JOURNAL OF BACTERIOLOGY, Apr., 1965
Vol. 89, No. 4

Printed in U.S.A.

Nickel-Dependent Chemolithotrophic Growth of Two *Hydrogenomonas* Strains[1]

R. BARTHA[2] AND E. J. ORDAL

Department of Microbiology, University of Washington, Seattle, Washington

Received for publication 20 November 1964

ABSTRACT

BARTHA, R. (University of Washington, Seattle), AND E. J. ORDAL. Nickel-dependent chemolithotrophic growth of two *Hydrogenomonas* strains. J. Bacteriol. **89**: 1015–1019. 1965.—The trace element requirements for growth of facultative chemolithotrophic *Hydrogenomonas* strains H1 and H16 were investigated under both autotrophic and heterotrophic conditions. The organisms were grown in a mineral medium, rendered deficient in trace elements by extraction with 8-hydroxyquinoline and chloroform, and, in some cases, by coprecipitation with copper. The organic substrates, succinate and fumarate, used for heterotrophic growth were treated in a similar fashion. Acetate and butyrate were purified by redistillation. It was found that iron alone was required for heterotrophic growth (optimal concentration, 1.5×10^{-6} M Fe^{+++}), but cells grown chemolithotrophically on molecular hydrogen required the addition of nickel. The yield of protein was proportional to the nickel added, reaching a maximum at 3×10^{-7} M Ni^{++}. Manganese, cobalt, copper, and zinc, alone or in combination, failed to substitute for nickel in the experiments with *Hydrogenomonas*. Although nickel is required specifically for the chemolithotrophic growth of *Hydrogenomonas*, nickel deficiency did not affect: (i) the synthesis or activation of hydrogenase, (ii) the Knallgas reaction, (iii) the assimilation of CO_2 by resting cells, or the synthesis of the storage material poly-β-hydroxybutyric acid. It is suggested that nickel participates in some reaction involved in CO_2 fixation by growing cells.

Two facultatively chemolithotrophic strains of *Hydrogenomonas*, H1 and H16, isolated and characterized as described in an earlier paper (Bartha, 1962), showed a definite requirement for trace elements that was satisfied by the addition of ferrous ammonium citrate and Hoagland's trace element solution (Bergmann, 1958). Since biochemical abilities are sometimes associated with specific trace elements (e.g., nitrogen-fixation with molybdenum) and the specific requirements of hydrogen bacteria had not been reported, it was decided to identify and compare the trace elements essential for growth of *Hydrogenomonas* under chemolithotrophic and heterotrophic conditions.

MATERIALS AND METHODS

A mineral medium consisting of 0.4% Na_2HPO_4, 0.15% KH_2PO_4, 0.1% NH_4Cl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, and 0.05% $NaHCO_3$ was rendered deficient in heavy metals by a method based on the data of Gentry and Sherrington (1950). Na_2HPO_4 (8 g), 3 g of KH_2PO_4, and 4.5 ml of 38% HCl were dissolved in 800 ml of water. The resulting solution at *p*H 5.0 was extracted three times with 50-ml amounts of 1% 8-hydroxyquinoline and chloroform. The *p*H was then adjusted to 7.2 with trace element-free NH_4OH prepared from NH_3, and the extraction was repeated. Residual 8-hydroxyquinoline was extracted with chloroform, and any remaining chloroform was boiled off. $MgSO_4$, $CaCl_2$, and $NaHCO_3$ were made up in concentrated stock solutions and extracted in a similar fashion. The described method did not assure the quantitative removal of molybdenum. Therefore, in some experiments designed specifically to test a possible molybdenum requirement, solutions were rendered deficient by coprecipitating molybdenum with copper sulfite (Hewitt, 1952) prior to the extraction with 8-hydroxyquinoline and chloroform. The organic substrates, succinic and fumaric acids, were extracted with 8-hydroxyquinoline and chloroform at *p*H 5 and 7.2 after partial and complete neutralization with $KHCO_3$ solution previously extracted with 8-hydroxyquinoline and chloroform. The volatile acetic and butyric acids were redistilled twice and neutralized with

[1] Presented in part at the 64th Annual Meeting of the American Society for Microbiology, 3–7 May 1964, Washington, D.C.

[2] Present address: Department of Agricultural Microbiology, Rutgers, The State University, New Brunswick, N.J.

$KHCO_3$ which had been extracted with 8-hydroxyquinoline and chloroform. The organic substrates were added to the mineral medium at a final concentration of 0.2%, and trace elements were added in the form of analytical-grade reagents without further purification. In all experiments, glass-distilled water and acid-cleaned Pyrex glassware were employed.

Cells were cultivated chemolithotrophically in test tubes, the lower ends of which were blown into bulbs each with a volume of about 25 cc. Each tube contained 10 ml of medium and was stoppered with foam rubber, cleaned previously with ethylenediaminetetraacetic acid (EDTA). The tubes were placed in anaerobic jars with an atmosphere of 70% H_2, 20% O_2, and 10% CO_2, and the jars were incubated on a reciprocating shaker at 30 C for 3 days. Cells were cultivated heterotrophically in unmodified test tubes containing 5 ml of medium. These tubes were incubated in an inclined position on a shaker at 30 C for 24 hr.

Growth was measured as milligrams of protein per milliliter of cell suspension (Layne, 1957). The actual iron content of the hygroscopic $FeCl_3$ was determined by the method of Diehl and Smith (1960). Hydrogenase activity was measured by the Knallgas reaction in an atmosphere that contained 70% H_2, 20% O_2, and 10% CO_2, with use of a Warburg respirometer at 30 C. The cells (0.1 mg of protein per ml and 3.0 ml of suspension per reaction flask) were suspended in trace metal-deficient mineral medium.

For studies of $C^{14}O_2$ incorporation and poly-β-hydroxybutyric acid synthesis, cells were grown chemolithotrophically with a limited supply of nickel (one-tenth of the optimal concentration). The cells were washed and suspended in 0.066 M phosphate buffer (*p*H 7.0), which was rendered deficient in trace elements by extraction with 8-hydroxyquinoline and chloroform. Radioactive carbon was provided as $Na_2C^{14}O_3$. At appropriate intervals, excess carbonate was expelled by acidification with HCl, the cells were collected on Millipore filters, and incorporated radioactivity was measured with a Nuclear-Chicago gas-flow counter. Poly-β-hydroxybutyric acid was measured by the method of Williamson and Wilkinson (1958). Synthesis of the compound was determined by analyzing cells for protein and poly-β-hydroxybutyric acid before and after they were incubated with agitation (magnetic stirrer) for 12 hr under an atmosphere of 70% H_2, 20% O_2, and 10% CO_2 at 30 C.

RESULTS

Trace element requirements. The influence of concentration of iron on growth of *Hydrogenomonas* strain H16 is summarized in Table 1. Results obtained with strain H1 did not differ significantly from those obtained with strain H16.

Both organisms, when grown heterotrophically on acetate, butyrate, succinate, and fumarate, required the addition to the medium of iron only. This was added as $FeCl_3$ from concentrated stock solution containing EDTA to prevent precipitation. The final EDTA concentration in the medium varied according to the iron concentration but never exceeded 0.00001%. The minimal iron concentration permitting optimal growth was 1.5×10^{-6} M. Growth was considered optimal when limited only by the carbon source in heterotrophic culture and by the nitrogen source in chemolithotrophic culture.

The iron-supplemented medium supported heterotrophic growth of *Hydrogenomonas* without change through 10 transfers, but it proved inadequate for chemolithotrophic development, which, in three transfers, first decreased and then ceased completely. However, the addition to the medium of Hoagland's solution rendered it nutritionally suitable for growth on hydrogen, indicating that iron was not the only metal supplement needed when the organism developed chemolithotrophically. Hoagland's solution contains 12 cations and 5 anions, and, to identify the required element, it was necessary to perform a series of

TABLE 1. *Iron requirement for growth of Hydrogenomonas strain H16*

Heterotrophic Fe requirement*		Chemolithotrophic Fe requirement†	
Fe concn	Protein	Fe concn	Protein
M	*mg/ml*	*M*	*mg/ml*
0.00	0.20	0.00	0.20
5×10^{-7}	0.47	5×10^{-7}	0.75
1×10^{-6}	0.66	1×10^{-6}	1.02
1.5×10^{-6}	0.78	1.5×10^{-6}	1.27
2×10^{-6}	0.77		
4×10^{-6}	0.76	2.5×10^{-6}	1.28

* Heterotrophic growth on 0.2% succinate.

† All cultures provided with optimal (3×10^{-7} M) $NiCl_2$.

TABLE 2. *Nickel requirement for chemolithotrophic growth of Hydrogenomonas strain H16**

Nickel concn	Protein
M	*mg/ml*
0.00	0.05
1×10^{-7}	0.54
2×10^{-7}	0.75
3×10^{-7}	1.26
4×10^{-7}	1.00
5×10^{-7}	1.05
1×10^{-6}	1.02

* All cultures provided with optimal (1.5×10^{-6} M) $FeCl_3$.

omission and replacement experiments. The results of these tests established that nickel was essential for chemolithogrophic growth. It substituted completely for Hoagland's solution. The influence of nickel concentration on growth of *Hydrogenomonas* strain H16 is shown in Table 2. Optimal growth was obtained at a level of 3×10^{-7} M $NiCl_2$, and continued transfer in medium supplemented with iron and nickel at optimal concentrations has not revealed additional requirements.

No nickel requirement was demonstrated by *Hydrogenomonas* strains grown on organic substrates that were rendered deficient in nickel by various methods. Purified substrates were ashed, and the ash, in an amount equivalent to the usual substrate concentration of 0.2%, failed to support significant chemolithotrophic growth of cells in nickel-deficient medium. This test provided evidence that the purified organic substrates were not contaminated with sufficient nickel to support growth, and that no nickel supplement is needed by cells growing heterotrophically. The requirements for nickel when *Hydrogenomonas* grows chemolithotrophically is specific. It was not possible to substitute manganese, cobalt, copper, or zinc, or any other component of Hoagland's trace element solution for nickel.

Role of nickel. The most plausible explanation of the nickel requirements of *Hydrogenomonas* is possible involvement of the metal in enzymatic reactions essential for chemolithotrophic metabolism. Nickel may serve as the active center of a metal enzyme or as activator for some enzyme reaction. Since the most characteristic features of the chemolithotrophic metabolism of *Hydrogenomonas* are the oxidation of hydrogen and the fixation of CO_2, the effect of nickel on these processes was investigated.

The hydrogenase activity of chemolithotrophically grown nickel-deficient cells, as measured by the rate of Knallgas reaction, did not differ significantly from a cell suspension having similar optical density but grown with nickel. Moreover, the addition of nickel to nickel-starved resting cells had no effect on the hydrogenase activity. Since it was possible that growth ceased when nickel was depleted, but that cells retained their normal hydrogenase supply and activity, the effect of nickel on the synthesis of the enzyme was investigated. Cells grown on nickel-deficient succinate medium having no hydrogenase activity were adapted to hydrogen oxidation in trace element-deficient mineral medium under a $H_2/O_2/CO_2$ atmosphere. It is evident that the rate of hydrogenase synthesis, measured manometrically as the rate of hydrogen oxidation, was identical with and without added nickel (Fig. 1). Therefore, nickel does not appear to be required for the synthesis or the activation of hydrogenase.

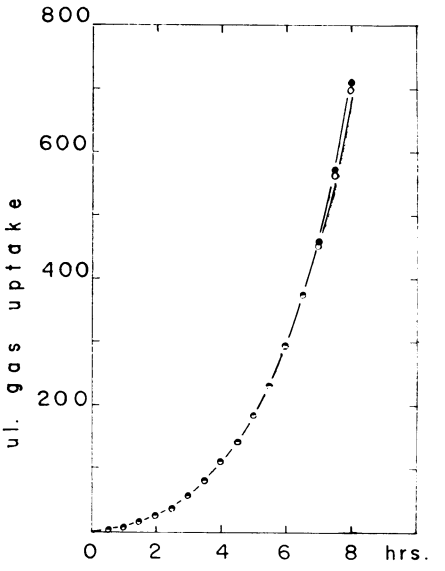

FIG. 1. *Hydrogenase adaptation with and without nickel added. Hydrogenomonas H16 was grown on trace element-deficient succinate medium. The cells were washed and suspended in trace element-deficient mineral medium provided with* 2×10^{-6} M $FeCl_3$. *Some of the Warburg reaction flasks were provided, in addition, with* 3×10^{-7} M $NiCl_2$. *Atmosphere: 70% H_2 + 20% O_2 + 10% CO_2. Temperature: 30 C; pH, 7.0. Symobls: $+NiCl_2$ = ●; $-NiCl_2$ = ○.*

The rate of CO_2 fixation by chemolithotrophically grown, nickel-deficient resting cells was not increased by the addition of nickel. Cells of *Hydrogenomonas* demonstrate a relationship between CO_2 fixation and the rate of hydrogen oxidation. When CO_2 fixation was inhibited by iodoacetic acid, the rate of the hydrogen oxidation decreased (Bartha, 1962). However, nickel deficiency failed to cause a secondary inhibition of hydrogen oxidation, and this and results of radioisotope experiments both indicate that CO_2 fixation by resting cells was not dependent on nickel. Resting, nickel-deficient cells accumulated, by means of chemolithotrophic CO_2 incorporation, large amounts of poly-β-hydroxybutyric acid, but the process was not influenced significantly by the addition of nickel (Table 3).

DISCUSSION

The experiments described here clearly establish a specific nickel requirement for the chemolithotrophic growth of *Hydrogenomonas*. Although the role of nickel has not been identified, various

TABLE 3. *Accumulation of poly-β-hydroxybutyric acid (PHBA) in resting cells of Hydrogenomonas in the presence and absence of nickel**

Sample	Protein	PHBA
	mg/ml	*mg/m*
Starting material	0.72	0.03
After 12 hr −Ni	0.72	0.40
After 12 hr +Ni	0.75	0.42

* Optimal (1.5×10^{-6} M) $FeCl_3$ was provided.

tests were performed and the number of possibilities decreased appreciably. Nickel was not essential for activation or synthesis of hydrogenase or for the Knallgas reaction. Moreover, carbon dioxide fixation and poly-β-hydroxybutyric acid synthesis by resting, nickel-deficient cells was not favorably influenced by the addition of nickel. It is possible that cells grown chemolithotrophically on limited nickel may have retained sufficient active nickel-enzyme complex to function metabolically. However, the activity of these cells would be minimal, and a substantial increase in carbon dioxide fixation and poly-β-hydroxybutyric acid synthesis would be expected to occur upon addition of nickel, provided that these processes were nickel-dependent.

Iron had a marked favorable influence on both the Knallgas reaction and CO_2 fixation, but the effect is considered to be of a secondary nature. Iron deficiency in this aerobic organism inhibits electron transport, and, thus, hydrogen oxidation is decreased. If electron transport is inhibited, the supply of energy and reducing power for CO_2 fixation is decreased also. Studies of partially purified hydrogenase from *Hydrogenomonas* H16 (Bartha, *unpublished data*) did not indicate that nickel or iron was directly involved in hydrogen activation.

It was not possible to locate a previous report describing nickel as essential for bacterial growth. It is not known whether the nickel requirement of *Hydrogenomonas* is unique, or if traces of nickel that contaminate commercially available chemicals are sufficient to satisfy the apparently very low demand by bacteria for nickel. Nickel and other bivalent metals activate in vitro the following enzymes: arginase (Hellerman and Perkins, 1935; Edelbacher and Zeller, 1936; Hellerman and Stock, 1938; Stock, Perkins, and Hellerman, 1938; Mohamed and Greenberg, 1945); β-keto acid decarboxylase (Speck, 1948; Kornberg, Ochoa, and Mehler, 1948); phosphomonoesterase (Neumann, 1949); phosphoprotein phosphatase (Paigen, 1958); uridine diphosphoglucose phosphorylase (Turner and Turner, 1958); enolase (Wold and Ballon, 1957); and carboxypeptidase (Coleman and Valle, 1960). In some of these enzymes, nickel and other bivalent metal activators appear to be strongly associated with the enzyme protein and may possibly take part in the catalyzed reactions. In other cases, the metal ions seem to orient the enzyme-substrate complexes, or to maintain the active configuration of the enzymes. Warren, Wacker, and Valle (1959) reported the presence of higher concentrations of nickel and other metals in ribonucleic acid than in other cellular components, and they suggested that those metals may maintain the structure of nucleic acid polymers or participate in protein synthesis. Nevertheless, nickel has not been previously demonstrated to have an essential role. On the contrary, it is usually a poor substitute for Mg^{++}, Mn^{++}, or other metal activators. It is only in the case of chemolithotrophic growth of *Hydrogenomonas* that other bivalent metals cannot replace nickel. Here nickel is essential, but its mode of action remains to be identified.

The fact that *Hydrogenomonas* cannot develop chemolithotrophically in the absence of nickel, but can, under that condition, synthesize poly-β-hydroxybutyric acid and grow heterotrophically, is understandable only if nickel has an essential function in carbon dioxide fixation by cells growing chemolithotrophically. The pathway of carbon dioxide fixation by resting *Hydrogenomonas* cells was described by Hirsch and Schlegel (1963), Hirsch (1963), Hirsch, Georgiev, and Schlegel (1963), and Schlegel and Gottschalk (*personal communication*); carbon dioxide is fixed via the Calvin cycle and poly-β-hydroxybutyric acid is synthesized from acetyl coenzyme A via acetoacetate. These reactions appear to be independent of nickel; however, little is as yet known about the mechanism of carbon dioxide fixation by growing cells, and it is possible that nickel acts in the transformation of assimilated carbon dioxide to nitrogenous compounds.

ACKNOWLEDGMENT

This investigation was supported in part by National Science Foundation grant GB-908. The senior author was a postdoctoral trainee on National Institutes of Health Training grant 5T1GM511.

LITERATURE CITED

BARTHA, R. 1962. Physiologische Untersuchungen über den chemolithotrophen Stoffwechsel neu isolierter Hydrogenomonas-Stämme. Arch. Mikrobiol. **41**:313–350.

BERGMANN, W. 1958. Methoden zur Ermittlung mineralischer Bedürfnisse der Pflanzen, p. 37–89. *In* W. Ruhland [ed.], Encyclopedia of plant physiology, vol. 4. Springer Verlag, Berlin.

COLEMAN, J. E., AND B. L. VALLE. 1960. Metal-

locarboxypeptidases. J. Biol. Chem. **235**:390–395.

DIEHL, H., AND G. F. SMITH. 1960. The iron reagents: bathophenanthroline, 2,4,6-tripyridyl-s-triazine, phenyl-2-pyridyl ketoxime. Section 3. The G. Frederick Smith Chemical Co., Columbus, Ohio.

EDELBACHER, S., AND A. ZELLER. 1936. Über die Natur der Arginase. Z. Physiol. Chem. **245**: 65–75.

GENTRY, C. H. R., AND L. G. SHERRINGTON. 1950. Extraction and photometric estimation of some metals with 8-hydroxyquinoline. Analyst **75**:17–21.

HELLERMAN, L., AND M. E. PERKINS. 1935. Activation of enzymes. III. The role of metal ions in the activation of arginase. The hydrolysis of arginine induced by certain metal ions with urease. J. Biol. Chem. **112**:175–194.

HELLERMAN, L., AND C. C. STOCK. 1938. Activation of enzymes. V. The specificity of arginase and the non-enzymatic hydrolysis of guanidino-compounds. Activating metal ions and liver arginase. J. Biol. Chem. **125**:771–793.

HEWITT, E. J. 1952. Sand and water culture methods, p. 191–193. Tech. Comm. 22, Commonwealth Bureau of Horticulture and Plankton Crops, East Malling, England.

HIRSCH, P. 1963. CO_2-Fixierung durch Knallgasbakterien. II. Chromatographischer Nachweis der frühzeitigen Fixierungsprodukte. Arch. Mikrobiol. **46**:53–78.

HIRSCH, P., G. GEORGIEV, AND H. G. SCHLEGEL. 1963. CO_2-Fixierung durch Knallgasbakterien. III. Autotrophe und organotrophe CO_2-Fixierung. Arch. Mikrobiol. **46**:79–95.

HIRSCH, P., AND H. G. SCHLEGEL. 1963. CO_2-Fixierung durch Knallgasbakterien. I. Einbau und Fraktionierung. Arch. Mikrobiol. **46**:44–52.

KORNBERG, A., S. OCHOA, AND A. H. MEHLER. 1948. Spectrophotometric studies on the decarboxylation of β-keto acids. J. Biol. Chem. **174**:159–172.

LAYNE, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 447–454. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.

MOHAMED, M. S., AND D. M. GREENBERG. 1945. Liver arginase. I. Preparation of extracts of high potency, chemical properties, activation-inhibition and *p*H-activity. Arch. Biochem. **8**: 349–364.

NEUMAN, H. 1949. Dependence of the pH-optimum of the phosphomonoestherase I on the substrate concentration and on inhibitors and activators. Biochim. Biophys. Acta **3**:117–124.

PAIGEN, K. 1958. The properties of particulate phosphoprotein phosphatase. J. Biol. Chem. **233**:388–394.

SPECK, J. F. 1948. The effect of cations on the decarboxylation of oxalic acid. J. Biol. Chem. **178**: 315–324.

STOCK, C. C., M. E. PERKINS, AND L. HELLERMAN. 1938. Activation of Enzymes. IV. The Jack bean arginolytic enzyme. J. Biol. Chem. **125**:753–769.

TURNER, D. H., AND J. F. TURNER. 1958. Uridine diphosphoglucose phosphorylase of pea seeds. Biochem. J. **69**:448–452.

WARREN, E., C. WACKER, AND B. L. VALLE. 1959. Nucleic acids and metals. I. Chromium, manganese, nickel, iron and other metals in ribonucleic acid from diverse biological sources. J. Biol. Chem. **234**:3257–3268.

WILLIAMSON, D. H., AND J. F. WILKINSON. 1958. The isolation and estimation of the poly-β-hydroxybutyric acid inclusions of Bac. species. J. Gen. Microbiol. **19**:198–209.

WOLD, F., AND C. E. BALLON. 1957. Studies on the enzyme enolase. I. Equilibrium studies. J. Biol. Chem. **227**:301–328.