \text{H}_2\text{O} + \text{OH}^- )</td>
<td>39.4</td>
<td>+0.23</td>
</tr>
<tr>
<td>Osmium dioxide</td>
<td>( \text{OsO}_4 + 2 \text{H}_2 \rightarrow \text{Os} + 2 \text{H}_2\text{O} )</td>
<td>2.6</td>
<td>+0.27</td>
</tr>
<tr>
<td>Nitrite</td>
<td>( \text{NO}_2^- + 3 \text{H}_2 \rightarrow \text{NH}_4^+ + 2 \text{OH}^- )</td>
<td>1.1</td>
<td>+0.32</td>
</tr>
<tr>
<td>Cupric hydroxide</td>
<td>( \text{Cu(OH)}_2 + \frac{3}{2} \text{H}_2 \rightarrow \text{Cu(OH)} + \text{H}_2\text{O} )</td>
<td>10.4</td>
<td>+0.32</td>
</tr>
<tr>
<td>Osmium tetroxide</td>
<td>( \text{OsO}_4 + \text{H}_2 \rightarrow \text{OsO}_3^- + 2 \text{H}^+ )</td>
<td>21.0</td>
<td>+0.36</td>
</tr>
<tr>
<td>Manganese dioxide</td>
<td>( \text{MnO}_3 + \text{H}_2 \rightarrow \text{Mn}^{2+} + 2 \text{OH}^- )</td>
<td>10.4</td>
<td>+0.40</td>
</tr>
<tr>
<td>Nitrate</td>
<td>( \text{NO}_2^- + \text{H}_2 \rightarrow \text{NO}_3^- + \text{H}_2\text{O} )</td>
<td>1.6</td>
<td>+0.41</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>( \text{Fe(CN)}_4^{2-} + \frac{3}{2} \text{H}_2 \rightarrow \text{Fe(CN)}_3^{2-} + \text{H}^+ )</td>
<td>54.0</td>
<td>+0.41</td>
</tr>
<tr>
<td>Tellurite</td>
<td>( \text{HTeO}_3^- + 3 \text{H}_2 \rightarrow \text{Te} + 3 \text{H}_2\text{O} + \text{OH}^- )</td>
<td>2.6</td>
<td>+0.51</td>
</tr>
<tr>
<td>Dithionite</td>
<td>( \text{S}_2\text{O}_5^- + \text{H}_2 \rightarrow \text{S}_2\text{O}_4^- + \text{H}_2\text{O} )</td>
<td>7.7</td>
<td>+0.52</td>
</tr>
<tr>
<td>Lead dioxide</td>
<td>( \text{PbO}_2 + \text{H}_2 \rightarrow \text{Pb(OH)}_2 )</td>
<td>0.7</td>
<td>+0.63</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>( \text{N}_2\text{H}_5\text{OH} + \text{H}_2 + \text{H}_2\text{O} \rightarrow 2 \text{NH}_4^+ + 2 \text{OH}^- )</td>
<td>0.3</td>
<td>+0.69</td>
</tr>
<tr>
<td>Oxygen</td>
<td>( \text{O}_2 + 2 \text{H}_2 \rightarrow 2 \text{H}_2\text{O} )</td>
<td>15.4</td>
<td>+0.78</td>
</tr>
<tr>
<td>Bismuthate</td>
<td>( \text{BiO}_2^- + \frac{3}{2} \text{H}_2 \rightarrow \text{Bi} + 2 \text{H}_2\text{O} + \text{OH}^- )</td>
<td>12.3</td>
<td>+0.89</td>
</tr>
<tr>
<td>Hydroxylyamine</td>
<td>( \text{NH}_3\text{OH}^+ + \text{H}_2 \rightarrow \text{NH}_4^+ + \text{H}_2\text{O} )</td>
<td>8.5</td>
<td>+0.90</td>
</tr>
</tbody>
</table>

* The equations show the various ions and compounds in the form that predominates at the pH where reduction was observed (Charlot, 1954), except where data have been obtained suggesting that a minor component was the actual species reduced (i.e., metabisulfite, \( \text{MoO}_4^{2-} \)).

** Expresssed as \( \mu \text{moles of } \text{H}_2 \times 10^9 \text{ per min per mg of protein.} \)

* The \( E_\theta \) values for pH 7.0 given in this table are calculated from the normal oxidation potentials given by Latimer (1952). The potential for the molybdate-molybdemnum blue couple was approximated with the aid of information given by Saxena and Saxena (1961).

* Value of Michaelis and Hill (1933).
TABLE 6. Reduction of inorganic compounds with molecular hydrogen by extracts of several hydrogenase-containing bacteria\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(M. lactilyticus)(^b)</th>
<th>(M. aerogenes)(^b)</th>
<th>(D. desulfuricans)</th>
<th>(C. pasteurianum)</th>
<th>(E. coli)</th>
<th>(A. vinelandii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl viologen, methyl viologen, or methylene blue(^f)</td>
<td>440</td>
<td>238</td>
<td>1,006</td>
<td>1,170</td>
<td>20.8</td>
<td>132</td>
</tr>
<tr>
<td>Nitrate</td>
<td>1.6</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>10.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Nitrite</td>
<td>1.1</td>
<td>0.0</td>
<td>43.0</td>
<td>85.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>8.5</td>
<td>3.2</td>
<td>65.5</td>
<td>31.8</td>
<td>4.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Arsenate</td>
<td>2.7</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Oxygen</td>
<td>15.4</td>
<td>0.6</td>
<td>17.4</td>
<td>38.0</td>
<td>47.0</td>
<td>52.6</td>
</tr>
<tr>
<td>Metabisulfite</td>
<td>83.0</td>
<td>0.3</td>
<td>37.0</td>
<td>230.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Tetrathionate</td>
<td>1.4</td>
<td>1.1</td>
<td>31.2</td>
<td>9.9</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Selenite</td>
<td>39.4</td>
<td>0.7</td>
<td>24.8</td>
<td>141.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Tellurite</td>
<td>15.1</td>
<td>0.4</td>
<td>23.4</td>
<td>31.8</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Vanadate</td>
<td>50.0</td>
<td>—</td>
<td>57.5</td>
<td>171.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sulfate(^c)</td>
<td>0.0</td>
<td>—</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) Results are expressed as \(\mu\)moles of \(H_2 \times 10^3\) per min per mg of protein.

\(^b\) Data for \(M. lactilyticus\) taken from Table 5 and included for comparative purposes.

\(^c\) Values for \(M. aerogenes\) are for cell suspensions. Specific activities were computed on a mg (dry wt) basis.

\(^d\) Hydrogenase activity was measured as hydrogen evolution from reduced methyl viologen for \(M. aerogenes\), as hydrogen uptake with benzyl viologen for \(M. lactilyticus\), \(D. desulfuricans\), and \(C. pasteurianum\), and methylene blue for \(E. coli\) and \(A. vinelandii\).

\(^e\) Adenosine triphosphate (10 \(\mu\)moles) and MgCl\(_2\) (10 \(\mu\)moles) were added.

observed with extracts of \(M. aerogenes\) or with suspensions of older cells.

**DISCUSSION**

The inorganic reductions catalyzed by \(M. lactilyticus\) are summarized in Table 5. A number of sulfur and nitrogen compounds can be reduced by other bacteria (Nason, 1962; Peek, 1962), both in assimilatory and dissimilatory reactions, and the mechanism of certain of these resembles the reductions mediated by \(M. lactilyticus\). The intermediates in the conversion of sulfur compounds by the latter organism are described in the following paper (Woolfolk, 1962); the reduction of nitrogen compounds will be described elsewhere. The reductions of selenite, tellurite, ferricyanide, ferric ion, manganese dioxide, oxygen, and benzyl viologen have also been observed for other bacteria. The hydrogenase-coupled reductions of selenite, selenium, molybdate, tellurate, tellurite, phosphotungstate, arsenate, vanadate, osmium dioxide and tellurite, bismuthate, hydroxides of uranyl, bis-muthyl, ferric, and cupric ions, and manganese and lead dioxide have not been described previously. In most of the reactions, a quantitative one-electron or two-electron reduction was observed. In some (bismuthate, bismuth hydroxide, tellurate, tellurite, selenite, and osmium dioxide), the anion was reduced completely to the free metal. The stoichiometry and rates of reduction of bismuthate and tellurate are consistent with the formation of bismuth hydroxide and tellurite, respectively, as intermediates, and selenium was shown to be an intermediate in the conversion of selenite to selenide. Neither the rates of the various reactions nor the extent of reduction were related to the oxidation-reduction potential.

The present investigation has shown that many of the above reactions can also be catalyzed by extracts of \(D. desulfuricans\) and \(C. pasteurianum\). The reactions with \(Desulfovibrio\) may result from the combined action of cytochrome \(c_1\) and hydrogenase, as reported by Senez and Pichinoty (1958a, b) for nitrite and hydroxylamine. It is also possible that other naturally occurring carriers, such as the brown protein of Hori (1961), may participate in some of the reactions. Although \(M. lactilyticus\) does not possess cytochromes, evidence has been presented (Whiteley and Ordal, 1957) that an iron-containing intermediate electron carrier, probably a protein, may function in another hydrogenase-
coupled reduction, that of xanthine. This intermediate electron carrier has recently been isolated and characterized (Mortenson, Valentine, and Carnahan, 1962; Valentine, Jackson, and Wolfe, 1962). Possibly there are a number of "electron transport" proteins in this anaerobe which may be conveniently investigated by means of an appropriate inorganic reduction. The role of electron-transport proteins and the mechanisms of some of the reductions catalyzed by M. lactilyticus are currently under investigation.

The possible function of the reactions shown in Table 5 in the metabolism of M. lactilyticus is of interest. Certain of the reactions (e.g., the reduction of sulfur and nitrogen compounds) may be of significance in providing reduced compounds utilized for growth. The reactions might also be coupled to the oxidation of reduced coenzymes such as flavin adenine dinucleotide, and thus influence over-all metabolism. It is also possible that certain of these reductions are catalyzed nonspecifically by enzymes and coenzymes which have another function in metabolism. Thus, these enzymes or coenzymes could be isolated and studied by virtue of an inorganic reduction which is catalyzed only incidentally. Of interest in this connection is the observation that the well-known diaphorase of animal tissues has been identified as lipoyl dehydrogenase (Massey and Veeger, 1960).

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LITERATURE CITED


Bromfield, S. M. 1954. Reduction of ferric comp-


Lascelles, J., and J. L. Still. 1946. Utilization


